M13 bacteriophage/single-walled carbon nanotube interactions for chirality selection and multifunctional materials

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**M13 BACTERIOPHAGE/SINGLE-WALLED CARBON
NANOTUBE INTERACTIONS FOR CHIRALITY SELECTION
AND MULTIFUNCTIONAL MATERIALS**

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Abstract

Filamentous M13 bacteriophages are excellent display systems and nanoscale building blocks. Single-walled carbon nanotubes (SWCNTs) have promising prospects in a wide range of applications, from molecular electronics to artificial muscles, owing to their fascinating electronic and mechanical properties. The interaction between these two novel materials was studied and exploited in this dissertation.

In the first part of this thesis, phage display screening was performed for SWCNT chirality separation. As a display system, the combinatorial M13 phage display library with a variable dodecapeptide on pIII protein was utilized to provide a new path for SWCNT chirality separation, which is a prerequisite for implementing SWCNTs in applications in electronics as chirality controls the electrical properties of SWCNTs and they are always produced in batches with diverse chirality.

After an evolutionary screening process for (7, 5) and (7, 6) enriched SWCNT samples, respectively, binding peptides were identified from phage display library based on the assumption that binding affinity between peptides and SWCNTs is sensitive to amino acid sequences. Structural similarities of (7, 5) and (7, 6) binding peptides were found, such as high contents of aromatic amino acids, histidine as the head of peptides, and overall hydrophobicity, etc. The SWCNT-peptide interaction was also investigated by molecular dynamics (MD) simulations to reveal the binding conformation and to calculate the binding energy. All the selected peptides bind to SWCNTs in an extended conformation with ring groups attaching to the SWCNT surface in a π -stacking form, which was also experimentally verified by circular

dichroism (CD) spectroscopy. Peptide HSNWRVPSPWQL was selected for chirality separation of large-diameter SWCNTs owing to its best distribution of binding energy and favorable energetic interaction with large-diameter tubes. It was able to disperse HiPco SWCNTs into small bundles and individual tubes without losing their intrinsic electronic properties. Large-diameter SWCNTs, such as (10, 3), (9, 5), (12, 2), (11, 4), and (10, 6), were specifically dispersed by this peptide.

In the second part, M13 phages, as nanoscale building blocks, were fabricated into centimeter-long liquid-crystalline microfibers (10 – 30 μm in diameter) with inherent fluorescence by crosslinking reaction. SWCNTs and magnetic nanoparticles were integrated into the fibers as functional fillers. SWCNTs oriented themselves parallel to the long axis of the phage composite fibers due to the well-ordered alignment of phage in the fibers. 1 wt% (weight percentage, wt%) SWCNT loading rendered significant improvements on mechanical properties to the fibers: 94% increase in tensile strength, 65% increase in Young's modulus, and 115% increase in failure strain were found. At 45 wt% SWCNT loading, the electrical conductivity of the fibers was $1790 \text{ S}\cdot\text{m}^{-1}$, 10 orders of magnitude higher than that of pure phage fibers and comparable to most of other as-produced microfibers spun by biopolymers. Magnetic phage fibers could be produced by attaching magnetic nanoparticles to M13 phage surface before fabrication. Moreover, the phage composite fibers were non-cytotoxic and suitable for further surface modifications because no filler materials covered on their outer surface.

This work achieves chirality-preferential dispersion of SWCNTs via peptides selected by phage display technique for the first time, and provides a new conceptual scheme for SWCNT chirality separation. This work also presents an all-in-one tool,

M13 phage multifunctional composite fibers for the first time. Such phage fibers, integrating fluorescence, electrical conductivity, magnetism, improved mechanical properties, biocompatibility, and surface functionalization sites, are promising in applications ranging from tissue engineering to biomedical devices by carrying out different tasks in parallel.

List of Abbreviations

ACCVD	Alcohol Catalytic Chemical Vapor Deposition
AFM	Atomic Force Microscopy/Microscope
BSA	Bovine Serum Albumin
C ₆₀	Fullerene
CD	Circular Dichroism
CNT	Carbon Nanotube
CoMoCAT	Co-Mo Catalyst
CVD	Chemical Vapor Deposition
DGU	Density Gradient Ultracentrifugation
DI	Deionized
DMEM	Dulbecco's Modified Eagle Medium
DOS	Density Of State
dsDNA	double-stranded DNA
<i>E.coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FBS	Fetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscopy
FET	Field Effect Transistor
FMN	Flavin Mononucleotide
GB	Generalized Born
HiPco	High-pressure Carbon Monoxide
HRTEM	High Resolution Transmission Electron Microscopy

HUVEC	Human Umbilical Vein Endothelial Cell
<i>I-V</i>	Current-Voltage
IEC	Ion-Exchange Chromatography
IPTG	Isopropyl β -D-thiogalactoside
MD	Molecular Dynamics
MSA	Mercaptosuccinic Acid
MWCNT	Multi-Walled Carbon Nanotube
NHS	N-hydroxysuccinimide
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PFO	Poly(9,9- dioctylfluorenyl-2,7-diyl)
pfu	plaque forming unit
PLE	Photoluminescence Excitation/Emission
POM	Polarized Optical Microscope
QD	Quantum Dot
RBM	Radial Breathing Mode
RCA	Rolling Circle Amplification
SC	Sodium Cholate
SDBS	Sodium Dodecylbenzene Sulfonate
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy/Microscope
SPC	Single Point Charged
ssDNA	single-stranded DNA
SWCNT	Single-Walled Carbon Nanotube

SWNH	Single-Walled Carbon Nanohorn
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20
TMV	Tobacco Mosaic Virus
TNT	Trinitrotoluene
UV-vis-NIR	Ultraviolet-visible-Near-Infrared
wt	weight percentage
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside
XPS	X-ray Photoelectron Spectroscopy

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Chapter 1

Introduction

In this work, the interaction between two nanometer scale materials – bacteriophage and carbon nanotube is focused. On one hand, chirality enrichment of carbon nanotubes was achieved by peptides deciphered from bacteriophages; on the other hand, new functionalities were attained on multi-functional bacteriophage fibers by integration of carbon nanotubes.

1.1 Background

Viruses, the ultimate parasites on the Planet Earth, have always been considered as highly feared predators because of the epidemic diseases caused by them. Fortunately, with the development of techniques for cultivating^[1] and identifying viruses^[2, 3], much has been learned about these minuscule infectious agents and now people are making friends with the ancient foes. Traditionally, viruses have been extensively used in life sciences and medicine for molecular and cellular biology research^[4, 5], molecule delivery^[6], anti-microbial treatments^[7], and development of vaccines and antibodies^[8, 9], etc. In recent years, the emerging bionanotechnology extends the usage of viruses to more fields and makes them versatile nanoscale platforms^[10], either as latent “organisms at the edge of life”^[11], or as nonliving proteinaceous biopolymers.

Bacteriophages (also known as phage), the most abundant form of biological entity in aquatic environments^[12], are a group of viruses that infect bacteria, with a variety

of shapes ranging from spheres to rods and sizes spanning from tens to hundreds of nanometers. λ , T4, and M13 are some common phages. They are fascinating organisms not only because of their contributions to areas such as basic biology research^[13, 14] and phage therapy^[15], but also their roles as screening display systems^[16] and nanosized building blocks^[17].

