Chapter

Targeting Peptides Derived from Phage Display for Clinical Imaging

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Abstract

Phage display is a high-throughput technology used to identify peptides or proteins with high and specific binding affinities to a target, which is usually a protein biomarker or therapeutic receptor. In general, this technique allows peptides with a particular sequence to be presented on a phage particle. Peptides derived from phage display play an important role in drug discovery, drug delivery, cancer imaging, and treatment. Phage peptides themselves can act as sole therapeutics, for example, drugs, gene therapeutic, and immunotherapeutic agents that are comprehensively described elsewhere. In this chapter, we discuss phage selection and screening procedures in detail including some modifications to reduce nonspecific binding. In addition, the rationale for discovery and utilization of phage peptides as molecular imaging probes is focused upon. Molecular imaging is a new paradigm that uses advanced imaging instruments integrated with specific molecular imaging probes. Applications include monitoring of metabolic and molecular functions, therapeutic response, and drug efficacy, as well as early cancer detection, personalized medicine, and image-guided therapy.

Keywords: peptides, membrane receptors, imaging, phage display, endoscopy

1. Introduction

One of the most important practices in modern era clinical imaging is imaging at the molecular level which can help characterize and measure in vivo biological processes at the cellular level [1]. Thus, the technique provides unambiguous and high-resolution real-time information for disease diagnoses and therapies. In addition, molecular imaging is usually noninvasive as a biologically active, receptor-specific, targeting vector conjugated to a radioligand, nanoparticle, and/or fluorescent/magnetic resonance imaging (MRI) probe is administered first, and then the probe signals can be quantified by positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), fluorescence imaging, or ultrasound. The specificity of probes may be contributed from targeting peptides, small proteins, and antibodies linked to the probes [2].

One of the most important aspects in successful molecular imaging is the development of imaging probes. Initial efforts focused on probes that are radiolabeled small molecules or macromolecules, e.g., monoclonal antibodies and their fragments [3]. Most such probes are unsuccessful because small molecules provide low specificity, whereas the antibodies have low target permeability. Taken into account altogether, these probes have low contrast between target tissues and background, leading to poor imaging qualities. Compared to small molecules and antibodies,

peptide imaging probes are more promising. The peptide length varies from several to approximately 50 amino acids [4]; thus they are usually more specific than small molecules and also more permeable than antibodies. The peptides have high capillary permeability, which allows efficient penetration into tissues. In addition, they also have high uptake rates in the target and rapid clearance from blood [1]. These distinctive advantages facilitate peptides as popular imaging probes. Such a probe is usually composed of a targeting peptide, a linker, and an imaging moiety. The linkers commonly are organic spacers, macrocyclic or branched chelators, and polymers, which link peptides with appropriate moieties. Different moieties render the probes observable by various devices, e.g., near-infrared (NIR) fluorescent dyes or quantum dots for optical imaging, radionuclides for PET or SPECT, and paramagnetic agents for MRI.

Phage display technology is a powerful approach to screen for peptides with high affinities and specificities to biomarkers. This technology was established by Smith et al. in 1985 to display polypeptides on the surface of filamentous M13-derived bacteriophage (phage) [5]. This technique modifies the phage genome to fuse the deoxyribonucleic acid (DNA) encoding a peptide to a gene encoding a protein comprising the phage coat; thus the peptide appears on the surface of the phage. In this way, each phage contains a single-peptide variant and its encoding DNA sequence, thus retaining a genotype-phenotype linkage. A library or pool of phages normally contains 10^9–10^{11} peptide variants for screening. The selection procedure consists of three main steps: (1) panning the pool of phages on the immobilized biomarker, (2) removing unbound phages by washing, and (3) elution of bound phages. After several rounds of such selections, the peptide sequences with high affinities to the biomarker are determined by sequencing the encoding DNAs in the phages. Specificity of the peptides can be also improved by adding extra negative selection steps [6].

Since its inception nearly 45 years ago, phage display has been widely used in thousands of research papers to isolate peptides that bind various targets [7]. The phage peptides are labeled with imaging agents such as radioactives, fluorescences, and nanoparticles. These probes have been successfully used to image tumors and cancers [8–10]. Moreover, phage display also influenced many other scientific fields such as drug discovery, vaccine development, and targeted drug delivery and gene therapy. With advances in molecular biology, the number of disease-associated biomarkers at the molecular level is ever-increasing. These new discoveries are motivating the applications of phage display to diagnostic imaging and targeted drug delivery.

2. Phage biology and phage selection screening methods

2.1 Phage biology

Bacteriophages (or phages) are viruses that infect bacteria. Phage virions vary widely in size, shape, and complexity, and phage genomes range in size from 3.4 kb to almost 500 kb [11]. Most phage genomes (>95%) discovered to date are linear, double-stranded DNAs (dsDNAs), but there are also genomes that are single-stranded DNAs (ssDNA), circular DNAs, and RNAs [12]. The phage genome is packed into a protein capsid, which together form a phage particle. Some phages, such as pleomorphic phages, are further covered by bacterial lipoprotein membrane during budding [13]. As a parasite, the phage life cycle is intertwined with that of the host cell, i.e., bacteria. The phage particle first attaches itself to a host cell by specific recognition of a receptor or other surface moiety of the host; then the phage

nucleic acids are inserted into the host cell. Inside the host cell, the phage genome shuts down defense mechanisms of the host and hijacks host cellular components to replicate phage genome and express capsid genes from the phage genome. Eventually, phage genomes and capsids are assembled into progeny phage particles. These phage particles emerge from the host cell, which usually results in cell lysis by phage proteins (**Figure 1**).

Phages were discovered by Twort in 1915 and d'Hérelle in 1917 [12], respectively. Now, it is clear that phages are the most abundant organisms on the earth; they have been found in every environment with bacteria. An estimated 10^{31} phage particles exist on earth. Since the 1940s, phages have been model organisms and have contributed to molecular biology substantially. The most remarkable contributions include revealing the random nature of mutation, the discovery of DNA as the genetic material, and the understanding of gene expression control. With advances in biotechnology, phage display was established by Smith in 1985.

In phage display, the most widely used phage is the M13 strain of filamentous bacteriophage. This type of phage infects F plasmid-containing gram-negative bacteria, such as *Escherichia coli*. Besides M13, other members of filamentous phages such as f1 and fd strains have also been used for phage display [14]. These phages have circular single-stranded DNA (ssDNA). The genome is composed of 11 genes [15], which are classified into three groups by functions. The first group comprises

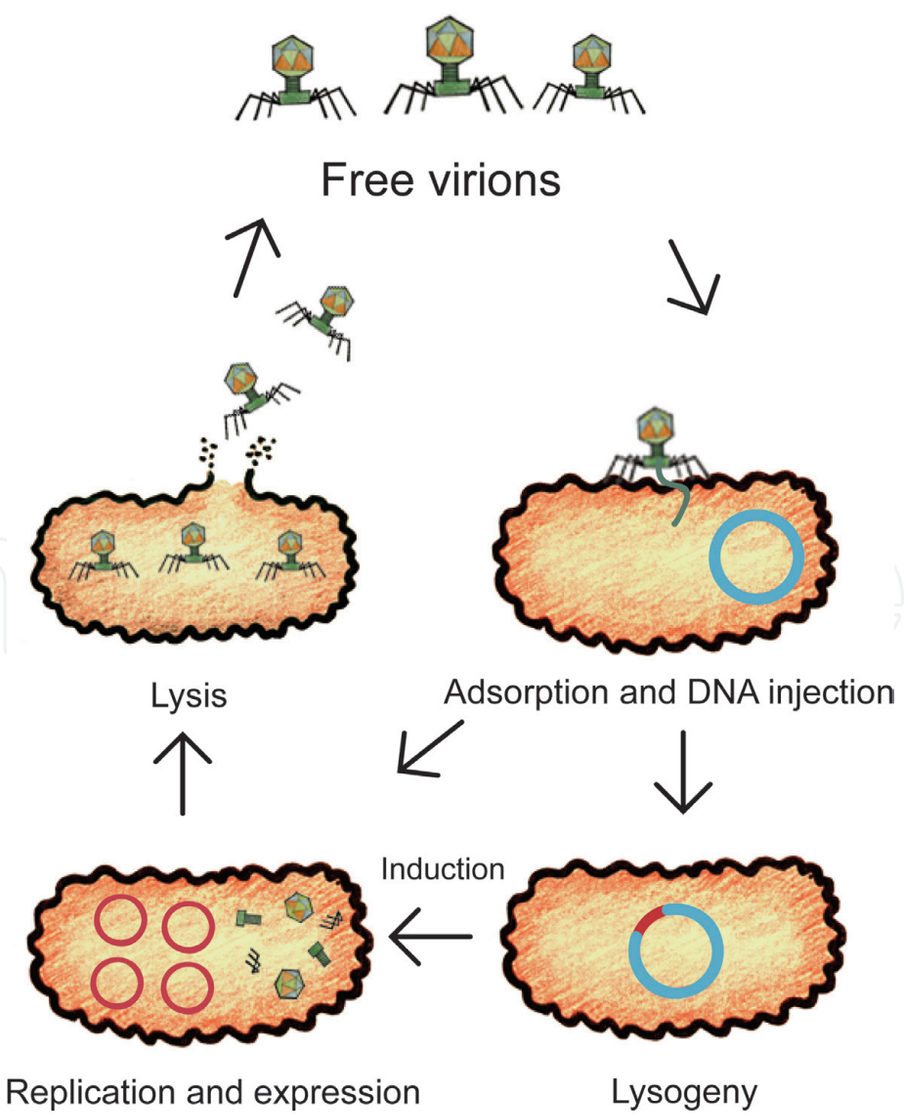

Figure 1.
A scheme presents lytic and lysogenic stages of phage.

