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Phage Display as a Strategy for Designing Organic/Inorganic Biomaterials

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To extend and optimize the performance of biomaterials, better control of biofunctionality is needed. In this chapter, we focus on the integration of peptides into biomaterials as a strategy for providing a biomaterial with greater ability to control subsequent protein, cell, and tissue responses. The focus of this chapter is on phage display, a high-throughput selection technique used to identify peptides that have preferential affinity to a specific material or cell type. The use of phage display provides a genetic engineering platform for designing new materials at the nanoscale. The basics of the phage display technique are presented, and postprocessing approaches to analyze the combinatorial data derived from phage display are discussed. Specific examples of the use of phage display with calcium phosphate biomaterials are presented, as are examples from the use of phage display to define amino acid sequences that preferentially bind to specific cell types. Data from multiple phage pannings can be used to create dual-functioning peptides that serve as linkers between the organic and inorganic worlds.

Abbreviations

BLM	bone-like mineral
CN	carbon nanotubes
DNT	2,4-dinitrotoluene
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FASTA	DNA and protein alignment software
PEG	polyethylene glycol
PPyCl	chlorine-doped polypyrrole
RELIC	REceptor Ligand Contacts

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6.1. Introduction: Biomaterials Development and the Need for More Robust Approaches to Control Protein, Cell, and Tissue Responses

Historically, most biomaterials have had their origins in other fields and only secondarily found utility in medicine and dentistry. For example, high-strength alloys used in joint replacements were first developed in the aerospace field, and acrylic bone cement was developed in the paint industry. Although many such materials have functioned ~~tolerably~~ well, few interact with their surrounding host environment or promote integration with host tissue in an intelligent and proactive fashion. The desire to implement more biological approaches toward biomaterials design such that materials can provide instructions ~~to cells of surrounding tissues~~ has led to an expansion and paradigm shift in the field of biomaterials in the last two decades. Many biomaterials are now being rationally designed to interact with the biological milieu they will encounter in vivo and, in some cases, facilitate tissue regeneration.

Biomaterial systems that can promote tissue regeneration should satisfy the following design requirements [1, 2]: (1) biocompatibility; (2) conductivity for attachment and proliferation of committed cells or their progenitors, and production of new, functional extracellular matrix (ECM); (3) ability to incorporate inductive factors to direct and enhance new tissue growth; (4) support of vascular ingrowth for transport of oxygen and biomolecules; (5) mechanical integrity to support loads at the implant site; (6) controlled and predictable degradation into nontoxic species that are easily metabolized or excreted; and (7) simple and inexpensive processing into irregular three-dimensional (3D) shapes of sufficient volume to fill clinically relevant tissue defects. Integration of these criteria into a single material presents design challenges that require more biomimetic complexity than many of the current simplified ECM mimics can provide.

The first generation of biomaterials that mimicked structural and/or functional aspects of ECMs and satisfied a subset of the design requirements listed earlier in this section included both organic and inorganic biomaterials: copolymers of poly(lactic–glycolic acid) [3], collagen [4], polyphosphazenes [5], polyurethanes [6], polycaprolactone [7], polyethylene glycol (PEG) [8], poly(propylene fumarate) [9], starch-based materials [10], alginate [11], silk [12], bioactive glasses and glass ceramics [13, 14], calcium–phosphate ceramics [15–17], and composites of calcium–phosphates and collagen [4] or synthetic polymers [18–21]. Varying the material properties of a biomaterial, such as composition, topology, and crystallinity can lead to a significant variation in a number of cell functions in vitro, including cell attachment, cell proliferation, RNA transcription, and protein synthesis [1, 13,14, 22–28]. The material properties of a biomaterial can also significantly affect cell differentiation, the rate and amount of tissue formation, and the duration and magnitude of inflammatory responses in vivo [1, 16, 29, 30].

To extend the performance of biomaterials beyond the capabilities of what these first-generation materials can provide and to incorporate more of the above design criteria into a single material, better control of biofunctionality is needed. The specific microenvironment that interacts with the material must be considered in the design process, such that in vivo functionality and tissue remodeling can be maintained in the long-term.

Biomaterial modification can take on different levels of complexity, resulting in increasing levels of physiological replication and functionality. One approach is to capitalize on the instructive cues inherent in natural ECMs; in fact, variations of ECM molecules serve as one basis for formulating biomaterials [31, 32]. Surface and bulk chemical modifications of

synthetic materials can also help control cell–material interactions and enhance tissue integration. These chemical modifications include changes in hydrophilicity and surface functionalization with charged groups [33, 34], supramolecular self-assembly [35–38], and development of materials that bind and release soluble factors [39–43]. Incorporation of insoluble ligands and cell recognition sequences from peptides, as well as attachment of larger proteins, are also key strategies to impart communication between a material and cells [33, 44–47]. Strategies based on physical, rather than chemical cues, include reproduction of the nanoscale topology of natural ECMs [31, 32, 48] and superposition of mechanical cues [49].