The technique involving artificial chemical evolution by phages is named phage display, which allows rapid selection of receptors based on binding affinity to given ligand molecules. To realize this *in vitro* evolutionary process, a variable peptide is linked to a surface protein of each phage in a phage library by standard recombinant DNA technology. Here this peptide can be regarded as a receptor, which specifically binds its ligands. The sufficient diversity of phages in the library is the prerequisite for the “blinding” screening process, so that no *a priori* knowledge of desired receptors is required. When the phage library is exposed to a ligand, specific receptors may be selected and individuals who fail to bind tightly to the ligands are removed from the library once appropriate selection conditions are used. Multiplying phages in their host strain and decoding their DNA sequences can retrieve the peptide information of the selected receptors for further studies. Rational design is an alternative way to obtain receptors to certain ligands, however, insufficient knowledge about protein-folding predictions and surface-binding chemistries often impedes this straight-forward method. Therefore, phage display, a simple and fast way outperforming rational design, finds broad applications in discovering new drugs, enzymes, antibodies, DNA-binding proteins, and diagnostic agents^[16, 18-21].

Phage display stems from a reverse biology concept, but does not confine itself within biological research and applications. Since Stanley Brown first provided seminal inspiration by using a bacterial combinatorial peptide library to isolate gold-

binding peptides^[22], phage display has been expanded to selections of peptides that bind to a wide range of inorganic materials and nanostructures^[23]. Following Brown's steps, Belcher and co-workers reach great heights on this concept. Phages or peptides isolated in these selections have been exploited to template inorganic nanomaterial synthesis^[24], align nanomaterials^[25], and produce lithium batteries^[26], etc. It seems that the utility of phage display technique will be limited only by our own imagination.

In addition to phage display, recently fabrication of composite materials by phages as nanoscale building blocks has offered these organic nanoparticles promising scopes as well. Phages have been assembled into structures including micro- and nanofibers^[27], films^[25], and three-dimensional scaffolds^[28, 29]. Such hybridized materials have shown their potential in energy storage^[30], anti-microbial textiles^[31], and sensing^[32], etc.

Nanotechnology, which has been widely known since Richard Feynman's enlightening talk "There's Plenty of Room at the Bottom" in 1959^[33], is one of the most promising technological areas that may transform the world. With the development of tools to manipulate atoms and molecules, nanotechnology has received exponential attention. Materials downsized to nanometer scale possess unique properties over their macroscopic states^[34, 35]. Numerous nanoscale materials have been synthesized and characterized in order to utilize their remarkable properties, which are often determined by their structures. Therefore, controlling the structures of nano-materials is prerequisite to fulfilling their properties.

Single-walled carbon nanotube (SWCNT), an important variety of carbon nanotubes (CNTs), is one of the most attractive nano-materials emerged in recent

years owing to its fascinating electronic, mechanical, structural, optical, and thermal properties. However, the full potential of SWCNTs cannot be realized because they are always fabricated in batches with diverse diameters and band gaps. The unavailability of SWCNTs with specific chiral structures prevents further SWCNT-based electronic applications. Thus pure metallic or semiconducting SWCNTs with particular band gaps are highly desired.

It is this requirement that links phage display and SWCNT together. Based on the fact that phages not only have affinity to various inorganic materials, but also can recognize the difference in the crystalline face, i.e., the spatial arrangement of the atoms, it is believed that phage may also recognize different chiral structures of SWCNTs. In this thesis, phage display technique has been used to discover peptides that can selectively bind to certain chiral structures of SWCNTs, thereby providing a new method and perspective for SWCNT separation. It is the first topic of this thesis. In the second part, owing to their outstanding properties, especially mechanical and electronic properties, we found that SWCNTs could be also beneficial to phages when phages are used as building blocks for microfibers. Fabrication of multi-functional phage microfibers involving phage, SWCNTs, and magnetic nanoparticles is the other main topic of this thesis.

1.2 Significance

1.2.1 Phages Beneficial to Carbon Nanotubes

An M13 bacteriophage display library has been used to discover peptides with different binding affinity to various SWCNT chiral structures. In this library, a

variable dodecapeptide (twelve amino acids) with 2.7×10^9 possibilities is expressed on the tip of the pIII minor coat protein of M13 bacteriophage. After a “blinding” screening process, peptides with affinity to SWCNTs were selected. Analyses, in respect of sequences similarity, binding consensus, hydrophobicity, and binding energy, were performed on these peptides to reveal the binding mechanism and perceive best candidates for chirality selection.

In fact, a few research groups have done selection of peptides binding to CNTs by phage display technique, either on multi-walled carbon nanotubes (MWCNTs)^[36] or SWCNTs with a wide chirality/diameter distribution^[37-39]. However, none of them aimed at chirality selection. We aim at chirality selection, and the outperformance of our phage display screening depends on two points. First, the SWCNTs used in our study were single-chirality enriched samples (>30%), thereby higher probability to obtain peptides with specifically high affinity to certain chirality. Second, molecular dynamics (MD) simulations were conducted on the selected peptides to calculate the binding energy of SWCNT/peptide complexes so as to advise strongest binders and peptides with the best chiral discernment for the subsequent chirality separation experiments. It was, to some extent, a correction to experimental results, and shed light on how peptides differentiate SWCNTs with different chiralities.

We describe the chiral selection of SWCNTs via peptides selected by phage display technique in this thesis based on the aforementioned conception. To our best knowledge, it is the first time that chirality-preferential dispersion of SWCNTs was achieved via peptides selected by phage display technique. It offers new prospect of understanding of the mechanism of peptide-CNT binding and producing “designer” peptides of SWCNT chirality recognition. One big obstacle of this work is the availability of large amount of peptides with high purity. We expect that if the mass

production of phage-displayed peptides is attainable by genetic engineering, the conception and methodology of this work will pave a new path to SWCNT chirality separation.

1.2.2 Carbon Nanotubes Beneficial to Phages

In addition to traditional applications in biology research and phage therapy, bacteriophages have been actively employed as nanoscale platforms in a variety of fields, such as molecular biomimetics^[40], chemical and biological sensing^[32], and display systems^[41], etc. Phage-based nano-/microfibers^[27, 42], which have well-ordered molecular structures and high potential to be functionalized, are built by filamentous phage particles assembling in a liquid crystalline form. Recently, such fibers were fabricated and decorated to eliminate bacterial^[31] and orientate cell growth^[43]. We have realized that although some functionalities were added to the phage fibers, the phage fibers reported previously were still relatively brittle and weak, while the functionalities were insufficient.

Carbon nanotube is a good choice to be integrated into liquid crystalline phage fibers for the following reasons. First, these nanosized tubes possess a similar geometry to filamentous phage, so they are geometrically compatible with phages and can be simply aligned along phages in the fibers. Second, they are ideal reinforcement fillers for composite materials^[44]. After incorporated into phage fibers, CNTs can improve the mechanical properties of phage fibers. Third, their excellent conductivity can impart electrical conductivity to phage fibers. Lastly, the integrated CNTs do not appear on the surface of phage fibers, thus more functional materials can be added onto the exterior phages. On top of properties brought by CNTs, we have

found that the phage fibers are also naturally fluorescent and non-cytotoxic. Magnetism can be also imposed to phage fibers by involving magnetic micro-/nanoparticles.