capsid proteins: protein III (pIII), pVI, pVII, pVIII, and pIX; the second group is for DNA replication, pII, pV, and pX; the last group consists of proteins for assembly, pI, pIV, and pXI. This genome is contained in a protein coat to form a phage particle of 6.5 nm in width and ~900 nm in length. Normally, filamentous phage is not lytic; thus phages are released from bacteria without bacterial lysis. Instead of phage M13, phage display can also use phagemid, which is simply a plasmid with a phage origin of replication so that the plasmid can be replicated and packaged into phage particles. The phagemid as a cloning vector needs helper phage to complete its infection process and virion packaging [16].

2.2 Phage selection screening methods

2.2.1 Peptide library

Peptide library construction is the first step in selecting peptides with high affinities to the target of interest. Each of the 20 amino acids is encoded by codons, and each codon consists of three consecutive nucleotides. There are four types of nucleotides, denoted as A (adenine), G (guanine), C (cytosine), and T (thymine). A random peptide is constructed by synthesizing an oligonucleotide containing $(NNK)_n$, where N stands for any of the four nucleotides, K stands for either G or T, and n indicates the desired length of the peptide. Note that only G or T is introduced to the third position of a codon because this reduces the frequency of stop codons (NNN generates three stop codons: TAA, TGA and TAG, whereas NNK generates only one codon: TAG). To add a site with N, simply provide an equimolar mixture of A, T, G, and C, and randomly one of them is added at the end of the nucleotide chain. As for a site with K, just provide a mixture of G and T to the reaction [17]. In this way, numerous oligonucleotides are synthesized in parallel, and each oligonucleotide encodes a random peptide. Note that the NNK codon pattern is generated by controlling nucleotide types to be added in reactions. This simple rule can be modified to create particular codons, e.g., allowing no stop codon or creating codons of charged residues.

Peptide libraries often have short peptide lengths, approximately less than 50 residues. The optimum length required for the randomized displayed peptide is often unknown before selection and varies with many factors including the folding properties of the displayed peptide and the target of interest. For a library of random peptides with seven residues, the maximum number of different peptides is 20^7. However, this number is usually unapproachable due to codon degeneracy and early stop codon. In other words, a library usually contains redundant peptides and peptides shorter than desired. On the other hand, the capacity of selection is limited by transfection, i.e., only 10^8–10^{10} phages, each encloses one peptide, can be transformed into *E. coli* by electroporation or other techniques. Taken together, the diversity of a library is important for the success of selection and screening for high affinity peptides. A typical commercially available library archives peptide diversity at the level of 10^9 [4].

In M13 phage, the oligonucleotides encoding random peptides are mostly fused to the N-terminus of pIII, with a spacer as a linker to generate a phage library. Another widely used gene for peptide display is the one encoding pVIII. Both pIII and pVIII are the major and minor capsid proteins, accessible from the outer surface of the phage. pIII has 406 residues, and for each phage, there are in total 3–5 pIII proteins which form a knob-like structure at one end of the phage. This structure is responsible for infection of bacteria via the F-pilus, virion stability, and assembly termination. The peptides linked to pIII for display almost have no restriction on length, facilitating pIII as the mostly targeted for peptide display applications. As for pVIII, it is a short helical protein (50 residues) [18], and about 2700 pVIII molecules are present on the capsid. However, only the three residues

at the N-terminus are accessible from the outer surface of the phage. Unlike pIII, pVIII can tolerate only short peptides with less than ten residues to be linked and successfully displayed, which is likely due to interrupted assembly by long peptides. However, this problem can be alleviated by reducing the density of pVIII mutants [19]. Interestingly, some other capsid proteins such as pV1, pVII, and pIX have poorer accessibilities on the phage surface but nevertheless successfully display peptides for screening [19].

2.2.2 In vitro screening

In a phage library, each of the trillion phages displays a single variant of random peptides on its surface. Many methods have been developed to identify the peptides that have high affinities to the target of interest, e.g., a biomarker protein. To this end, biopanning is the most commonly used method, and it has many variations to improve the performance of selection and screening or to accommodate for special targets [20].

Biopanning consists of four basic steps: (i) target immobilization, (ii) phage binding, (iii) washing, and (iv) phage elution. In the first step, the target of interest is purified and immobilized on plates. Some targets are not able to maintain structural integrity after being separated from cells, e.g., some transmembrane proteins. Therefore, whole cells or engineered cells may instead be immobilized on plates or suspended in solutions in this step [21]. In phage binding, the phages in a library are incubated with the plate, and appropriate buffers are also added to facilitate the binding reactions between the displayed peptides on phages and the targets on plates. After that, the plates are washed to remove unbound phages. Phage elution is to acquire the bound phages by disrupting the interaction between the peptides and the targets. This disruption is conducted by changing pH or adding competing ligands, denaturant, or protease. The eluted phages are amplified by infecting *E. coli*. The phages may go through another round of biopanning or are subject to DNA sequencing to determine the peptide sequences (**Figure 2**). These peptides are considered to possess high affinities to the target.

Following several rounds of biopanning, the selected peptides have high affinities to the target but may not have high specificities, i.e., the peptides may also bind to nontargets with high affinities. To this end, subtractive screening can be added to the basic biopanning steps. The subtractive strategy allows phages to interact with nontargets, and thus the unbound phages are candidates that are specific to the target of interest. For example, to identify the peptides that specifically bind to esophageal cancer cells, Zhang et al. use normal human esophageal epithelial cells as the nontarget to perform subtractive screening, followed by screening against esophageal cancer cells. They repeated this procedure for three rounds and discovered two peptides that exhibited higher binding affinities and specificities for the cancer cells, which were validated by enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays, and immunohistochemistry assays [6].

Improvement of peptide library screening could be performed through small modifications. A stepwise reduction of elution buffer pH in the final round of biopanning reduces low affinity phages, thus effectively further enriching for high affinity phages. Optimized commercial kits are also available, for example, solid-phase screening, solution-sorting screening, kinetic antibody binding screening, and capture-sandwich ELISA screening [22, 23].

2.2.3 In vivo screening

In vivo screening of phage display is designed to isolate tissue-specific binders in living animals. Unlike in vitro screening, this in vivo approach considers the

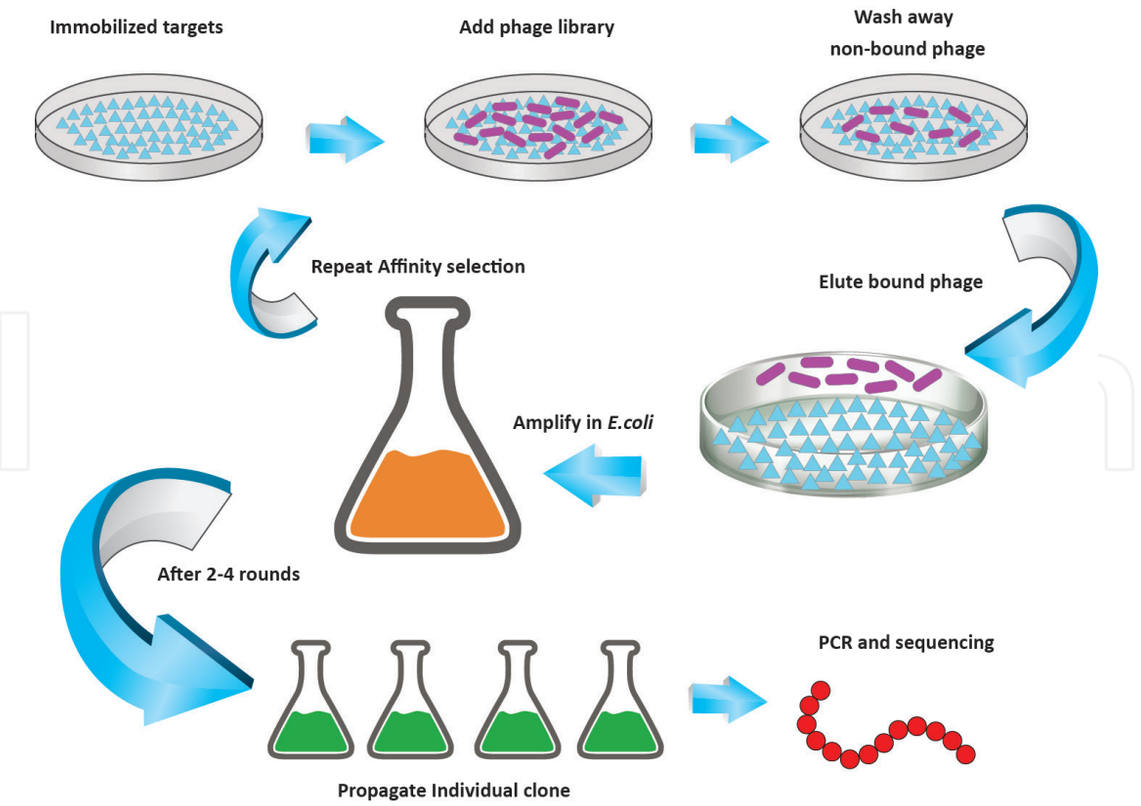

Figure 2.
General scheme of affinity selection of target-specific peptides.