In this chapter, we focus on the integration of peptides into biomaterials as a strategy for providing a biomaterial with greater ability to control protein, cell, and tissue responses. Starting with a discussion of the rationale for utilizing peptides over larger proteins and the physiological importance of using peptides as a component of a biomaterial, we next focus on the high-throughput technique of phage display as a tool to discover peptides that have preferential affinity to specific materials. Following a general presentation of the phage display technique and postprocessing approaches to analyze the combinatorial data derived from phage display, we present specific examples of the use of phage display with calcium phosphate biomaterials. We next discuss the analogous use of phage display to define amino acid sequences that preferentially bind to progenitor cells. Finally, we discuss how data from multiple phage pannings can be used to create dual-functioning peptides that serve as linkers between the organic and inorganic worlds. The objective of this chapter is to discuss the use of the phage display technique as a means of linking cells to biomaterials in a more rational manner via the discovery of linker peptides ~~(Table 6.1)~~.

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Table 6.1. List of peptides discussed in this chapter and their proven functions

Peptide sequence	Derived from	Proven function	Reference
RGD	Multiple proteins	Relevant to bone engineering, RGD enhances cell adhesion and differentiation into bone, cartilage, neural, and endothelial tissue	[53–59]
FHRRIKA	Heparin-binding domain	Increases osteoblast adhesion and mineralization	[60]
KRSR	Heparin-binding domain	Increases osteoblast adhesion and mineralization	[53]
YIGSR	Laminin	Increases human foreskin fibroblast adhesion	[66]
IKVAV	Laminin	Increases neurite extension	[61]
REDV	Fibronectin	Increases endothelial cell adhesion	[67]
KHIFSDDSSE	Neural cell adhesion molecules	Increases astrocyte adhesion	[68]
VPGIG	Elastin	Increases stiffness of synthetic matrices	[64]
SVSVGMKPSPRP	Phage display	High selectivity toward hydroxyapatite and tooth enamel	[117]
VTKHLNQISQSY	Phage display	High selectivity toward hydroxyapatite and ~~BML~~	[47]

(continued)

Table 6.1. (continued)

Peptide sequence	Derived from	Proven function	Reference
CRKRLDRNC	Phage display	Interacts with IL-4 receptor on endothelial cells, macrophages, and smooth muscle cells	[131]
THRTSTLDYFVI	Phage display	High selectivity toward polypyrrole that increases adhesion of PC12 cells	[81]
STFTKSP	Phage display	Homes to primitive hematopoietic progenitor cells in bone marrow	[98]
WYRGRL	Phage display	Binds to collagen IIα1	[93]
ASSLINA	Phage display	Binds to both skeletal and cardiac tissue	[97]
CAGALCY	Phage display	Targets brain tissue	[94]

6.2. Peptide–Biomaterial Interactions

Functionalizing a surface via either protein or peptide adsorption or attachment can increase binding of specific cell receptors. Incorporation of proteins or their subsequences into the backbone of a polymer can control processes, such as cell differentiation and matrix synthesis. Proteins, growth factors, and peptides have been either ionically or covalently attached to biomaterial surfaces to promote cell adhesion, and, ultimately, the amount of tissue regenerated [33, 40, 47, 50]. While several proteins enhance cell adhesion, proteins are challenging to isolate and prone to degradation [51]. Proteins can also change conformation or orientation because they possess sections with varying hydrophobicities that modulate cellular functions other than adhesion. On the other hand, peptides can mimic the same response as a protein while being smaller, cheaper, and less susceptible to degradation. Peptides may, therefore, have a greater potential for controlling initial biological response to a material, because they can contain specific target amino acid sequences and can permit control of hydrophilic properties through specific sequence design [52].

In addition to its structural role, the ECM contains adhesive ligands, such as fibronectin, vitronectin, and, laminin that direct cell function. Identification of peptide sequences within proteins that are responsible for cell adhesion led to the development of peptide-functionalized biomaterials [44]. Incorporation of peptide motifs containing sequences, such as the arginine–glycine–aspartic acid (RGD)-based sequences, that are recognized by integrin receptors on cell membranes is now a common strategy to enhance the biological functionality of substrates [33, 45, 46]. Materials with appropriate concentrations of RGD-containing sequences can enhance cell adhesion and direct differentiation into cells of the bone [53–55], cartilage [56, 57], neural [58], and endothelial [59] tissues.