After all of the functionalities are achieved on one phage fiber, a new all-in-one tool, which integrates several different functions in one single hybrid material, is now available. And different tasks can be carried out in parallel by such a phage fiber. Many other materials, especially small molecules and nanoparticles, can be also added into the phage fiber body or attached to the superficial phages, thereby making the phage fiber a versatile system for undertaking new functionalities. Consequently, we expect this phage-based all-in-one tool will contribute to future applications in tissue engineering, microelectronics, or hybrid devices as a new multifunctional material.

1.3 Scope of the Work

The work of this thesis encompasses two major subjects - M13 phage and carbon nanotube. How M13 phage and carbon nanotubes can benefit to each other will be demonstrated. First, a combinatorial M13 phage display library has been used to isolate peptides that specifically bind to SWCNTs and have chiral recognition for several chiralities of SWCNTs. Second, M13 phage, a nanoscale versatile building block, has been used to assemble into liquid crystalline multifunctional microfibers, in which SWCNTs lead to improved mechanical properties and excellent electrical conductivity as nanofillers.

The thesis is composed of six chapters. In **Chapter 1**, a brief introduction as well as initial motivation and significance of this work, followed by an outline for the

scope and organization of the thesis, are provided. In **Chapter 2**, phage display technique and carbon nanotube are introduced systematically, particularly phage display for selecting inorganic substrate binders and chirality separation of carbon nanotubes. **Chapter 3**, which lays groundwork for Chapter 4, focuses on phage display screening. The selection method is introduced in detail. Statistical analyses for peptides isolated from biopanning experiments and the evidence of binding are then presented. **Chapter 4** describes how the peptides selected from phage display experiments can be used on SWCNT chirality selection. MD simulations confirm the experimental results and guide the subsequent experiments. A peptide isolated from phage display experiment and selected by MD simulations shows preference to large-diameter SWCNTs, thereby demonstrating that phage-displayed peptides can be used for SWCNT chirality separation for the first time. In **Chapter 5**, a facile and versatile approach to fabricate liquid crystalline multifunctional M13 phage composite microfibers is introduced. Results demonstrating that such fibers are naturally fluorescent, non-cytotoxic, and compatible with SWCNTs and micro-/nanoparticles are presented. SWCNTs bring new functionalities to the fibers, such as higher mechanical strength and electrical conductivity, without interfering the superficial phages for further functionalities. The potential of phage fibers to be a versatile all-in-one tool is then discussed. In **Chapter 6**, conclusions and suggested directions for future work related to SWCNT chirality separation by peptides and phage-based multifunctional composite materials are highlighted.

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Chapter 2

M13 Phage Display and Carbon Nanotube

In this chapter, M13 phage display technique and physical characteristics of carbon nanotube are reviewed. The structure and lifecycle of M13 phage are overviewed in Section 2.1.1 and 2.1.2, respectively. Phage display technique is introduced in Section 2.1.3. Representative examples of inorganic materials specific binding peptides, particularly carbon materials, are given in Section 2.1.4. Fabrication of composites with phages as building blocks is covered in Section 2.1.5. In Section 2.2.1 and 2.2.2, SWCNT's structure, characteristics, and synthesis methods are briefly introduced. The chirality selectivity methods, including selective growth methods and post-synthetic sorting schemes, especially those with respect to biomolecules, are reviewed in Section 2.2.3.

2.1 Phage Display

How to get proper peptides binding to specific targets? Conceptually, rational design is a straightforward and efficient way, however, the current knowledge of protein-folding predictions and surface-binding chemistries does not support the rational design of proteins/peptides that specifically bind to each target of interest. An alternative is screening, in which a highly diverse population of peptides are available and their “fitness” is determined by targets, i.e., only the strongest target binders are retained in the end. No comprehensive understanding of binding mechanism required during the selection is another unique advantage of screening.

Phage display is a chemical evolution technique in which polypeptides are expressed as coat proteins of a bacteriophage, and these polypeptides with desired binding specificities to a variety of target molecules or substrates are later identified. Relying on the fact that foreign DNA fragments inserted into filamentous bacteriophage gene by using standard recombinant DNA technology can encode a fusion protein which is incorporated into the virion and displayed on the surface of bacteriophages, phage display technology was first described by George Smith in 1985^[1]. Since then, a large number of phage display libraries (collections of phages having variable fusion peptides displayed on their coat protein) have been constructed^[2-6] and this powerful method has been conventionally utilized in many fields such as design/discoveries of new drugs, enzymes, antibodies, DNA-binding proteins, and diagnostic agents^[7-11].

Many types of bacteriophage, including M13, f1, fd, T7, T4, and λ , have been used for phage display systems. Herein the background of filamentous bacteriophage M13 will be reviewed because they are used for screening experiments and nanoscale building blocks in this thesis, and play the leading role in phage display technology.

2.1.1 Structure of M13 Bacteriophage

Wild-type M13 bacteriophage is a male *Escherichia coli* (*E.coli*)-specific filamentous virion with specific infection receptors for a host bacterium strain presenting an F-factor (F^+ strain). A schematic diagram of the structure of M13 bacteriophage is shown in Fig. 2.1. The viruses are approximately 880 nm in length and 6-7 nm in diameter. The M13 particle consists of a circular, 6407-base, single-stranded DNA (ssDNA) encapsulated by various major and minor coat proteins. The

genome contains 11 genes: five of them encode structural proteins while the rest encode non-structural proteins which contribute to viral replication and assembly^[12]. The rod-like viral capsid is composed primarily of 2700 copies of pVIII major coat protein. The four minor coat proteins are located at each end of the phage: pVII and pIX cap one end while pIII and pVI cap the infectious end. The length of the virion depends on the length of genome and the electrostatic balance between the coat proteins and ssDNA: commonly longer DNA generates longer phage^[13].

Figure 2.1. Schematic diagram of the structure of an M13 filamentous bacteriophage^[11]. The circular ssDNA is encapsulated by a proteinaceous coat. The coat comprises 5 types of structural proteins and 6 types of non-structural proteins.

All of the five coat proteins, pVII, pIX, pVIII, pVI, and pIII, are responsible for the structural stability of phage particles, but pIII is also necessary for attaching to the F pilus of a male *E.coli* and for initiating infection. The pIII protein (Fig 2.2) comprises more than 400 residues and contains three distinct domains (D1, D2, and D3),

separated by glycine-rich tetra- and pentapeptide repeats, as well as a C-terminal transmembrane region^[14]. The crystal structure of the first two domains, D1 and D2, have been solved by X-ray crystallography and NMR spectroscopy^[14-16], although the C-terminal region and D3 domain are poorly understood. A schematic diagram of pIII protein is shown in Fig. 2.2.

Figure 2.2. Schematic illustration of the pIII protein showing the domains and their functions^[17].