complexity and heterogeneity of the living organism and thus is one step closer to clinical applications. The phage library is administered directly into a living animal and allowed to circulate for a period of time, and then the animal is sacrificed with the desired organ extracted and homogenized in saline. The lysates or pelleted cells of the organ are used to infect *E. coli* so that the phages with high-affinity binding peptides are amplified. This procedure is repeated several rounds, and the resultant phages are sequenced to determine the binding peptide sequences. Similar to in vitro screening, in vivo screening can have a step to wash away unbound phages. It is achieved usually by perfusion of the left ventricle of the animal with saline [24].

In vivo screening faces a more complicated environment than in vitro screening and thus requires extra considerations. Filamentous bacteriophages are often used for in vitro screening; however these viral particles are quite long (>500 nm) and thus may have problems during extravasation to some tissues. For example, M13 phage cannot be used to target liver parenchymal cells due to the impermeability problem [25]. Instead, T7 bacteriophage is used to identify peptides targeting livers because it is smaller in size. An even more challenging issue for in vivo phages is how to avoid immune surveillance for accurate phage displays. Host immune system, particularly reticuloendothelial system, degrades phages quickly and increases nonspecific uptake in the liver and spleen. As expected, the severity of these phenomena reduces within immune-compromised nude and SCID mouse strains. Glycosylation and succinylation to wild-type M13 phages substantially reduce its half-life in murine bloodstream from 4.5 hours to 18 and 1.5 minutes, respectively [26]. This indicates that phages modified to display peptides should have a similarly short half-life, which is consistent with existing data. Therefore, the optimal time of phage library circulation needs to be determined before the actual in vivo phage display experiments. The time varies with phage modifications and targeted tissues.

Phage library administration is another concern for in vivo screening. Administration approaches determine circulation routes of phages in living animals.

The most widely used approach is intravenous delivery. It enables rapid exposure of the phages to vascular receptors of any organ or tissue. However, this approach is inappropriate to the discovery of peptides targeting brain tissues due to the blood-brain barrier. To this end, Wan et al. administered phage library intranasally and identified a peptide targeting the brain, which performs 50-fold better than random control peptides. Other "bloodless" approaches including site-directed phage administrations and transdermal delivery have been used successfully to identify targeting peptides [27].

3. General considerations for phage display to target membrane receptors

Membrane proteins are the most popular targets for diagnostic and therapeutic applications. The structures of membrane proteins are generally composed of three parts, i.e., the extracellular, transmembrane, and intracellular domains. The extracellular domains are the primary targets of drug discovery and diagnosis. About 60% of drug targets are membrane proteins [28]. More specifically, membrane proteins are targeted by the 61% monoclonal antibodies approved or under review as therapeutic drugs throughout Europe and the United States. This prevalence is all attributed to the unique properties of membrane proteins. First, they have various important biological functions such as in signaling and cell channels; thus drugs targeting them can manipulate cellular functions effectively. Second, membrane proteins are presented on cell surface and thus are more accessible than cellular proteins. Some membrane proteins can define cell types. For example, various cluster of differentiation (CD) markers are membrane proteins and define immune cell types. More importantly, diseases usually alter expression levels of membrane proteins. A well-known example is the overexpression of human epidermal growth factor receptor 2 (HER2) membrane protein in 20-25% of breast cancers.

Despite being popular targets, membrane proteins present a limitation for phage display. The key to a successful phage display is the presentation of correctly folded targets, i.e., native structures of extracellular domains (ectodomains) in membrane proteins. This is fairly easy for soluble proteins because they fold correctly in solution while being immobilized on a surface, whereas membrane proteins usually fail to fold into their native structure in solution without membranes [29]. For example, G protein-coupled receptors (GPCRs) constitute the largest class of drug targets but have had limited success in phage display due to their hydrophobic regions and complicated ectodomains, which are usually comprised of the N-terminal chain, parts of the seven transmembrane helices, and their connecting loops. To stabilize the membrane proteins, several methods have been developed. The principle of this method is to engineer the membrane proteins by mutations or adding hydrophilic domains so that they maintain stability in solution for phage display. Note that such engineering must not interfere with the native fold of the ectodomain of membrane proteins.

Another way to circumvent the limitation of unstable membrane proteins in solution is directly biopanning on whole cells instead of immobilizing membrane proteins on a surface as targets for phage display screening. Although this whole cell panning provides membrane proteins in native state as screening targets, it causes extra difficulties. First, the membrane protein may be present at low density on the cell surface; thus other nontargeted membrane proteins generate a high background noise. Second, other components on the surface of cells, such as sugar or lipid polymers, may sequester phage particles for nonspecific uptake into a cell. Taken together, both of these possibilities facilitate the selection of nonspecific ligands, rendering phage display inefficient. To address these newly introduced problems, Jones et al. have reported a modified phage display screening for antibodies. The key innovation is that the cell is

transfected so that the target membrane protein and green fluorescent protein (GFP) are highly expressed simultaneously. For phage display, the green fluorescence provides a means to select only the cells with the target membrane protein highly expressed on cell surface using fluorescence-activated cell sorting (FACS) [29]. In other steps of phage display, small but nontrivial modifications are also adopted to improve or overcome the problems of using whole cells. For example, a low pH wash is found effective in removing phage that is present through nonantibody binding. Details of these many small modifications in phage display protocols are reviewed by Alfaleh et al. [30].

A third category of methods has also been developed to create cell membrane-like microenvironments that preserve the stability and integrity of membrane proteins for phage display. In the first method, the membrane protein is purified in the presence of detergents, which forms micelles after exceeding their critical micellar concentration. These micelles mimic cell membrane and have been used in phage display to identify antibodies to the sodium-citrate cotransporter and the fluoride ion channel. However, detergent micelles themselves are unstable and heterogeneous in size, which may cause membrane proteins to unfold or aggregate. In another method, the membrane protein is inserted into a liposome, which is a bilayer structure that more closely mimics the cell membrane than detergent micelles. Recently, nanodiscs and virus-like particles (VLP) have been developed to mimic host membrane protein. Nanodiscs are macromolecular structures that spontaneously assemble when lipids and apolipoprotein A1 or B are mixed. In general, one nanodisc structure contains a lipid bilayer of ~1000 phospholipids bundled by two apolipoproteins. The diameter of a nanodisc is ~10 nm and thus houses only one membrane protein approximately. Nanodiscs can take in a membrane protein through coupled in vitro transcription-translation. A VLP contains viral capsid proteins, lipids, and membrane proteins on its surface, but not a viral genome. It is much more stable than native cells, micelles, and liposomes and thus can withstand wash buffers with detergents, which decreases nonspecific binding in phage display.

In summary, some simple ectodomains of membrane proteins can be directly immobilized on plastic surfaces and treated as soluble proteins in phage display, whereas some other membrane proteins need to be engineered to increase ectodomain stability and integrity before phage display. Various nanoparticles such as detergent micelle, nanodisc, and VLP have been developed to create membrane-like environments to house membrane proteins. Panning directly on wild-type cells or on engineered cells, e.g., with membrane proteins highly expressed, is another way to represent membrane proteins for phage display. Such methods have to manage high background noise from cells. However, these methods have a unique advantage, i.e., consistent with native binding mechanisms. Many membrane receptors, e.g., cytokine receptors, exert their biological functions through ligand binding and dimerization. The ligand may interact with the monomer first and then mediate dimerization for function or bind to the dimer directly and then carry out its function. These two scenarios may both occur depending on the ligand concentrations [31]. Therefore, in those methods without whole cells, the monomer immobilized in phage display may not mimic the targets in vivo, and thus the ligand selected by phage display may not bind to in vivo receptors with high affinities.