Using recombinant DNA technology, synthetic proteins can be designed to mimic specific ECM constituents. In addition to the ubiquitous RGD sequence, sequences derived from the heparin-binding domain, such as FHRRIKA [60] and KRSR [53], increase osteoblast adhesion and mineralization. Peptide sequences that mimic sections of collagen [50] and of the noncollagenous proteins laminin [61], bone sialoprotein [62], osteopontin [63],

statherin [63], elastin [64], and osteonectin [65] also increase cell adhesion, proliferation, and lineage-specific progenitor cell differentiation. A noninclusive list of synthetic sequences used in tissue engineering and their functions includes: YIGSR, derived from laminin, increases human ~~foreskin~~ fibroblast adhesion [66]; IKVAV, derived from laminin, increases neurite extension [61]; REDV, derived from fibronectin, increases endothelial cell adhesion [67]; KHIFSDDSSE, derived from neural cell adhesion molecules, increases astrocyte adhesion [68]; and VPGIG, derived from elastin, increases stiffness of synthetic matrices [64].

In addition to using recombinant technologies to synthesize peptide sequences found within proteins known to promote a specific biological function, domains within a protein can be deleted to investigate the effect of targeted sequence deletions on the function of the protein. Subsequently, sequences deemed to control a specific function could be synthesized for integration with a biomaterial. The focus of this chapter is, however, on another discovery technique, phage display, a high-throughput selection technique in which a bacteriophage library expressing combinations of either linear or cyclic peptide inserts is used to identify amino acid sequences that have high affinity to either a substrate or a cell type. The use of phage display provides a genetic engineering platform for designing new materials at the nanoscale.

6.3. Phage Display as a Selection Technique

Typically, phage display technologies introduce a combinatorial library (on the order of 10^9 sequences) of 7-mer or 12-mer peptide sequences to a molecule, ligand, or material. The phage display technique has been utilized to identify amino acid sequences that recognize specific substrates, serving as a strategy to create biological linkers to bridge biomolecules and synthetic materials at the nanoscale. Targeted inorganic substrates include $BaTiO_3$ for electronic applications [69], SiO_2 [70], TiO_2 [70], aluminum [71], steel [71], semiconductors [72–74], platinum [75], and silver [76, 77]. Organic substrates include carbon ~~for applications of ink binding [78], carbon structures [79],~~ helical wrapping of DNA [80], the electrically conductive chlorine-doped polypyrrole (PPyCl) [81], plastics [82], and poly(methyl methacrylate) [83]. The use of phage display is not limited to solid surfaces. To detect volatile organic compounds, phage libraries have been used to identify peptide recognition motifs for 2,4,6-trinitrotoluene and 2,4-dinitrotoluene (DNT) and to develop gas-binding assays [84].

In addition to creating functional materials via the adhesion of phage motifs to a synthetic material, M13 phage can be assembled into a material itself. The M13 phage is a nanofiber-like virus that can self-align to form higher-order structures [74, 85–87]. Phage fibers can also be fabricated via wet spinning and electrospinning techniques [88]. The fiber-like organization and specific recognition motifs also allow this phage to coassemble with other molecules. Ordered phage-based materials can be constructed by controlling the concentration of virus and ionic strength of the solution in which the virus is suspended, as well as exogenous forces [86–88].

In the biomedical sector, phage display has been used to identify biological species that selectively bind to polystyrene (*Saccharomyces cerevisiae*; [89]), poly(hydroxybutyrate) (immunoglobulin variable regions of human antibodies; [90]), catheters (*Staphylococcus aureus*; [91]), and carbon nanotubes (CN) for drug delivery [92]. Peptides that target specific tissues, including cartilage [93], brain microvasculature [94], kidney tubules [95], breast vasculature [96], muscle [97], bone marrow [98], cell/organ targets in vivo [99], and malignant cell types [100, 101] have also been identified. In vivo tissue screening via phage display

allows targeted delivery of drugs [102] and imaging agents [103]. Furthermore, phage displays have identified sequences with high affinity to cell lines cultured in vitro [104, 105], enzymes and their inhibitors, DNA, proteins, and specific tissues [52, 106, 107].

Although phage display has been mostly used in identifying enzyme substrates and inhibitors, DNA and protein-binding peptides, tissue-specific peptides, and receptors [52, 106, 107], the principle of the technique can be applied to identifying peptides that have high affinity and specificity to biomaterials.

Introduction of phage display libraries to the research fields of cancer, tissue engineering, and molecular biology has proven fruitful since its debut in 1990 [108]. The concept behind phage display is to create an oligonucleotide insertion mutation on the gene of a virus allowing a library of sequences to be expressed on the exterior protein coat of the phage [109]. After several rounds of panning, or expanding and reintroducing the sequences that adhere, consensus sequences emerge and are identified by DNA sequencing (Figure 6.1). The peptide sequences physically presented on the exterior of the bacteriophage (or phage) coats are the result of a genetic modification within the virion DNA encapsulated within the phage.