2.1.2 The M13 Lifecycle

The lifecycle of M13 phage (Fig. 2.3) is briefly reviewed here. Phage infection begins with D2 domain binding to the bacterial F pilus. Then D2 undergoes a conformational change, making the TolA binding site on D1 become accessible, which initially interacts with D2. The F pilus retracts until the D1 domain can interact with *E.coli* membrane protein, TolA^[18]. The subsequent steps have not been elucidated. Eventually, the phage DNA is transferred into the host bacterium, where the phage replication and assembly occur.

Once inside the host bacterium, the ssDNA is converted to a double-stranded form by bacterial enzymes. The double-stranded DNA (dsDNA) is further duplicated and used as the templates for the synthesis of phage proteins, including the five structural proteins (pIII, pVI, pVIII, pVII and pIX) and the six non-structural proteins (pII, pX, pV, pI, pIV and pXI)^[12]. The phage assembly begins with the formation of ssDNA-pV complex, in which dsDNA conversion is prevented by pV. Under a packaging signal (78-nucleotide hairpin), phage packing is initiated by pVII and pIX. Subsequently pVIII coats the phage DNA while pV is removed. It is thought that pI, pXI, and pIV interact to form an open channel that is not blocked by the phage tip, and phage is extruded from the bacterium from this channel. Phage replication is terminated by addition of pIII and pVI. pIII and pVI initially anchor in the bacterium membrane and interact with pVIII. After the pIII and pVI membrane interaction is disrupted by a conformational change of pIII, the virion is released. Because M13 is a non-lytic bacteriophage, newly assembled phage particles are continuously released while the infected bacteria can continue to grow and divide, albeit at half of the rate of normal bacteria^[19].

Figure 2.3. Schematic diagram of the non-lytic lifecycle of M13 bacteriophage, including infection, replication, assembly, and secretion^[11].

2.1.3 Phage Display Systems

Currently, pIII protein is most commonly used to display engineered peptides/proteins. Exogenous segments are fused to the N-terminal of pIII, i.e., the first residue of the mature protein is the first randomized position of the fusion peptide. The peptide is followed by a short spacer and then the wild-type pIII D1 domain^[20]. Although it is believed that the addition of fusion peptides will not interfere pIII function to replicate and release from the bacterial membrane, we cannot

presume that all the engineered phage will replicate with the same efficiency, since one cannot test all the fused peptides for their capability to incorporate into the membrane and release from bacteria.

Phage display libraries, each of which has one type of random fusion oligo-peptide displayed on their coat proteins, such as Ph.D.-12, Ph.D.-C7C, and Ph.D.-7 Phage Display Peptide Library, are commercially available. The phage display library used in this thesis, Ph.D.-12 Phage Display Peptide Library, and the other two libraries are commercially available from New England Labs (NEB, Beverly, Massachusetts, USA). In Ph.D.-C7C library, all displayed peptides are structurally constrained in a 7-residue disulfide loop. Ph.D.-7 library presents peptides in the form of 7 linear amino acids. The reason that Ph.D-12 library was chosen is that longer peptides of this library can provide more potential contacts with targets^[20]. In the Ph.D.-12 library, five copies of a same random dodecapeptides are displayed on the N-terminus of pIII protein. The library contains a highly diverse population of peptides, approximately 2.7×10^9 different sequences, on the average 37 copies of each sequence in one screening experiment.

Conceptually, phage display screening is simple, although it can be fraught with technical difficulties. In its simplest form, phage display screening is a process where phages with desired peptides are retained while phages with undesired peptides are eliminated. In a typical phage display screening experiment, a target is exposed to a phage display peptide library for a period of time. After the unbound and non-specifically bound phages are washed away, elution buffer recovers the specific binders. The eluted phages then undergo amplification and purification, and subsequently the purified phages are used as a “new library” and taken through additional binding/elution/amplification/purification cycles until consensus sequences

are yielded. After several rounds (at least 3 rounds), the eluted phages grow as single plaques for further characterization by DNA sequencing. In Fig. 2.4, a schematic diagram of a typical phage display screening process is shown.

Figure 2.4. Biopanning process to identify specific binding peptides against desired targets. The probability curves, in which the x-axis represents possible peptide sequences and y-axis represents their probability, depict phage population as it goes through several rounds of selection, indicating that phage display is to eliminate unwanted sequences and enrich desired sequences from the initial random library.

This *in vitro* selection process is known as biopanning, also referred to as a directed evolution process when the undesired peptides are eliminated and the desired ones are retained. The superiority of phage display technology is based on the fact that it is unnecessary to know *a priori* information about the desired sequences, as they can be selected and enriched in the panning process if the population of the random

sequences is sufficiently large^[21]. Even peptides with artificial functionalities, that is, not present or at least not yet known in the biological realm, can be selected. This method also provides a linkage between genotype and phenotype. This linkage implies the colocalization of the genotype and phenotype in every single phage particle and the isolation of a functional peptide with its encoding DNA sequences without any knowledge of the sequence-function relationship^[22]. The properties of the peptides on the surface of the virion can simply be modified by changing the underlying genomic information through basic genetic engineering approaches. In addition, the replication of phage through *E.coli* infection and amplification provides a rapid and economical way to obtain a large monodisperse population of desired phages for further studies and applications.

2.1.4 Phage-displayed Peptides to Inorganic Materials

Biological systems possess inherent molecular recognition capability. Conventionally, phage display technique has been used to identify protein epitopes, antibodies, and enzymes, and to study protein interaction and to discover new drugs. In 1997, Stanley Brown first provided seminal inspiration by using a bacterial combinatorial peptide library to isolate gold-binding peptides^[23]. Since then, phage display has been extensively utilized to evolutionarily select specific peptide sequences for a wide range of inorganic materials and nanostructures, such as noble metals, metal alloys, metal oxides, semiconductors, and carbon allotropes including fullerenes, carbon nanohorns (SWNHs), and carbon nanotubes. Phage-displayed peptides with affinity to materials referred to in this thesis are listed in Table 2.1.

Table 2.1. Examples of phage-displayed peptide sequences exhibiting affinity to various inorganic materials.

Target	Peptide sequences [Ref.]
Ag	NPSSLFRYLPSD [24] WSWRSPTPHVVT [25] GPGVSSAPPFSK [26]
Pt	CDRTSTWRC, CQSVTSTKC, CSSSHLNKC [21]
Pd	CSVTQNKYC, CSQPHPGPYC, CHAPTPMLC [21]
Ti	RKLPDAPGMHTW [27]
FePt	HNKHLPSTQPLA [28]
CoPt	CNAGDHANC [29]
Fe ₃ O ₄	CNSPHRHSC [30]
α -Fe ₂ O ₃	CPMLHNSRC [30]
GaAs	AQNPSDNNTHTH, RLELAIPLQSG, TPRPIQYNHTS [31]
GaN	SVSVGMKPSRP, HATPQPLHSRM [30]
InP	CTDTSSRSC, TTQHHAVWTSQH, ALPELSSLPESA [30]
ZnS	CNNPMHQNC [32] LRRSSEAHNSIV [33]
CdS	SLTPLTTSHLRS [33]
C ₆₀	CNMSTVGRC [34]
Carbon nanohorns	DYFSSPYEQLF [35]
Carbon nanotubes	HWSAWWIRSNQS [36] LLADTTHHRPWT, CGHPWTKC [37] DDWSHWWRAWNG [38] HGHPYQHLLRVL, DMPRTTMSPPPR [39]

2.1.4.1 Metal-binding Peptides

Sarikaya *et al.* utilized platinum (Pt) and palladium (Pd) as targets in screening

experiments by a constrained heptapeptide library. The selected peptides having affinity to noble metals possess some similar properties in terms of amino acids. For example, they are rich in hydroxyl amino acids such as serine and threonine, and they participated in hydrogen bonding by nitrogen atoms. Additionally, they have very few cysteines, histidines and aromatic amino acids. Instead, hydrophobic and hydroxyl-containing polar amino acids appear predominately in these noble-metal-binding peptides^[21]. Another two examples are briefly discussed as follows.