4. Phage display provides potential therapeutic and diagnostic agents

Peptide phage display has played an important role in the development of clinically useful therapeutics and diagnostic agents. Peptide-based therapeutics have attracted a significant level of interest in the drug discovery and development industry. First, phage particles themselves can be used as the therapeutic agent. For

example, the M13 bacteriophage was used successfully to treat a bacterial infection by delivering DNA encoding for bactericidal toxin proteins [32].

Second, peptides derived from phage display can be used as therapeutic drugs. In 2017, peptide drug annual market was approximately $300–500 million and is estimated to increase 25% each year [33]. Compared to proteins and antibodies, peptides have numerous advantages, for example, low manufacturing costs, better activity and stability, negligible immunogenicity, and superior organ penetration. A number of peptide drugs developed from phage display technique have been approved or are currently in clinical trials. For example, DX-890, an inhibitor of human neutrophil elastase, with potential application in the treatment of pulmonary diseases such as cystic fibrosis and chronic obstructive pulmonary disease, was originated from phage display [34]. Ecallantide, a highly potent inhibitor of human plasma kallikrein, has been approved by the US Food and Drug Administration for the treatment of acute hereditary angioedema [35].

Filamentous phage has also been used as an immunogenic carrier useful in vaccine development, with high immunogenicity, low production cost, and high stability. In addition, phage can also act as a gene-delivery vehicle. For example, phage can deliver functional genes to mammalian cells through receptor-mediated endocytosis.

Phage-derived peptides that bind protein targets with high affinity and specificity can be used as molecular imaging probes. The classic example is octreotide, an eight amino acid cyclized peptide that binds the somatostatin receptor. ^{111}In-DTPA-octreotide (OctreoScan®) has been used successfully to image somatostatin receptor-positive tumors in humans [36].

5. Applications of targeting peptides derived from phages in clinical imaging

Targeted molecular imaging of disease processes, particularly tumor growth and metastasis, has been a focus of many investigations recently. Molecular imaging probes have assisted in the understanding of fundamental biological processes, disease pathologies, as well as pharmaceutical development. Enormous progress has been made in both discovery of imaging probes and development of imaging instruments. Additionally, optical imaging methods provide many advantages over other imaging modalities that include high sensitivity, the use of nonradioactive materials, and safe detection using readily available instruments at moderate cost. Today, in vivo imaging can be applied at preclinical and clinical settings due to significant improvements in engineering technologies, optical systems, and advanced imaging instruments. These technologies in a combination with cutting-edge optical imaging probes provide noninvasive, real-time imaging at macroscopic and cellular levels. Indeed, the combination of numerous NIR probes and targeted ligands, such as antibodies, aptamers, and engineered peptides, has significantly enhanced the performance of optical imaging systems. Recent progress in clinical imaging and the utilization of phage-derived targeting peptides are reviewed below.

5.1 Magnetic resonance imaging

MRI is a medical imaging technique used in radiology to form pictures of the anatomy and the physiological processes of the body, including in healthy and diseased tissues and organs. MRI scanners use strong magnetic fields, electric field gradients, and radio waves to generate images of the organs in the body. After a radiofrequency pulse, MRI detects the relaxation times of magnetic dipoles, such as hydrogen atoms in water and organic compounds, and generates MR signals. MRI offers spatial resolution

on the millimeter scale with simultaneous physiological and anatomical correlation. However, MRI requires long scan and postprocessing times and has relatively low sensitivity, thus requiring high doses of magnetic contrast agents.

In a reparative or reactive process, excess fibrous connective tissue (type I collagen) or fibrosis could be formed in a tissue. This is a common consequence in many chronic heart, kidney, liver, lung, or vasculature diseases. The high levels of collagen in fibrosis make it an attractive MRI target. Therefore, a collagen-specific MRI contrast agent was developed. A type I collagen-specific peptide was identified using phage display and subsequently modified to improve affinity for collagen. Conjugate EP-3533 consists of a peptide of 16 amino acids, with 3 amino acids flanking a cyclic peptide of 10 amino acids that is formed through a disulfide bond. The peptide was modified with biphenylalanine and gadolinium to improve collagen binding and sensitivity. EP-3533 was evaluated in a mouse model of aged myocardial infarction. From MR images, EP-3533 was able to enhance the collagen-rich scar, providing specific, high-contrast images compared to adjacent viable myocardium tissues and blood vessels [37].

Another example of the utilization of peptides from phage with MRI in early detection of colorectal cancer (CRC) was reported. Human gastric mucin (MUC5AC) is secreted in the colonic mucus of cancer patients. MUC5AC is a specific marker of precancerous lesions called aberrant crypt foci. MRI can detect the accumulation of MUC5AC in xenograft and mouse stomach. To enhance MRI visualization, peptides that specifically bound MUC5AC were developed using an M13 phage library. Once, the peptide binding MUC5AC (C-PSIYPLL-C, 60C) was identified; it was synthesized and conjugated to biotin and finally to ultra-small particles of iron oxide (USPIOs). The ability of USPIO-60C to detect MUC5AC in vivo was investigated on two xenograft mouse models. A heterogeneous but significant negative enhancement was observed in MUC5AC-secreting tumor postcontrast images 1 hour after intravenous USPIO-60C administration [38]. The results provided in this study supply a proof of concept that targeted contrast agents can be used to detect pathologies earlier than allowed by conventional MRI approaches or clinical assessment.

5.2 Positron emission tomography/single-photon emission computed tomography

PET and SPECT are nuclear medicine tomographic imaging techniques using gamma (γ) rays. Both techniques require the injection of a radioactive tracer. There are three main tracers used in SPECT imaging: technetium-99m, iodine-123, and iodine-131. The radioactive tracer emits gamma rays from the patient. PET and SPECT record high- and low-energy γ-rays emitted from within the body. These imaging modalities have very high sensitivity but relatively low spatial resolution.

VRPMPLQ (VQ) is a heptapeptide sequence first identified by Hsiung and colleagues by screening phage display peptide libraries against fresh human colonic adenomas [39]. In a later study, Shi and team synthesized and evaluated ^{99m}Tc-HYNIC-VQ (HYNIC 5,6-hydrazinonicotinamide) as a SPECT radiotracer for tumor imaging in five different xenograft mouse models (HT-29 human colon cancer, CL187 human colon cancer, BGC823 human gastric cancer, U87MG human glioma, and UM-SCC-22B human head and neck cancer). The images were acquired 1 and 2 hours postinjection. The tumors were clearly visualized at 1 hour postinjection with excellent target-to-background (T/B) contrast. The studies demonstrated that ^{99m}Tc-HYNIC-VQ could provide high-contrast images in different tumor models and an inflammation model [40].

In another study, a radioactive probe targeted to a dysplastic lesion in inflammatory bowel disease (IBD) or early CRC was developed. The cyclic peptide

c[Cys-Thr-Pro-Ser-Pro-Phe-Ser-His-Cys]OH (TCP-1) was originally identified in an orthotopic mouse CRC model using phage display selection [41]. TCP-1 peptide was labeled with radioisotope technetium-99 m (^{99m}Tc) and the NIR fluorophore cyanine-7 (Cy7) for molecular imaging. The in vivo images of ^{99m}Tc-TCP-1 in xenografted HCT116 and PC3 prostate cancer models were collected using dynamic or static SPECT. The ^{99m}Tc-TCP-1 or control peptides were administered via intravenous or tail vein injection. Dynamic images of ^{99m}Tc-TCP-1 in HCT116 colon cancer xenograft mice exhibited that the tumor could be detected in 15–30 minutes after injection and remained visible until 180 minutes. The data demonstrated the feasibility of TCP-1-targeted detection of colorectal tumor [42].

5.3 Photoacoustic tomography

Photoacoustic tomography (PAT) is an emerging imaging technique that demonstrates great potential for preclinical research and clinical applications. PAT is a hybrid system that detects the acoustic energy of endogenous chromophore or exogenous contrast agent optical absorption. PAT generates high-resolution images in both the optical ballistic and diffusive regimes due to less ultrasound scatter in tissue. Over the past decade, the photoacoustic technique has been developing rapidly, leading to exciting findings and applications.

Epidermal growth factor receptor (EGFR) is highly overexpressed in hepatocellular carcinoma (HCC). Therefore, it is a potential cell surface molecule for in vivo targeted imaging of HCC. A peptide specific for EGFR previously reported by Zhou and team was conjugated to Cy5.5 dye. Nude mice were injected with EGFR overexpressed human HCC cells. A 2D ultrasound scanner and MRI system were used to monitor tumor growth in the mice. Cy5.5-labeled EGFR and control peptides were injected to the mice separately. Photoacoustic images were recorded periodically for 24 hours. At 3 hours postinjection, the maximum photoacoustic signal in tumors was seen and results in high-contrast images of tumors beneath the skin. The T/B ratio was significantly different between the EGFR and control peptide. The signal was diminished by 24 hours [43]. From the data, a peptide specific for EGFR can detect HCC xenograft tumors in vivo with photoacoustic imaging.