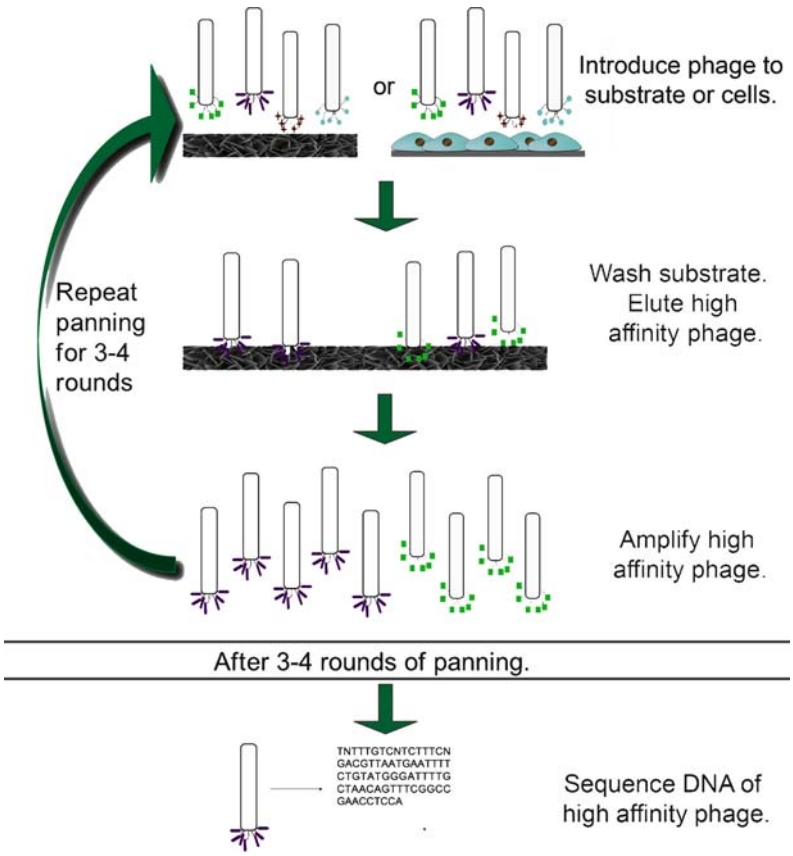

Figure 6.1. Schematic showing the phage display panning technique in vitro on either a substrate or a cell population. One round of panning includes introducing the parent library (~10^9) to the substrate or cell population, washing off the phage that do not adhere, and eluting the phage that have high affinity. Multiple rounds of panning can yield consensus sequences identified via DNA sequencing.

This provides the user with a link between the physically presented peptide and the encoded DNA within the virion, allowing simultaneous testing of a vast number of possible peptide sequences (e.g., 10^9 sequences). This simple biopanning approach allows researchers to investigate either stabilized molecules or substrates in vitro in addition to organ and tissue targeting in vivo. Once the phage display library is introduced in vitro, multiple rounds of rigorous washing ~~disregard~~ nonadherent phage, allowing highly adherent phage to be eluted and amplified, constituting one round of phage panning. Multiple rounds of panning, typically between three and five rounds, can elucidate phage that physically bind to either the substrate or biological constituent of interest. DNA sequencing of the adherent phage reveals the peptide sequence presented on the phage protein coat. This sequence information is often used to synthesize a peptide, and its affinity to either the material or biological target is verified via peptide adsorption and/or immunohistochemical assays.

If the phage display library is introduced in vivo, targeted organs are often harvested, the bound phage are carefully eluted and amplified, and the amplified phage are reintroduced in vivo, constituting one round of in vivo panning. Similar steps as in in vitro studies are then taken to achieve peptide sequences that home to the targeted tissue.

The biological vehicle that is oftentimes used in phage display combinatorial libraries is the filamentous M13 bacteriophage. M13 bacteriophage are a well understood and characterized strain with three main structural protein regions on the virion, pIII, pVIII, and pVI, which have been manipulated to display random peptides [109]. The most commonly used region is the pIII, which allows presentation of approximately five copies of the randomly generated peptide expressed in either a linear or cyclic manner. In commercial phage display libraries (e.g., from New England Biolabs), the random peptide is fused to the pIII protein coat and is expressed at the N terminus of the pIII, typically with a Gly–Gly–Gly–Ser spacer (Figure 6.2). Commercial libraries contain ~10^9 sequences amplified such that ~55 copies are presented in 10 μL of the parent library. The bacterial host used for M13 bacteriophage is typically a strain of *Escherichia coli*.