Peptides specifically binding to silver particles were selected from a 12-mer combinatorial phage display library. NPSSLFRYLPSD was obtained by regular biopanning method^[24], while WSWRSPTPHVVT was yielded by a polymerase chain reaction-driven elution process^[25]. Although both peptides reduced silver ions to metallic silver, leading to efficient silver nanoparticle synthesis, the latter exhibit superior capability. Therefore, it is thought that some better binders were lost during regular biopanning experiments and optimization of biopanning protocols gives rise to higher affinity.

After selecting a titanium-binding peptide, Sano *et al.* used molecular biology tools such as alanine substitution mutation and deletion mutation to investigate the binding effect of each amino acid. They found that positively charged amino acids were necessary and the first half of the peptide (hexapeptide RKLPDAP) was sufficient for Ti binding. Although they presented a model to explain the phenomenon, the underlying mechanism was not fully understood. The fact that the binding ability of the peptide can be enhanced by modifying the Ti particles with hydroxyl groups implies that hydroxyl group are the target of the phage recognition^[27].

2.1.4.2 Semiconductor-binding Peptides

Belcher and co-workers have performed a series of excellent biological selections through phage display technique to find peptides that specifically recognize various metallic alloy and semiconducting materials^[40, 41]. The binding specificity was confirmed by various tools such as X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), scanning electron microscopy (SEM), high resolution transmission electron microscopy (HRTEM), and fluorescence microscopy. In their early work, peptides with high affinity to GaAs were selected. Chemical composition specificity was shown as peptides bound to GaAs but did not bind to Si. Furthermore, they demonstrated some peptides had specific affinity to GaAs (100) but not to GaAs (111) only because of the difference in the crystalline face, i.e., the spatial arrangement of the atoms^[31], demonstrating that phage display technology is suitable to distinguish surfaces having very close structures.

Besides surface recognition, selected phage or synthetic peptides can be further utilized to direct nano-architecture assembly. For example, after phages that are capable of specific recognition to ZnS and CdS were obtained, ZnS-specific phage was exposed to ZnS precursor solutions to form phage-ZnS nanocrystal liquid crystalline suspension. Using proper concentration of phage and a magnetic field, the hybrid materials were patterned into films containing phage aligned along their long axis and ZnS crystal located at the tip of phage particles (pIII protein)^[32]. It has also been demonstrated that the selected phages possessed different nucleation ability for ZnS. For instance, A7 and Z8 are both ZnS-specific phages and able to nucleate wurtzite ZnS and zinc blende ZnS, respectively. This was re-confirmed by displaying A7 and Z8 peptides on pVIII major coat protein. Similar phenomenon was also observed on the nucleation of CdS by CdS-specific phage reported by Flynn *et al.*^[33].

Assembly of nanowires is another extensively studied subject by Belcher's group. ZnS, CdS, FePt, CoPt-specific binding phage/peptides were used to realize magnetic and semiconducting nanowire synthesis, which was, to some extent, a genetically controlled biological route to nanoscale semiconductor heterostructures^[28, 29, 33, 42]. In addition, the selected virus/peptides were used to assemble nanoarchitectures of various geometries and materials^[43-45], direct mineralization of selected inorganic materials^[46], and recognize crystal defects^[47].

2.1.4.3 Carbon Materials Binding Peptides

Because of their unique properties, novel carbon materials, such as fullerenes, carbon nanohorns, and carbon nanotubes, have been the subjects of intense research. The combinatorial phage display libraries were exposed to these materials for selecting specific binders.

Wang *et al.* performed a screening experiment to find CNT-binding peptide sequences by 12-mer phage display library against MWCNTs^[36]. Strong consensus sequences were obtained after the fifth round of biopanning, showing that these peptides were rich in histidine and tryptophan. The importance of tryptophan for binding was investigated by site-directed mutagenesis, through which tryptophan at position 6 in HWKHPWGAWDTL and HWSAWWIRSNQS was replaced by serine, leading to marked impairment to the affinity. Conformations of the peptides were characterized by computational simulations, showing that the binding sequences held a more folded structure and a more flexible chain than the controls^[36]. However, the underlying mechanisms are still not well understood, even though the CNT-binding peptides were somewhat featured by the powerful information-mining tool.

Su *et al.* reported that, through both 12-mer linear peptide library and 7-mer

disulfide-constrained library, peptides with stronger binding affinity to SWCNTs than Wang's can be found^[36], and a new binding motif was proposed. Interestingly, the selected peptide sequences, LLADTTHHRPWT and CGHPWTKC, have less aromatic amino acids but more hydroxyl amino acids compared with Wang's result. So it seems that although aromatic moieties in the sequences are critical for binding, other types of amino acids also play important roles in the interaction. They also examined the conformation of the identified peptides by circular dichroism (CD), fluorescence, NMR spectroscopy, and molecular modeling, and revealed that the peptides exhibited a β -turn (β -hairpin) structure when binding to SWCNTs^[37]. Subsequently, the importance of tryptophan in the binding sequences was investigated by using a single-point mutation (i.e., replacing tryptophan by alanine) and unnatural amino acid substitution (i.e., replacing tryptophan by 5-fluorotryptophan, 5-hydroxytryptophan and 7-azatryptophan). The importance of aromatic moieties in the sequences was confirmed. Moreover, the affinity was attributed to the interaction between the highest occupied molecular orbital of the tryptophan indole ring and the lowest occupied molecular orbital of the SWCNTs^[48].

Naik and co-workers identified SWCNT-binding peptides rich in aromatic, charged, and hydroxyl-containing amino acids. Peptide HSSYWYAFNNKT (P1) is demonstrated to disperse SWCNTs into homogenous suspension while peptide YTTHVLPFAPSS does not have such capability because P1 has more aromatic residues which are located in the central hydrophobic domain. Additional peptide domains or epitopes were engineered into P1 to obtain new functionalities. For instance, silica or titania could be precipitated onto SWCNTs by a bi-functional peptide, in which one end is P1 and the other end can bind silica or titania^[49]. In another example, a bi-functional peptide containing a trinitrotoluene (TNT)-binding

domain and P1 could be used to fabricate SWCNT-FET (field effect transistor) device capable of detecting TNT^[50]. Such SWCNT-binding bi-functional peptides represent a new and broad route to specifically integrate functional materials to SWCNTs.