5.4 Optical endomicroscopy

Optical imaging offers several unique advantages. Optics is nonionizing and provides resolution on the micron scale. Another advantage of optical imaging is the ability to collect images in real time in comparison to other imaging modalities, such as MRI and PET.

Endoscopes are thin, flexible instruments that provide a macroscopic view of the large mucosal surfaces in hollow organs internal to the human body. Endomicroscopy employs high numerical aperture (NA) optics to provide a small field-of-view (FOV) with micron-level resolution for observing subcellular features. It commonly requires scaling down the size of a conventional microscope design into a miniature package. Novel optical designs and scanning mechanisms have been developed to improve imaging performance for both endoscopy and endomicroscopy. These instruments provide a unique opportunity for early cancer detection and prevention by allowing biopsy or resection to be performed concurrently with diagnosis.

5.4.1 Wide-field fluorescence endoscopy

Previously, all CRCs were believed to arise from adenomas that progress through the traditional adenocarcinoma sequence. Recently, this pathway has been found

to account for approximately 60% of CRCs, and up to 35% are now attributed to the serrated pathway [44]. White-light endoscopy (WLE) that is normally used in colonoscopy is sensitive to gross morphologic abnormalities, such as polyps. Dysplasia that is flat in morphology, focal in size, and patchy in distribution appears "invisible" on conventional wide-field endoscopy. Therefore, imaging methods with improved contrast and sensitivity to molecular rather than morphological properties that could improve early detection and prevention of CRC are in needed. There are several reports on discovery and validation of targeted peptides derived from phage display for early detection of CRC [45].

One group used phage display to identify a peptide that binds to dysplastic colonic mucosa in vivo in a genetically engineered mouse model of colorectal tumorigenesis, *CPC;Apc* [46, 47]. A peptide, QPIHPNNM, was isolated after several rounds of in vivo T7 library biopanning. The peptide was synthesized, fluorescently labeled, and purified. The peptide was sprayed topically in mouse distal colon. The wide-field fluorescent videos were recorded. After quantitative image analysis, the fluorescent-labeled peptide was significantly bound twofold greater to the colonic adenomas when compared to the control peptide. The target peptide also showed minimal binding to an activated $Kras^{G12D}$ mutant mouse model that demonstrates hyperplastic polyp-like features used as a control hyperplastic model that does not progress to carcinoma (**Figure 3A**) and the lumen of a *CPC;Apc* bred mouse negative (**Figure 3B**).

c-Met overexpression has been shown to occur as an early event in colorectal adenocarcinoma. A peptide that has high affinity for the extracellular domain of human c-Met was discovered using an M-13 phage display library. GE-137 is a water-soluble cyclic peptide (AGSCYCSGPPRFECWCYETEGT) labeled with a cyanine dye with a high affinity for human c-Met. The quantitative biodistribution, pharmacokinetics, binding specificity, and qualitative fluorescence of GE-137 were assessed in a CRC xenograft mouse model using subcutaneous injection of the c-Met-expressing human CRC cell line HT29. Intravenously administered GE-137 accumulated in the c-Met-expressing tumor xenografts and left a fluorescent signal in the tumors and kidneys 120 and 240 minutes

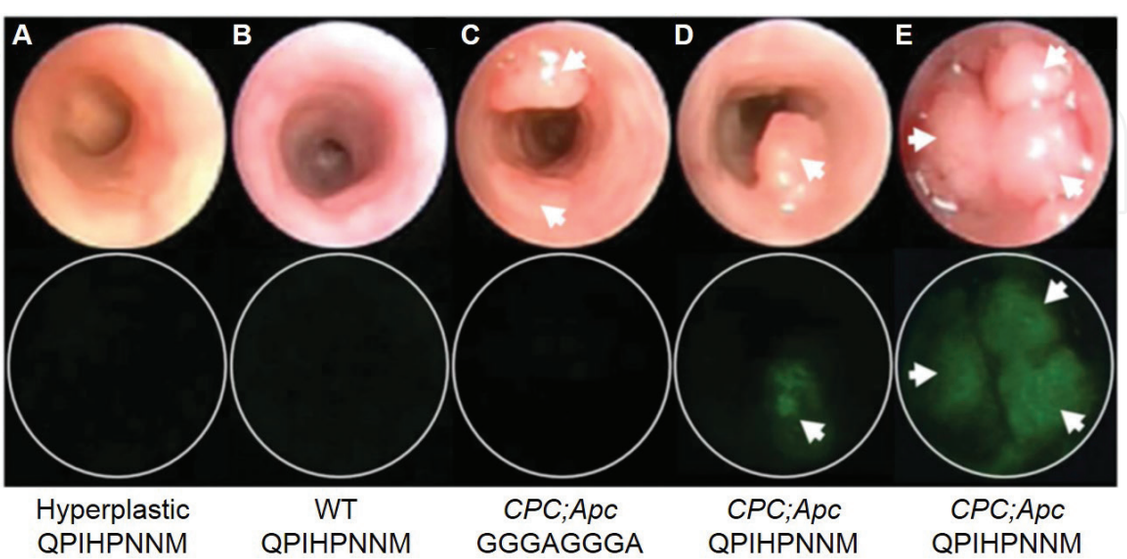

Figure 3.
Images from wide-field endoscopy videos after topical application of fluorescence-labeled peptides. The top and bottom panels represent frames from white light and fluorescence, respectively. (A) The hyperplastic epithelium after QPI peptide application, (B) the lumen of a CPC;Apc bred mouse negative for Cre recombinase (control litter mate), (C) single adenoma after control peptide application, (D) single adenoma, and (E) multiple adenomas in a CPC;Apc mouse after QPI peptide application. Used with permission [47].

after injection (**Figure 4**). In a pilot study in 15 patients at high risk of colorectal neoplasia, a total of 101 lesions were detected during first inspection with white light (WL), and an additional 22 were detected during second inspection with dual WL/fluorescence (FL). After immunohistochemical analysis, 36 hyperplastic lesions and 8 serrated polyps were identified. Most of these were visible in fluorescent mode (94 and 100%, respectively), and the majority (78 and 87%, respectively) showed increased fluorescence. From the data, GE-137 peptide showed some specificity to hyperplasia and serrated lesions in CRC mouse model and patients [48].

Overexpression of EGFR has been reported in as high as 97% of colonic adenocarcinomas, and it is a validated biomarker for CRC. In one study, the extracellular domain of EGFR (EGFR-ECD) was expressed and purified [49]. A library of M13 bacteriophage was used to select peptide candidates that bind specifically to EGFR-ECD. A peptide, QRHKPRE, that is specific for EGFR was developed and validated. Peptide binding to cells occurred within 2.46 minutes and had an affinity of 50 nM. A NIR fluorescence endoscope was used to perform *in vivo* imaging to validate peptide binding to spontaneous colonic adenomas in a *CPC;Apc* mouse model via topical administration (**Figure 5**). T/B ratios of polyps and flat lesions were 4.0 ± 1.7 and 2.7 ± 0.7, respectively. Subsequently, specific peptide binding to human colonic adenomas was assessed on immunohistochemistry and immunofluorescence. On human colonic specimens, greater intensity from peptide binding to dysplasia than normal was found with a 19.4-fold difference.

In another study, a phage-derived peptide was tested for specific binding to sessile serrated adenomas (SSAs) in proximal colon which accounts for 35% in CRC. Joshi and team used phage display to identify a peptide that binds specifically to SSAs. Many SSA cells have the V600E mutation in BRAF. Therefore, peptide selection was performed with an M13 Ph.D.-7 phage display library using a biopanning strategy with subtractive hybridization with HT29 colorectal cancer cells containing the V600E mutation in BRAF [45]. Binding of fluorescently labeled peptide, KCCFPAQ, to colorectal cancer cells was evaluated with confocal fluorescence