The technique of biopanning is not limited to combinatorial peptide libraries, as other polypeptide libraries such as immunological protein and cDNA libraries have been also utilized. In addition, the means of peptide display is not limited to bacterial display in eukaryotic

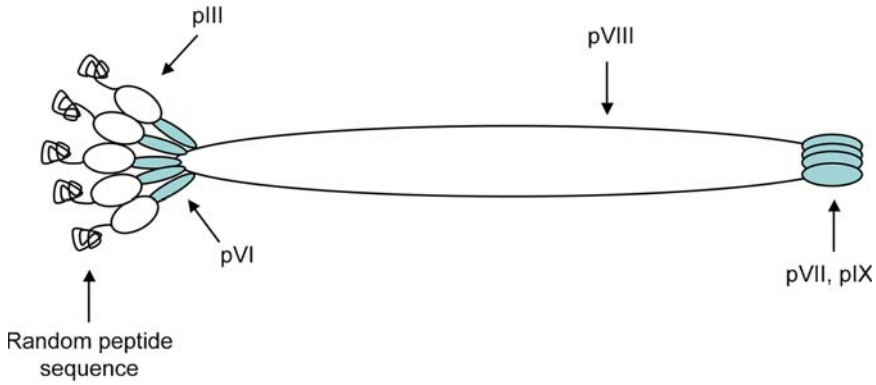

Figure 6.2. Schematic of a bacteriophage identifying the protein coat regions encoded in the virion DNA. The phage display library expresses the same random peptide sequence in three to five copies at the pIII end of the bacteriophage. A parent library can have on the order of 10^9 random sequences.

viruses, since yeast, mammalian cells, ribosomes, messenger RNA (mRNA), and ~~covalent~~ DNA display techniques have also been investigated [110].

Phage display combinatorial libraries offer potential in isolating binding peptide sequences from a vast library, making otherwise unfathomable experiments possible [106, 107, 111]. However, as with any biological system, limitations intrinsic to the technique do exist. Phage libraries can express an unequal representation of each amino acid within the library, but are not able to include every peptide combination possible, and oftentimes omit important post-translational modifications such as phosphorylation of serine residues in the parent library. Nonetheless, the array of peptides presented in these libraries provides the opportunity to identify sequences specific to biological molecules, tissues, as well as synthetic and natural materials. It is possible with phage display techniques to identify high adsorbing, nonmodified sequences that could achieve even higher affinity after synthetic phosphorylation.

6.3.1. Computational Analysis Tools

Because the phage display experiment involves a biological system that can produce biased results from broth growth conditions and/or ease of sequence fabrication by the bacteriophage, computational tools have been developed to supplement experimentation in order to further analyze phage display data. An example of such a tool is REceptor Ligand Contacts (RELIC), a database designed to advance functional genomics through identification of small molecule-binding regions on proteins (http://relic.bio.anl.gov/programs.aspx). This publicly accessible database allows the user to utilize a statistically based approach in analyzing peptide populations, searching for common motifs, and comparing homologous regions with protein sequences [112]. RELIC is broken into five main categories: translation, characterization of peptide populations, peptide motif identification, comparison of peptide population to ~~sequence known~~ structures, and analysis of either single or multiple FASTA (the DNA and protein sequence alignment software) sequences. Translation involves taking DNA sequences of individual phage plaques and translating them to protein code. Although the program is written for New England Biolabs' libraries, other libraries can be inserted if the start and end vector sequences are known. Characterization of the peptide population provides multiple programs that can provide analysis of amino acid frequency with position within a given peptide population, diversity of a peptide population, and the likelihood of observing sequences by chance based on amino acid composition and position for a given peptide population. Both continuous and discontinuous peptide motifs allowing conservative substitutions can be identified using the peptide motif identification programs. To better link the identified sequences with a protein–ligand interaction, peptides within a population that could be in contact with proteins listed in the Protein Data Base are listed and visually displayed. Sequence alignments between peptides and a given protein sequence list (from text only, no coordinates) can also be generated using the analysis of either single or multiple FASTA sequences. Depending on the aim of the study, one or many of the programs can facilitate data analysis.

Other computational tools exist for evaluating parent libraries and they can be used to estimate diversity [113]. Another computational approach uses genetic algorithms to assign sequence similarity scores for individual sequences in a population of peptides [114]. Computational programs are also available to facilitate the design and construction of randomized unbiased libraries [113]. With increased phage display research, we can only hope to mirror the evolution of a variety of computational tools now available in molecular biology to help us advance our abilities of identifying significant peptide sequences [115].

6.3.2. Characterization Techniques

Computational analysis tools are available to aid in the analysis of data sets resulting from phage display experiments; however, characterization techniques to verify that identified peptides are indeed specific binders include analyses ranging from biological assays to computer modeling. The purpose of this section is to give an overview of the techniques researchers are utilizing to support their phage display findings.

The enzyme-linked immunosorbent assay (ELISA), is a common technique used to determine the binding affinity of the isolated and amplified phage binders to both substrate materials and cell sources [47, 116]. High background noise on some substances, such as apatite-based materials, has been reported as one limitation of the ELISA [117]. Another common technique used to visually display the binding of either phage or synthetic peptides uses microscopy to image fluorescently tagged phage or peptides [81, 93, 95, 117]. While this technique can provide qualitative analysis, it is important to couple this technique with a quantitative measure. If a cell source or tissue is being panned against, immunohistochemistry methods can be employed to visually detect phage binding [96–98, 101, 104, 118]. Another powerful ~~technique~~ commonly used in imaging nanoscale features, the atomic force microscope, is used to determine peptide–substrate binding affinities [81, 116]. Less common techniques include quartz crystal microbalance [70, 90, 117], surface plasmon resonance [90], and circular dichroism [76], which are used to detect phage binding, binding of antibodies to a given substrate, and secondary structure before and after binding to particles, respectively.