In phage display experiments of Burke's group^[38], SWCNTs were immobilized on a silicon chip during screening, whereas in prior experiments SWCNTs were suspended in aqueous solution by surfactants. The freestanding SWCNTs avoided the use of surfactants hence a better interaction between peptides and nanotubes. Additionally, they have developed a new buffer to break the peptide-nanotube interaction instead of the conventional acidic elution buffer. They presented an SWCNT-binding motif SXWWXXW (where X is any amino acid), in which the importance of tryptophan is clearly seen. Many following-up analyses and characterizations on the selected peptides were conducted to reveal why and how peptides bound to SWCNTs. However, no direct evidence was provided to prove the peptides' affinity to SWCNTs as their peptides were unable to disperse SWCNTs^[38].

Carbon nanohorns have the same graphitic nanostructure as normal carbon nanotubes yet they have closed ends like a horn. Kase *et al.* identified the peptides binding to carbon nanohorns. The sequence DYFSSPYEQLF was selected from a 12-mer phage display library through a magnetic separation process. The peptide underwent some conformational change at different pH: at neutral pH, it adopts a random-coil-like structure while at low pH, it prefers an α -helical structure^[35]. Further analysis revealed that the peptide was conformationally unstable because of the D1-S5 segment (the first five amino acids) and the proline at position 6. They proposed that this instability was somehow stabilized by interactions between peptides and inorganic materials^[51].

A peptide selectively binding to fullerene (C₆₀) was found from a cyclic 7-mer phage display library. The selected sequence, CNMSTVGRC, is very different from the ones which bind to SWCNTs and SWNHs because it doesn't have any aromatic amino acids, although the three types of targets are all carbon structures^[34]. This indicates that, except amino acids with aromatic groups, other types of amino acids also contribute to the affinity for carbon structures. The binding mechanism is not discussed in that paper, however.

2.1.5 Fabrication of Composites with Phages as Building Blocks

Except display systems, phages have been widely used as building blocks for fabrication of composite materials with various morphologies, such as films and fibers. One exceptional feature of these nanoscale building blocks is the coupling sites on their surface, whose functionalities are tunable via genetic engineering techniques and post-translational modifications, thereby offering great possibilities to devise materials with sophisticated surface chemistry. Some examples of M13 phage-based composite materials are reviewed herein.

Lee *et al.* fabricated transparent M13 phage films (72 μm thick) doped with ZnS quantum dots on pIII minor coat protein, which expressed peptides with specific affinity to ZnS crystal surfaces. Such M13 phages were selected via phage display screening and were highly concentrated so as to form a liquid crystalline alignment^[32]. They used the same method to synthesize pink phage films (the thickness is less than 10 μm) by conjugating Au nanoparticles-streptavidin complex onto pIII protein that specifically binds to streptavidin^[43]. The latter approach is more versatile to form ordered nanomaterials because as long as the nanomaterials can be linked to

streptavidin, they can be conjugated to streptavidin-binding phages. This self-ordering viral system provides a new way to organize various nanomaterials.

Streptavidin-binding M13 phages with the same molecular order, i.e., the liquid crystalline form, were also fabricated into micro- and nanofibers by the same research group. The microscale fibers, with 10 – 20 μm in diameter and centimeter in length, were spun by extruding highly concentrated phage solution into a crosslinker bath, which was able to bind phages together via a crosslinking reaction. If the phages bound R-phycoerythrin conjugated streptavidin prior to spinning, the resulting viral microfibers would be fluorescent due to R-phycoerythrin. Fluorescent phage nanofibers were produced by electrospinning phages linked with R-phycoerythrin and using polyvinyl pyrrolidone as the spinning solvent^[52].

Chiang *et al.* further studied phage microfibers. They genetically modified M13 phages so that the engineered phages had 2 more carboxyl groups on the outer part of each pVIII major coat protein than wild-type phages. This modification made the M13 phages more efficient to covalently link with amine-terminated quantum dots and nucleate Au nanoparticles^[53]. Therefore, M13 phage fibers can be utilized as a toolkit to integrate various functionalities via genetic manipulation on their surface proteins. Moreover, the strength (a tensile strength of 33 MPa) and toughness (a Young's elastic modulus of 3 GPa) of pure phage microfibers are comparable to synthetic polymer fibers, which often lack functional versatility. Mao *et al.* followed Chiang's conceptual scheme to develop silver-coated phage microfibers. Such fibers exhibited excellent antimicrobial capability^[54]. However, scaling-up for industrial applications is the bottleneck of all the phage films and fibers discussed earlier.

In addition to films and fibers, M13 phages have been used as layer constituents for various applications. They can form self-assembled lamellar structure with cationic

lipid membranes as anionic polyelectrolytes. Such virus-membrane complexes have large pore size to package and organize large functional molecules, such as protein complexes^[55]. M13 phages can also self-assemble into liquid crystalline monolayers atop oppositely charged weak polyelectrolyte multilayers. The density of the viral monolayer can be altered by changing pH value because the assembly process is driven by competitive electrostatic binding^[56]. Nanoparticles can be deposited onto such viral monolayers if phages with specific binding affinity to certain materials are used. Hybrid gold-cobalt oxides have been attached to surface of engineered M13 phages to form nanowires that assemble to monolayers for an electrode of lithium batteries^[57]. Engineered M13 phages, whose pIII protein can bind to SWCNTs and pVIII protein can template α -FePO₄ growth, have been used to fabricate cathodes of lithium-ion batteries^[39].

Without gene manipulation, wild-type M13 phage also can be chemically modified to improve its binding affinity to certain materials, forming composites with these materials. For instance, Niu *et al.* derivatized lysine groups on the outer surface to carboxylate groups to enhance their binding affinity to aniline. Conductive composite fibers and films were fabricated by mixing modified M13 phages and aniline with poly(sulfonated styrene) as a dopant acid and a stabilizing agent^[58]. Another example is that M13 phages have been fabricated into films capable of orienting cell alignment after chemically decorated with RGD peptides^[59].

In sum, various materials can be integrated onto M13 phage surface either by specific binding or chemical modifications. Composites fabricated from M13 phages and add-on materials have different morphologies and wide applications in optics, electronics, energy storage, and biomedical engineering, etc.

2.2 Carbon Nanotube

2.2.1 Structure and Characteristics

Since the discovery of fullerenes in 1985 by Kroto, Curl, Smalley and co-workers at Rice University^[60], the study of carbon nanostructures becomes an explosively growing field. Doubtless, carbon nanotube is one of the most attractive subjects being studied to date. As the fourth form of carbon (the others are diamond, graphite, and fullerenes), its observation was first reported by Radushkevich and Lukyanovich in 1952, in the form of multi-walled carbon nanotubes^[61]. Single-walled carbon nanotubes, the fundamental structural unit of MWCNTs, were first observed experimentally by Iijima at NEC research laboratory in Japan and Bethune at IBM Almaden Laboratory in California^[62, 63].

The ideal carbon nanotubes can be considered as seamless hollow cylinders consisting of single or multiple atomic sheets of graphene (Fig. 2.5). These hollow cylinders can have a length to centimeter scale or more^[64], but a diameter less than 1 nm. In aqueous solution, they aggregate into bundles in parallel due to intertubular Van der Waals Force. These nanosized tubes exhibit fascinating electronic, mechanical, structural, and thermal properties^[65] that make them extremely promising for molecular electronics^[66, 67], optoelectronics^[68-70], electronic devices^[71, 72], composite materials^[73-75], catalytic support^[76-78], sensors^[79-82], probes^[83, 84], hydrogen storage^[85, 86], electrochemical energy storage^[39], cell and tissue imaging^[87], and drug delivery^[88, 89], etc.