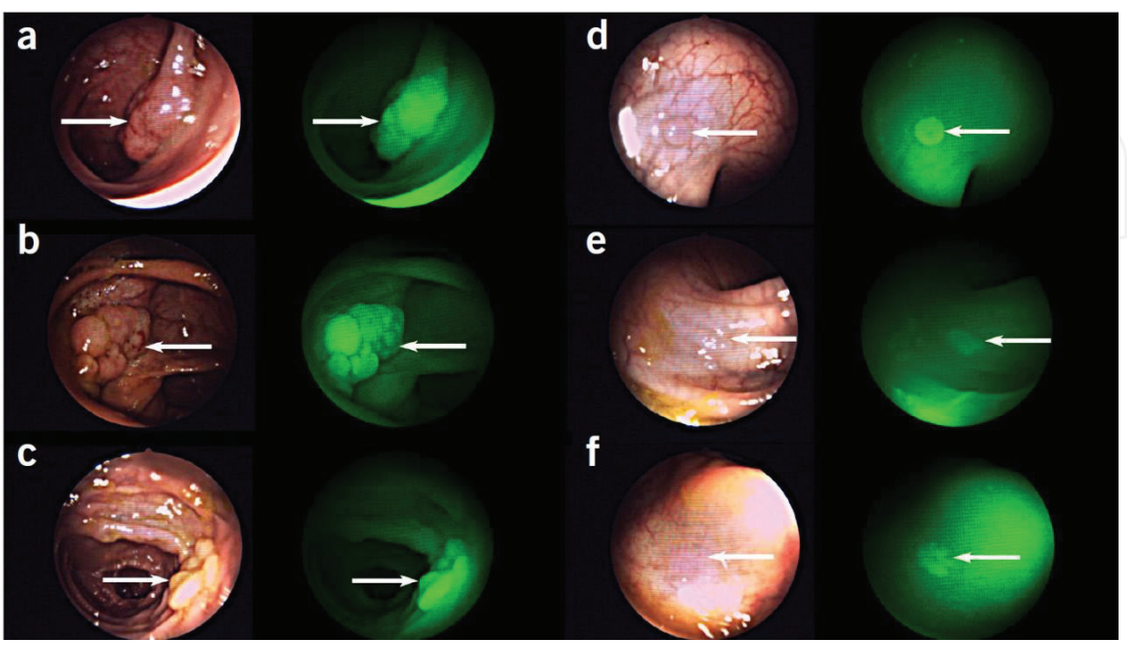

Figure 4.
WL and FL images of representative lesions. (a–c) The lesions that are visible in WL show increased fluorescence. (d) A lesion that is visible in WL has enhanced visibility in FL. (e, f) Flat lesions that were only visible in FL. Used with permission [48].

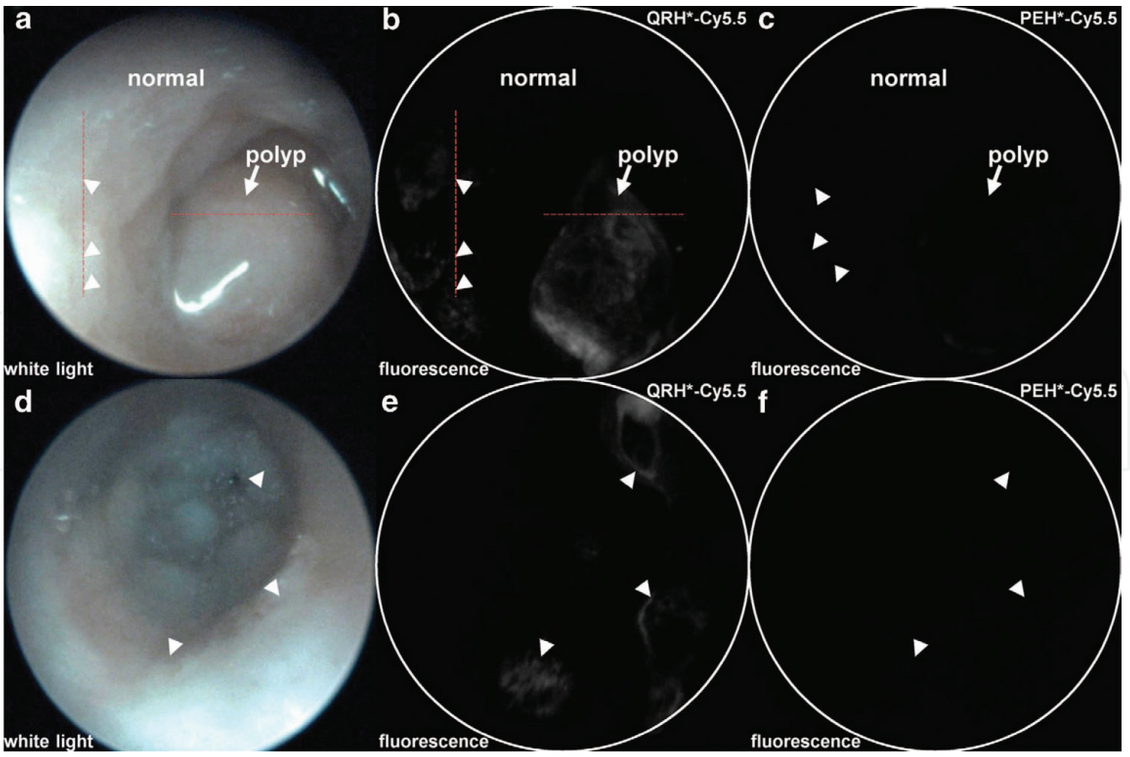

Figure 5.
In vivo imaging of colon in CPC;Apc mouse. *(a) WL image of colon in CPC;Apc mouse shows the presence of polyp (arrow). (b) NIR fluorescence image after topical administration of QRH*-Cy5.5 shows increased intensity from polyp (arrow) and several flat lesions (arrowheads). (c) Image with Cy5.5-control peptide (PEH*-Cy5.5) shows minimal signal. (d) WL image shows no grossly visible lesions (polyps). (e) NIR fluorescence image with QRH*-Cy5.5 shows the presence of flat lesions (arrowheads). (f) Image with Cy5.5-control peptide shows minimal signal. Used with permission [49].*

microscopy. The peptide showed an apparent dissociation constant of K_d = 72 nM and an apparent association time constant of K = 0.174/minute. Toxicity was also assessed in rats. In the clinical safety study, fluorescently labeled peptide was topically administered, using a spray catheter, to the proximal colon of 25 subjects undergoing routine outpatient colonoscopies. Subsequently, endoscopists resected identified lesions, which were analyzed histologically by gastrointestinal pathologists. Fluorescence intensities of SSAs were compared with those of normal colonic mucosa. During fluorescence imaging of patients during endoscopy, regions of SSA had 2.43-fold higher mean fluorescence intensity than that for normal colonic mucosa. Fluorescence labeling distinguished SSAs from normal colonic mucosa with 89% sensitivity and 92% specificity.

5.4.2 Confocal laser endomicroscopy

Confocal laser endomicroscopy (CLE) is an endoscopic modality that based on tissue illumination using a low power laser and the subsequent detection of fluorescent light that is reflected back from the tissue through a pinhole. The term "confocal" refers to the alignment of both illumination and collection systems in the same plane. This alignment dramatically increases the spatial resolution of CLE and enables cellular imaging and evaluation of tissue architecture at the focal plane. CLE can be used to guide biopsies and has been demonstrated in a number of clinical studies to detect cancer in the digestive tract, bladder, cervix, ovary, oral cavity, and lungs. CLE requires the use of a fluorescent contrast agent to enhance visualization of cells. Contrast agents can be administered intravenously or topically. Intravenous fluorescein sodium and acriflavine are widely used contrast agents.

CLE can be performed shortly following injection with its fluorescence lasting approximately 30 minutes. However, due to the lack of specificity of conventional contrast agents, there is an increasing use of tissue-specific binding molecular probes in CLE. The studies that used CLE in combination with a targeting peptide derived from phage display are summarized below.

Hsiung et al. used an M13 phage library to identify peptides that would specifically bind dysplastic colonic mucosa from fresh human colonic biopsies [39]. A peptide (VRPMPLQ) that bound to colonic dysplasia was identified, synthesized, and conjugated with fluorescein for in vivo testing in a pilot study in patients undergoing routine colonoscopy using a flexible-fibered confocal microscope. Fluorescence images and videos of bound topically administered peptide collected in vivo showed that the selected peptide bound more strongly to dysplastic colonocytes than to the adjacent normal mucosa in the same subject with 81% sensitivity and 82% specificity.

In another study, Qiu and team demonstrated vertical cross-sectional (XZ-plane) images of NIR fluorescence with a handheld dual-axis confocal endomicroscope that revealed a specific binding of a Cy5.5-labeled peptide (LTTHYKLGGGSK-Cy5.5) to premalignant colonic mucosa in mice [50]. This targeting peptide was selected using in vivo phage display technology in a *CPC;Apc* mouse model which developed adenomas spontaneously in the distal colon. NIR vertical cross-sectional fluorescence images of fresh mouse colonic mucosa demonstrate histology-like imaging performance as shown in **Figure 6**. The peptide showed specific binding and distinguished premalignant colonic mucosa from normal mucosa.