Synthetic peptide fabrication is a logical next step after identifying highly specific phage to either a material or cell of interest, and thus, the adsorption behavior of the peptide should also be thoroughly investigated. Being able to control the amount of peptide that adsorbs to the surface of a substrate is imperative when linking peptide concentration to subsequent cellular function. Controlling the amount of peptide on a surface can be as simple as covalently linking the peptide to a functionalized polymer, but mainly relies on peptide adsorption in the case of ceramic materials that do not have modifiable surface chemistries. Fluorescently tagged or untagged peptide adsorbed to a substrate can be quantified using an adsorption assay that detects the peptide via UV spectrophotometry [47]. Additionally, a fluorescein isothiocyanate tag is a common tag that can be added during or after peptide synthesis. Radiolabeling tyrosine is an alternative to fluorescently tagging a peptide that can also provide quantitative results [119]. Less commonly used peptide quantification methods include x-ray photoelectron spectroscopy and amino acid analysis [53, 120]. Once adsorbed peptide quantities are established, cell functions can be tested using various cell attachment assays [99, 121–123].

Synthetic peptide can also be useful in performing competitive binding studies between either phage and peptides or untagged and tagged peptides, to provide evidence that peptide binding to material, cell, or tissue is being established [93, 95, 101, 104, 117, 124]. Finally, computational modeling of identified peptide sequences on a substrate of interest, with or without water, is another tool useful in determining the utility of the phage data sets [47, 94, 125, 126].

6.4. Phage Display on Apatite-Based Mineral

A complete set of sequences known to selectively adhere to each of the variety of biomaterials used in bone tissue engineering would provide the opportunity to ~~functionalize~~ the material surfaces. An appropriately functionalized material surface could minimize

inflammation, deliver biological molecules, and direct tissue growth when implanted in vivo. To achieve this, combinatorial phage display technology has been utilized to elucidate specific sequences or sequence patterns that adhere to biomaterials of interest in orthopedic and dental applications.

Since the osteoconductivity of a bone implant material is improved if the material is either made or coated with an apatite-based material [25, 127], it is of interest to design peptide sequences with preferential adsorption to apatite. Compared with polymer-based materials, apatite-based materials are less ~~tolerant~~ to surface modification required for covalent molecular attachment; for this reason, these materials must rely on their inherent material properties to achieve consistent, ionic adhesion of peptides. Post-translational modifications and poly-acidic peptide strings are two strategies that have been used to encourage peptide adhesion to apatite-based materials [62, 65]. Phage display technology can be utilized to identify 12-mer peptide sequences with preferential binding abilities to apatite-based materials, including bone-like mineral (BLM) (Figure 6.3). It has also been suggested that 7-mer peptide sequences have been identified on hydroxyapatite substrates, but limited information on either the substrate preparation or pertinent properties is reported [128, 129]. Another study identified the peptide SVSVGMKPSPRP as having high selectivity toward hydroxyapatite and tooth enamel [117].

The limited ~~discovery of~~ peptides that selectively adhere to apatite led the pursuit of sequences that preferentially bind to BLM. Since B~~ML~~ is a synthetic form of carbonated apatite fabricated at benign physiologic conditions (pH = 6.8; 37°C), it is capable of biomolecular incorporation and positively influences cell spreading [24, 39, 43], making it an