Figure 2.5. Typical structures of single-walled carbon nanotubes. Graphene sheet rolls into seamless tubes at different chiral angles, θ . According to the graphene lattice vectors, i.e., chiral vectors, SWCNTs are categorized into zigzag, chiral, and armchair tubes.

Each SWCNT can be uniquely characterized by a so-called chiral vector $\mathbf{C}_h = n\mathbf{a}_1 + m\mathbf{a}_2 \equiv (n, m)$ which specifies the roll-up modes of a single graphene layer, where a_1 and a_2 are the graphene lattice vectors and (n, m) is a pair of integers^[90]. The chiral angle, θ , is defined as the one between $(n, 0)$ and (n, m) (Fig. 2.5). Based on the chiral vector, SWCNTs can be categorized into “zigzag” ($n = 0$; $\theta = 0^\circ$), “armchair” ($n = m$; $\theta = 30^\circ$), and “chiral” ($n \neq m$; $0^\circ < \theta < 30^\circ$). The metallicity of SWCNTs also depends on the chiral vector. Generally, SWCNTs are metallic with $n - m = 3j$ and semiconducting with $n - m \neq 3j$, where $j = 0, 1, 2, 3, \dots$. Approximately 33% of SWCNTs are metallic and the rest 67% of SWCNTs are semiconducting^[91].

2.2.2 Synthesis and Purification of Carbon Nanotubes

The synthesis of SWCNTs can be realized by several methods including arc discharge, laser ablation, chemical vapor deposition (CVD), and the so-called HiPco (high-pressure carbon monoxide). In an arc-discharge method, a high direct current

passes through opposing graphite anode and cathode in a gaseous atmosphere. Carbon atoms are evaporated and CNTs are generated in the soot materials^[63, 92]. With a metal catalysts inserted anode, SWCNTs can be synthesized. The laser ablation process utilizes a pulsed laser to ablate/vaporize a graphite target with trace of nickel and cobalt as catalysts in a tube-furnace at 1200°C. The as-produced SWCNTs are transported to cold surfaces by passing inert gas through the growth chamber^[93]. During chemical vapor deposition, SWCNTs grow over the metal catalyst particles on a substrate when carbon precursors, such as CO, CH₄, C₂H₅OH, CH₃OH, and C₂H₂, flow through the tube reactor for a period of time. In this process, carbon precursors dissociate at the surface of metal catalysts and dissolve in these particles. After the catalyst particles are saturated, carbon atoms precipitate to form carbon nanotubes^[65]. Particularly, with carbon monoxide serving as carbon precursor, SWCNTs as small as 0.7 nm in diameter can be synthesized at pressure of 1 – 10 atm and temperature of 800 - 1200°C^[94].

As-grown SWCNTs co-exist with contaminants which are mainly amorphous carbon, fullerenes, MWCNTs, graphite particles, graphitic polyhedrons, and metal catalyst particles. The side products can be removed by treatment with chemical (gaseous, acidic, and electrochemical) oxidation, physical purification such as microfiltration and centrifugation, and combination of the aforementioned methods^[95].

2.2.3 Chiral Selectivity of Single-walled Carbon Nanotubes

Although considerable progress has been accomplished in SWCNT synthesis and SWCNTs with few impurities can be obtained, the full potential of SWCNTs in areas such as nanoscale electronics and biological sensing still cannot be fulfilled because of the unavailability of samples that are either purely metallic or purely

semiconducting, or even with specific chirality. As mentioned earlier, the electronics of SWCNTs strongly depend on their chiral vector. However, generally, SWCNTs are fabricated in batches with a wide distribution of diameter and chirality. The problem of heterogeneity prevents further SWCNT-based applications. For instance, metallic SWCNTs will hinder effective electronic switching in a field-effect transistor^[91]. Even if enriched semiconducting SWCNT fractions with small amount of metallic species are assembled in single-nanotube transistor channels, the transistor will certainly be short-circuited. In addition, the same band gap is also required, whereas for semiconducting SWCNTs, the band gap varies inversely with diameter. Therefore, separation of SWCNTs with specific chirality is the Holy Grail in CNT related field and the prerequisite for their applications in nanoelectronics. Two general methods, selective growth and post-synthetic sorting, have been applied to achieve this ultimate goal.

2.2.3.1 Selective Growth Methods

An ultimate goal of the CNT field is the direct growth of SWCNTs with controllable length, metallicity, diameter, and chirality. Although the other selection scheme, post-synthetic sorting, can provide a higher degree of separation than selective synthesis currently, selective synthesis methods are irreplaceable and highly desired because the yield of selective growth is much larger and more cost-effective than that of post-synthetic purification, and narrow-diameter or narrow- (n, m) distributed starting materials, which are necessary for some post-synthetic processes, are produced by selective growth methods.

The (n, m) distribution of SWCNTs can be controlled by adjustment of several parameters, such as the type of catalysts and catalyst supports, carbon feedstock, and

the reaction conditions (temperature and pressure). Silica-supported Co-Mo catalyst (CoMoCAT) shows great promise for synthesis of SWCNTs with narrow (n, m) distribution. Bachilo *et al.* reported, with a Co:Mo molar ratio of 1:3, (6, 5) and (7, 5) SWCNTs comprised more than 50% of the semiconducting species in their samples^[96]. Except Co-Mo catalyst, FeRu bimetallic catalyst was also developed for selective SWCNT growth. At 600°C, (6, 5) SWCNTs were produced predominately from methane CVD on FeRu catalyst. As temperature increased, SWCNTs of larger diameter were obtained and the abundance of (6, 5) became lower. At 850°C, (8, 4), (7, 6), and (7, 5) were the dominant species, with few (6, 5) SWCNTs^[97]. The influence of catalyst supports for SWCNT (n, m) selectivity was also studied. Different CoMoCAT supports including SiO₂, Al₂O₃, MgO, and TiO₂ were examined for SWCNT growth by Wang *et al.* It was shown that SiO₂ and MgO exhibited good but similar (n, m) selectivity while Al₂O₃ and TiO₂ produced MWCNT contaminants^[98], whereas Lolli *et al.* reported a different trend that by changing catalyst support from SiO₂ to MgO, SWCNTs with similar diameter but different chiral angles were obtained^[99].

By CVD using C₂H₅OH as carbon precursor (alcohol catalytic CVD, ACCVD), Miyauchi *et al.* obtained SWCNTs with close to armchair structures, i.e., distributing predominately in the higher chiral angle region^[100]. Semiconducting (n, m) abundance was also realized by using four different carbon precursors including CO, C₂H₅OH, CH₃OH, and C₂H₂ on Co-Mo catalyst. Narrowest (n, m) distribution dominant at (7, 5), (7, 6) and (8, 4) can be produced with CO while more (8, 6), (8, 7) and (9, 5) SWCNTs can be synthesized with C₂H₅OH and CH₃OH^[101]. Further investigation was conducted in the control of CO pressure. 48% of (6, 5), 28% of (7,

5) and 26% of (7, 6) chirality enrichment could be achieved when CO pressure were at 18, 4, and 12 bars, respectively^[102].