Gastric cancer vessels may have many differentiating characteristics compared to normal vessels. However, identification of gastric cancer vascular endothelial cells is difficult. Co-culture of gastric cancer cells and vascular endothelial cells was suggested to simulate gastric solid tumor mass. In a study, GEBP11, a nine amino

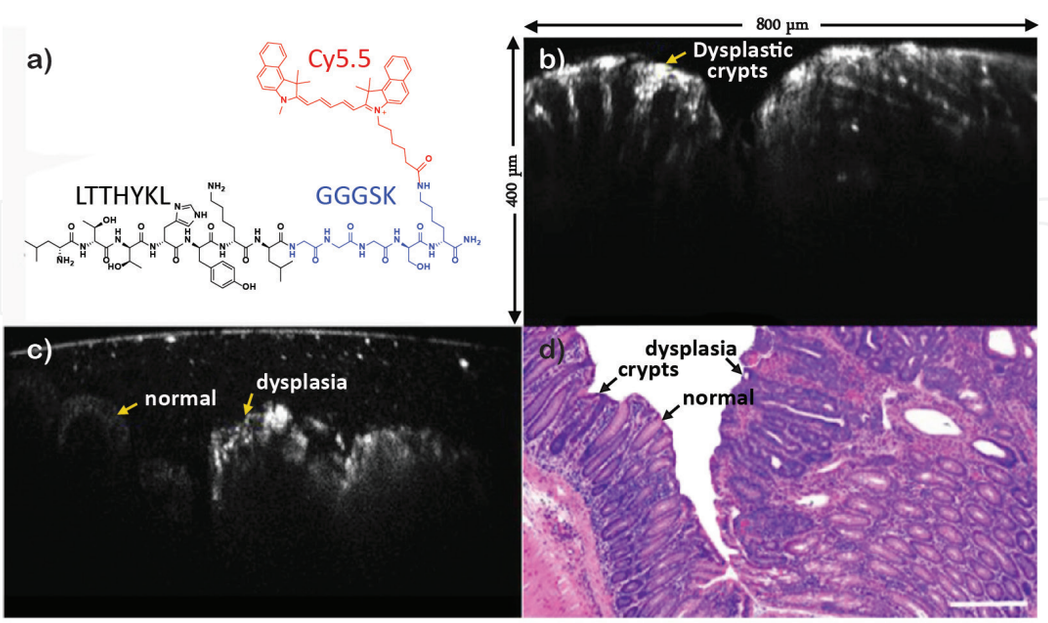

Figure 6.
Vertical cross-sectional image of colonic dysplasia. (a) Chemical structure of LTTHYKL peptide with GGGSK linker and Cy5.5 fluorophore. (b) NIR fluorescence image from CPC; Apc mouse colon ex vivo shows vertically oriented dysplastic crypts. (c) The border between normal colonic mucosa and dysplasia shows increased contrast from specific binding of the LTT-Cy5.5 peptide. (d) Corresponding histology (H&E), scale bar 200 µm. Used with permission [50].*

acid vascular homing peptide, was screened and identified using Ph.D.C7C phage display peptide library kit panning against Co-HUVECS cells [51]. Liu et al. used FITC-GEBP11 to identify gastric cancer in mouse model [52]. A whole-body fluorescent imaging was first used to screen for the strongest specific fluorescent signal in xenograft models after tail vein injection of FITC-GEBP11. A specific signal was observed in both subcutaneous and orthotopic xenograft models in vivo, whereas the group injected with FITC-URP, a control peptide, showed no fluorescent signals. In addition, neoplastic and nonneoplastic gastric mucosae obtained from the patients were incubated with FITC-GEBP11 or FITC-URP for 30 minutes and were scanned with CLE. A specific signal of GEBP11 was observed in 26/28 neoplastic human specimens and in 8/28 samples of nonneoplastic specimens ($p < 0.01$).

Sturm and team developed a peptide (ASYNYDA) that binds specifically to high-grade esophageal dysplasia and adenocarcinoma using phage display technology [53]. After peptide specific binding validation in human esophageal cancer specimens, they applied peptide topically and performed confocal endomicroscopy in 25 patients. The targeting peptide showed 3.8-fold greater fluorescence intensity for esophageal neoplasia compared with Barrett's esophagus and squamous epithelium with high sensitivity and specificity. The peptide revealed no toxicity in animals or patients.

In a pilot study, Palma and team identified dysplasia lesions in ulcerative colitis (UC) patients using CLE and a fluorescently labeled peptide [54]. A phage-derived peptide (VRPMPLQ) was synthesized and conjugated with fluorescein. Eleven suspected dysplasia specimens were collected from nine UC patients. Specimens were stained with the peptide and subsequently inspected by CLE. The CLE images were correlated to histological results from specialists. The peptide showed a different pattern on dysplastic mucosa compared to nondysplastic lesions. However, due to several restrictions of this study, further studies on larger UC patients are required for systematic validation.

ErbB2 expression in early breast cancer can predict tumor aggressiveness and clinical outcomes in patients. Up to 30% of all breast cancers express ErbB2, also known as HER2. Immunohistochemistry is commonly used to evaluate ErbB2 expression, but it has limitations due to tumor heterogeneity. Therefore, the use of a specific biomarker for ErbB2 is increasingly popular. One study used a NIR-labeled ErbB2 peptide and a handheld dual-axis CLE to detect in human xenograft breast tumors and human specimens [55]. As a result, they found significantly greater peptide binding to xenograft breast cancer in vivo and to human specimens of invasive ductal carcinoma that express ErbB2 ex vivo. From the data, a miniature dual-axis confocal fluorescence endomicroscope with ErbB2-specific peptide could be implemented to support future image-guided surgery.

5.5 Multimodality imaging

Radiation-induced pulmonary fibrosis (RIPF) is a serious side effect of radiation therapy, especially in lung and breast cancers. Computed tomography (CT) imaging is currently utilized to identify and monitor RIPF. However, anatomical change interference is a major limitation of CT. Therefore, RIPF detection and observing techniques need to be improved. Collagen accumulation is common in fibrosis. In one study, a collagen-targeting peptide was fabricated to maximize the visualization of fibrosis using fluorescence endomicroscope imaging [37]. The probe showed moderate binding ability to collagen in a fibrosis in vitro binding assay and on lung tissue specimens. The probe showed a similar binding pattern on lung specimens compared to antibody. But its sensitivity was not as good as the collagen-binding antibody.

In another study, Zhang and team evaluated the potential applicability of GEBP11 peptides in gastric cancer diagnosis and radiotherapy. They developed iodine 131-labeled GEBP11 peptides and derivatives [56]. The clinical potential of GEBP11 peptides was determined with multimodality imaging methods. Cerenkov and SPECT imaging showed significantly higher tumor uptake for ^{131}I-2PEG-$(GEBP11)_3$ trimer compared to monomer. Higher tumor accumulation and better T/B ratio of ^{131}I-2PEG-$(GEBP11)_3$ trimer were observed. Treating with ^{131}I-2PEG-$(GEBP11)_3$ trimer exhibited a significant tumor growth suppression compared to control and monomer groups. The tumor volume and vasculature decreased significantly after treatment with ^{131}I-2PEG-$(GEBP11)_3$ trimer, resulting in prolonged survival time. In addition, ^{131}I-2PEG-$(GEBP11)_3$ trimer showed no significant hepatic or renal toxicity. In conclusion, ^{131}I-2PEG-$(GEBP11)_3$ trimer could be a potential ligand used to identify gastric cancer and in antiangiogenic therapy.

6. Future prospects

The discovery and development of therapeutic drugs and diagnostic probes are a time-consuming, expensive, and complex process. The processes involve experts from a wide range of disciplines such as medicinal chemistry, biochemistry, molecular biology, medicine, and pharmacology. It has been estimated that from about 10,000 new chemical entities identified, only one will reach the market in an average time of 16 years. Phage display, and particularly peptide phage display, has played a major role in the development pipeline for bringing peptide therapeutics into the clinic. Phage-derived peptides play an important role in disease detection and therapy, including in clinical imaging. The potential of peptides in preclinical and clinical molecular imaging is tremendous. Molecular imaging offers invaluable opportunities to explore complex disease-related biological processes at the molecular level in vivo. The emergence of current molecular imaging technologies is dependent not only on the progress of imaging systems but, more importantly, also on molecular imaging probes. Peptide-based imaging has now become an established approach in nuclear imaging, and its application is expanding to other imaging modalities. Considering the emergence of novel library designs and innovative selection strategies, we are confident that phage-derived peptides will continue to be promising biomarkers for early cancer detection, in metabolic abnormalities, and in personalized medicine.