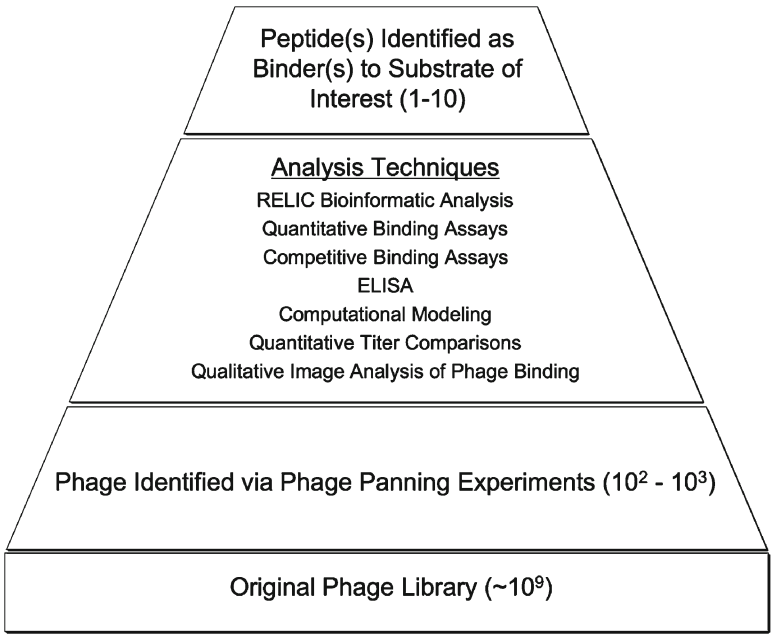

Figure 6.3. Representation of the steps utilized to identify peptide sequences with preferential adsorption to substrates via phage display. The analysis techniques listed are used to identify a small number of peptides from the hundreds of phage sequences identified after multiple rounds of phage panning.

important apatite-based substrate to investigate. Specifically, compared with bone sialoprotein-derived peptide E_7PRGDT, the sequence VTKHLNQISQSY was identified as having increased ability to bind to BLM and hydroxyapatite [47].

Understanding the conformation of peptides and proteins adsorbed to a material surface is imperative to control the presentation of cell adhesion sequences. Therefore, links between surface adsorption density, free peptide in solution, and peptide conformation must be established. For example, an optimal peptide surface density of $\rho \geq 0.62$ pmol/cm^2 has been reported for a bone sialoprotein-derived peptide covalently attached to quartz [54]. As another example, adsorption of proteins onto hydroxyapatite occurs preferentially on certain mineral faces, specifically the (100) ~~surface~~ [130]. Effects of peptide density and peptide conformation on subsequent cell adhesion have not been studied on mineral surfaces; such information is, however, critical in presenting concentrations and appropriate sequences that will promote cellular responses and functions pertinent to new tissue formation.

Phage display techniques can also be utilized to identify peptide sequences that bind to mineral deposits formed in vivo, such as in atherosclerotic plaques. Identifying peptides that home to calcified plaques in cardiovascular tissue can prove effective in delivering target drugs to the diseased areas. The peptide CRKRLDRNC has been reported as an atherosclerotic plaque-homing peptide that interacts with the ~~interleukin (IL) 4~~ receptor on endothelial cells, macrophages, and smooth muscle cells [131]. Attachment of nanoparticles acting as both drug delivery carriers and imaging probes could advance in vivo imaging of atherosclerotic lesions, as well as ~~having~~ therapeutic benefits. Other pathological mineral systems known to develop in vivo that could potentially benefit from this approach include kidney stones, gallstones, and dental calculus.

Phage display experiments are not limited to ceramics and have been used to identify peptide sequences on polymer substrates. An advantage of most polymer substrates is their modifiable surface chemistry that can be altered to covalently link drugs or therapeutic molecules. However, the polymer polypyrrole, mainly used in neural tissue engineering, does not have a surface group capable of modification; as a result, polypyrrole is a good candidate for surface modification via peptides. A 12-mer phage display library run on polypyrrole isolated the peptide, THRTSTLDYFVI, as a positive binder to polypyrrole that increased adhesion of PC12 cells when the identified peptide was conjugated with an RGD sequence [81].

6.5. Phage Display on Cells and the Role of Dual-Functioning Peptides

In order to improve guided tissue formation using peptides, the peptides identified to selectively bind to a material of interest need to be functionalized with a cell-instructive sequence that will promote appropriate cell function for the desired cell lineage needed. This approach encourages the idea of a dual-functioning peptide where one portion of the peptide attaches to the material and the other portion controls cell function, particularly cell adhesion. The usefulness of phage display in cell adhesion has been proven through the isolation of integrin receptor sequences that bind to RGD [99]. Understanding the steps involved in cell adhesion is imperative when trying to modulate function of anchorage-dependent cells using peptides. Cell adhesion progresses in four steps: cell attachment, cell spreading, production of organized actin filaments, and focal adhesion formation [132]. The importance of cell adhesion has been demonstrated in a variety of tissues including the differentiation of myoblasts [133], ~~terminal~~ keratinocytes [134], mammary epithelium [135], and osteoblasts [136]. Since

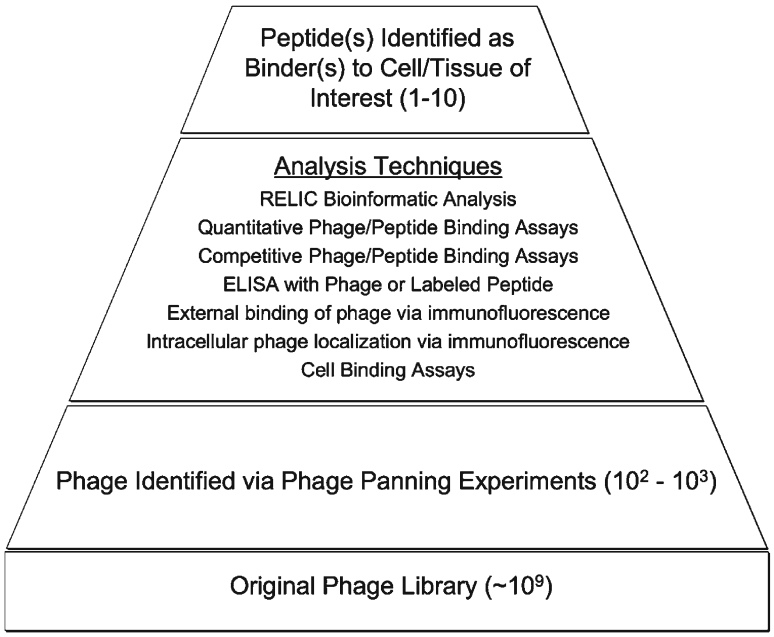

Figure 6.4. Representation of the steps utilized to identify peptide sequences with preferential adsorption to cell populations via phage display. The analysis techniques listed are used to identify a small number of peptides from the hundreds of phage sequences identified after multiple rounds of phage panning.

cell attachment is the first step of adhesion, dual-functioning peptides can aim to improve attachment of cells to the biomaterial. In the process of bone and other tissue repair, establishing cell attachment is essential, as subsequent phenotypic differentiation cannot occur if anchorage-dependent cells do not attach. Before a dual-functioning peptide can be constructed, both ends of the peptide need to be identified; phage display techniques, therefore, can be utilized on cells and tissues to identify appropriate peptide sequences (Figure 6.4).

The cell sequence can be identified either by panning against an in vitro population of cells, or by injecting the phage library in vivo and harvesting target organs. For example, the 7-mer peptide, STFTKSP, was identified via in vivo phage display panning to possess the specific ability to home ~~specifically~~ primitive hematopoietic progenitor cells ~~to~~ bone marrow [98]. For cartilage, the peptide sequence, WYRGRL, was found to bind to collagen IIα1 after biopanning against denuded cartilage. Functionalizing such peptides could provide a therapeutic approach in targeting avascular tissue [93]. In another example, phage display was able to identify the peptide, ASSLINA, as a muscle-specific sequence by panning against murine C2C12 myotubes both in vitro and in vivo ~~displaying the ability to bind~~ to both skeletal and cardiac tissue [97]. Phage display was also used to isolate the cyclic sequence, CAGALCY, that targets brain tissue [94]; most importantly, this study demonstrated that this sequence inhibits platelet adhesion to the brain microvasculature.

Ongoing work to identify peptide sequences that adhere to clonally derived human bone marrow stromal cells could benefit dual function peptide design for guided bone regeneration. Such an identified sequence can be attached to an apatite-binding sequence, providing an effective peptide that can increase initial cell attachment, the imperative first step in anchorage-dependent cell viability and function.

Identification of specific substrate-binding moieties has led to the development of dual-functioning peptides for a diverse range of applications, including digital printing. For this purpose, peptide elements are binding domains for a pigment (cellulose) and for a printing surface (carbon black). Incorporation of a hydrophilic linker in between the binding domains isolates the two-functional elements, allowing dual binding activity [78]. Another use of dual peptides is in drug delivery. By combining peptide motifs that have affinity to the surfaces of CN with peptides that can target specific organs, the CN can be functionalized, leading to CN drug carriers [92]. For cytophobic coatings, amphiphilic macromolecules containing a polystyrene-adherent peptide domain and a cell-repellent PEG domain have been designed [116]. Moreover, a dual-functioning peptide was formulated by linking a phage, with high binding capability, to the electrically conductive polymer PPyCl, to a cell-adhesive sequence to promote cell attachment to PPyCl [81].

6.6. Advancing Phage Display in Biomaterials Research – Summary

Developing multifunctioning biomolecules is necessary in biomaterials design if we are to better mimic the ability of nature to fabricate intricate tissues in situations that require either repair or restoration. In this chapter, we present phage display as a lesser-used discovery technique that can potentially identify useful peptide sequences to be used in drug targeting, establishing a union between two substrates, and increasing initial cell attachment on a biomaterial prior to implantation. Furthermore, identification of such peptides could lead to a better understanding of the behavior of biomolecules, including proteins, during tissue growth, disease, and repair. In order to capitalize on the powerful phage display technique, multiple bioinformatics, experimental, and analytical tools in addition to characterization techniques should be implemented in tandem with phage display.

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Author Queries

Chapter No.: 6 0000898293

Queries	Details Required	Author's Response
AU1	Is the PPyCl definition correct as edited to match the text definition?	
AU2	Please check the place of insertion of citation of Table 6.1	
AU3	Correct as edited: "collagen 11a1"?	
AU4	"specifically the (100) surface"?	
AU5	Correct definition for IL-4?	
AU6	"peptide elements are binding domains for a pigment (cellulose)"? The word "are" was not present in the original text.	