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References

[1] Lee S, Xie J, Chen X. Peptide-based probes for targeted molecular imaging. Biochemistry. 2010;**49**(7):1364-1376

[2] De Jong M et al. Tumor imaging and therapy using radiolabeled somatostatin analogues. Accounts of Chemical Research. 2009;**42**(7):873-880

[3] Wu AM, Olafsen T. Antibodies for molecular imaging of cancer. The Cancer Journal. 2008;**14**(3):191-197

[4] Derda R et al. Diversity of phage-displayed libraries of peptides during panning and amplification. Molecules. 2011;**16**(2):1776-1803

[5] Smith GP. Filamentous fusion phage: Novel expression vectors that display cloned antigens on the virion surface. Science. 1985;**228**(4705):1315-1317

[6] Zhang ZF et al. Screening and selection of peptides specific for esophageal cancer cells from a phage display peptide library. Journal of Cardiothoracic Surgery. 2014;**9**(1):76

[7] Witt H et al. Identification of a rhabdomyosarcoma targeting peptide by phage display with sequence similarities to the tumour lymphatic-homing peptide LyP-1. International Journal of Cancer. 2009;**124**(9):2026-2032

[8] Hui X et al. Specific targeting of the vasculature of gastric cancer by a new tumor-homing peptide CGNSNPKSC. Journal of Controlled Release. 2008;**131**(2):86-93

[9] Wang W et al. Near-infrared optical imaging of integrin αvβ3 in human tumor xenografts. Molecular Imaging. 2004;**3**(4):15353500200404148

[10] Ye Y et al. Design, synthesis, and evaluation of near infrared fluorescent multimeric RGD peptides for targeting tumors. Journal of Medicinal Chemistry. 2006;**49**(7):2268-2275

[11] Comeau AM et al. Exploring the prokaryotic virosphere. Research in Microbiology. 2008;**159**(5):306-313

[12] Ofir G, Sorek R. Contemporary phage biology: From classic models to new insights. Cell. 2018;**172**(6):1260-1270

[13] Drulis-Kawa Z, Majkowska-Skrobek G, Maciejewska B. Bacteriophages and phage-derived proteins, application approaches. Current Medicinal Chemistry. 2015;**22**(14):1757-1773

[14] Murphy FA et al. Virus Taxonomy: Classification and Nomenclature of Viruses. Vol. 10. New York: Springer Science & Business Media; 2012

[15] Russel M, Lowman HB, Clackson T. Introduction to phage biology and phage display. In: Phage Display: A Practical Approach. New York: Oxford University Press; 2004. pp. 1-26

[16] Barbas CF, et al. Phage Display: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press; 2001. pp. 1-24

[17] Pal G, Fellouse FA. Methods for the construction of phage-displayed libraries. In: Phage Display in Biotechnology and Drug Discovery. Florida: CRC Press; 2005. pp. 131-162

[18] Zeri AC et al. Structure of the coat protein in fd filamentous bacteriophage particles determined by solid-state NMR spectroscopy. Proceedings of the National Academy of Sciences. 2003;**100**(11):6458-6463

[19] Bratkovic T. Progress in phage display: Evolution of the technique and

its applications. Cellular and Molecular Life Sciences. 2010;**67**(5):749-767

[20] Pande J, Szewczyk MM, Grover AK. Phage display: Concept, innovations, applications and future. Biotechnology Advances. 2010;**28**(6):849-858

[21] Hamzeh-Mivehroud M, Mahmoudpour A, Dastmalchi S. Identification of new peptide ligands for epidermal growth factor receptor using phage display and computationally modeling their mode of binding. Chemical Biology & Drug Design. 2012;**79**(3):246-259

[22] Matz J, Chames P. Phage display and selections on purified antigens. In: Antibody Engineering. Heidelberg: Springer; 2012. pp. 213-224

[23] Smolarek D, Bertrand O, Czerwinski M. Variable fragments of heavy chain antibodies (VHHs): A new magic bullet molecule of medicine? Advances in Hygiene & Experimental Medicine. 2012;**66**:348-358

[24] Kolonin MG et al. Synchronous selection of homing peptides for multiple tissues by in vivo phage display. The FASEB Journal. 2006;**20**(7):979-981

[25] Ludtke JJ et al. In vivo selection and validation of liver-specific ligands using a new T7 phage peptide display system. Drug Delivery. 2007;**14**(6):357-369

[26] Molenaar TJ et al. Uptake and processing of modified bacteriophage M13 in mice: Implications for phage display. Virology. 2002;**293**(1):182-191

[27] Wu M et al. Mapping alveolar binding sites in vivo using phage peptide libraries. Gene Therapy. 2003;**10**(17):1429

[28] Arinaminpathy Y et al. Computational analysis of membrane proteins: The largest class of drug targets. Drug Discovery Today. 2009;**14**(23-24):1130-1135

[29] Jones ML et al. Targeting membrane proteins for antibody discovery using phage display. Scientific Reports. 2016;**6**:26240

[30] Alfaleh MA et al. Strategies for selecting membrane protein-specific antibodies using phage display with cell-based panning. Antibodies. 2017;**6**(3):10

[31] Schooltink H, Rose-John S. Designing cytokine variants by phage-display. Combinatorial Chemistry & High Throughput Screening. 2005;**8**(2):173-179

[32] Westwater C et al. Use of genetically engineered phage to deliver antimicrobial agents to bacteria: An alternative therapy for treatment of bacterial infections. Antimicrobial Agents and Chemotherapy. 2003;**47**(4):1301-1307

[33] Lau JL, Dunn MK. Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorganic & Medicinal Chemistry. 2017;**26**:2700-2707

[34] Rothe A, Hosse RJ, Power BE. In vitro display technologies reveal novel biopharmaceutics. The FASEB Journal. 2006;**20**(10):1599-1610

[35] Zuraw, B, Yasothan U, Kirkpatrick P. Ecallantide. Nature Reviews Drug Discovery. 2010;**9**:189-190

[36] Froidevaux S, Eberle AN. Somatostatin analogs and radiopeptides in cancer therapy. Peptide Science. 2002;**66**(3):161-183

[37] Caravan P et al. Collagen-targeted MRI contrast agent for molecular imaging of fibrosis. Angewandte Chemie International Edition. 2007;**46**(43):8171-8173

[38] Rossez Y et al. Early detection of colonic dysplasia by magnetic resonance molecular imaging with a contrast agent raised against the colon cancer marker MUC5AC. Contrast Media & Molecular Imaging. 2016;**11**(3):211-221

[39] Hsiung PL et al. Detection of colonic dysplasia in vivo using a targeted heptapeptide and confocal microendoscopy. Nature Medicine. 2008;**14**(4):454

[40] Shi J et al. Technetium 99m–labeled VQ peptide: A new imaging agent for the early detection of tumors or premalignancies. Molecular Imaging. 2013;**12**(5):7290.2012. 00047

[41] Li ZJ et al. A novel peptide specifically targeting the vasculature of orthotopic colorectal cancer for imaging detection and drug delivery. Journal of Controlled Release. 2010;**148**(3):292-302

[42] Liu Z et al. Characterization of TCP-1 probes for molecular imaging of colon cancer. Journal of Controlled Release. 2016;**239**:223-230

[43] Zhou Q et al. In vivo photoacoustic tomography of EGFR overexpressed in hepatocellular carcinoma mouse xenograft. Photoacoustics. 2016;**4**(2):43-54

[44] Snover DC. Update on the serrated pathway to colorectal carcinoma. Human Pathology. 2011;**42**(1):1-10

[45] Joshi BP et al. Detection of sessile serrated adenomas in the proximal colon using wide-field fluorescence endoscopy. Gastroenterology. 2017;**152**(5):1002-1013. e9

[46] Elahi SF et al. Targeted imaging of colorectal dysplasia in living mice with fluorescence microendoscopy. Biomedical Optics Express. 2011;**2**(4):981-986

[47] Miller SJ et al. In vivo fluorescence-based endoscopic detection of colon dysplasia in the mouse using a novel peptide probe. PLoS One. 2011;**6**(3):e17384

[48] Burggraaf J et al. Detection of colorectal polyps in humans using an intravenously administered fluorescent peptide targeted against c-Met. Nature Medicine. 2015;**21**(8):955

[49] Zhou J et al. EGFR overexpressed in colonic neoplasia can be detected on wide-field endoscopic imaging. Clinical and Translational Gastroenterology. 2015;**6**(7):e101

[50] Qiu Z et al. Targeted vertical cross-sectional imaging with handheld near-infrared dual axes confocal fluorescence endomicroscope. Biomedical Optics Express. 2013;**4**(2):322-330

[51] Liang S et al. Screening and identification of vascular-endothelial-cell-specific binding peptide in gastric cancer. Journal of Molecular Medicine. 2006;**84**(9):764-773

[52] Liu L et al. In vivo molecular imaging of gastric cancer in human-murine xenograft models with confocal laser endomicroscopy using a tumor vascular homing peptide. Cancer Letters. 2015;**356**(2):891-898

[53] Sturm MB et al. Targeted imaging of esophageal neoplasia with a fluorescently labeled peptide: First-in-human results. Science Translational Medicine. 2013;**5**(184):184ra61-184ra61

[54] De Palma GD et al. Detection of colonic dysplasia in patients with ulcerative colitis using a targeted fluorescent peptide and confocal laser endomicroscopy: A pilot study. PLoS One. 2017;**12**(6):e018050e9

[55] Gao Z et al. In vivo near-infrared imaging of ErbB2 expressing

breast tumors with dual-axes confocal endomicroscopy using a targeted peptide. Scientific Reports. 2017;**7**(1):14404

[56] Zhang J et al. Targeted radiotherapy with tumor vascular homing trimeric GEBP11 peptide evaluated by multimodality imaging for gastric cancer. Journal of Controlled Release. 2013;**172**(1):322-329