Another selective synthesis method with great potential is SWCNT “cloning” approach, which is particularly promising when SWCNT “seeds” with high chiral purity are provided as templates to grow tubes with the same chiral structures via open-end growth mechanism^[103-106].

2.2.3.2 Post-synthetic Sorting Schemes

As-synthesized SWCNTs can be further sorted to enrich specific chiral structures via various post-synthetic strategies. Typically, the chiral selection process comprises two main steps. The first stage of selection is adsorption of certain chemicals onto specific SWCNTs, such as metal particles, polymers, and biomolecules. Subsequently, some separation techniques, such as ultracentrifugation and chromatography, are utilized to divide the chemical-bound SWCNTs into different fractions, some of which may contain species with very high abundance of a single chirality.

Common aqueous surfactants, such as sodium dodecyl sulfate (SDS), sodium dodecylbenzene sulfonate (SDBS) and sodium cholate (SC), have shown diameter-dependent binding properties^[107]. Mixtures of competing cosurfactants were first demonstrated to exhibit capability of metallicity separation for SWCNTs^[108]. Selective enrichment of (6, 5) and (8, 3) SWCNTs to more than 85% was obtained from cosurfactants (typically two different surfactants) extraction on SWCNT starting materials with narrow chiral distribution, as the competitive SWCNT-binding of the two surfactants might assist the specific affinity to certain chiral structure^[108, 109]. In the extraction process, small-diameter SWCNT samples with narrow (n , m)

distribution were dispersed in different cosurfactant solutions by sonication and ultracentrifugation, resulting in chiral enrichment of SWCNTs in supernatants. Higher (n, m) abundance can be obtained by removing the surfactants and redispersing the nanotubes in the same cosurfactant solutions repeatedly. In particular, the $(6, 5)$ abundance reached up to 69% among semiconducting tubes after the third extraction iteration by using the mixture of SDS/SC (1:4). In addition, the $(7, 5)$ enriched SWCNTs up to 45% were obtained from SC dispersion^[109].

Porphyrin chemistry has also been applied on SWCNT selection through molecular recognition^[110-113]. Chiral gable-type diporphyrins were used as “nanotweezers” to select SWCNTs with certain chiralities and the degree of chiral selectivity was controlled by changing the dihedral angle between the two porphyrins. Peng *et al.* reported that 2, 6-pyridylene-bridged chiral diporphyrins preferentially extracted SWCNTs with small chiral angles, thereby enriching $(8, 3)$, $(8, 4)$, and $(9, 4)$ and decreasing the relative abundance of $(6, 4)$, $(6, 5)$, and $(7, 5)$ ^[112]. In their subsequent work, it was demonstrated that the chiral selectivity is precisely controlled by the structure of nanotweezers. In the presence of 3, 6-pyridylene-bridged diporphyrin instead of 2, 6-pyridylene-bridged diporphyrin, $(7, 5)$ SWCNTs were enriched simultaneously with $(8, 4)$ SWCNTs by sonication and ultracentrifugation in methanol, because these SWCNTs could enter the cavity of nanotweezers deeply and lead to the formation of stable complexes^[113].

Some other aromatic polymers also serve as effective chirality-selective materials. Meunier and co-workers designed a pentacenic-based amphiphile with two hydrophilic side chains to preferentially adsorb close-to-armchair tubes. High concentration of this amphiphile allowed predominant selection of $(8, 6)$ and $(8, 7)$ SWCNTs^[114]. A family of fluorene-based polymers, such as poly(9,9-

dioctylfluorenyl-2,7-diyl) (PFO), was reported to wrap SWCNTs with certain diameters or chiral angles more specifically. Nish *et al.* show that PFO is able to selectively extract six SWCNT species close to the armchair structure from HiPco samples having at least 22 different chiral species solubilized in toluene^[115]. Particularly, when CoMoCAT SWCNTs were used as starting materials, the (7, 5) abundance could reach around 60% among semiconducting tubes in which (6, 5) was the dominant species before PFO extraction. Chen *et al.* achieved enrichment of (7, 5) SWCNTs as high as 79% by using PFO to preferentially wrap (7, 5) SWCNTs in toluene from SWCNT starting materials with narrow (*n*, *m*) distribution produced by catalyst Co-MCM-41 because of its favorable binding to tubes with chiral angles larger than 24.5°^[116].

Biomolecules, especially DNA, have also been extensively studied for their excellent capability in SWCNT separation. After techniques commonly utilized in biological sciences, such as density gradient ultracentrifugation^[108, 117] and chromatography^[118, 119], are explored for sorting SWCNTs, separation of biomolecule-encapsulated SWCNTs has been achieved at chirality level. Since it was first introduced by O'Connell^[68], ultracentrifugation has been widely used in CNT purification and separation process. Based on sedimentation coefficient, conventional ultracentrifugation allows metallicity selection of SWCNTs^[120, 121]. As sedimentation coefficient depends on multiple parameters such as size, shape, density and orientation of a particle, hence hard to control, a more effective strategy called density gradient ultracentrifugation (DGU) was developed, which shows higher selectivity for SWCNTs because it exclusively depends on buoyant density. In DGU, SWCNTs are placed into a density gradient and deposited toward their respective isopycnic point under ultracentrifugal force. If SWCNTs are uniformly and identically wrapped by

encapsulating agents, the buoyant density becomes a function of only the SWCNT diameter^[122]. Therefore, SWCNTs with different diameters can be fractionated into different layers, thereby attaining rough chiral separation. SWCNTs encapsulated by surfactants such as sodium cholate led to diameter and chirality sorting by DGU, particularly when multiple ultracentrifugation iterations were utilized. After three cycles of DGU separation, the abundance of (6, 5) was enhanced nearly two times, from 43.1% to 83.6%, while (7, 5) was almost eliminated, abundance decreasing from 21% to less than 1%. Alternatively, (7, 5) could be enriched up to 58.6%, with reducing the abundance of (6, 5) to 24.3%^[108]. It should be noted that although DGU is based on diameter sorting which is able to realize the percentage of SWCNTs within 0.02 nm of the mean diameter exceeds 97%, it can concentrate certain individual (*n*, *m*) species when appropriate encapsulating chemicals and density gradient are employed. The drawback is that it is difficult to sort SWCNTs with very similar or identical diameter but different chiralities.

Compared with ultracentrifugation, DNA-encapsulated SWCNTs are separated at a higher purity level by chromatography according to their chiral structures. The combination of size exclusion chromatography (SEC) and ion-exchange chromatography (IEX) enabled enrichment of single-chirality SWCNTs from eluates at various salt concentrations. Oligo DNA d(GT)₃₀-wrapped SWCNTs were fractionated by SEC to first narrow length distribution, followed by IEX to obtain single chirality enriched SWCNTs in (6, 4), (9, 1) and (6, 5), which allowed the separation of (9, 1) from the same diameter but different chirality and much more abundant (6, 5) species^[123]. Dai and co-workers also used this combinatorial chromatography method to conduct chirality separation on HiPco SWCNTs. After ultrasonically dispersed in d(GT)₂₀ solution, three SWCNT fractions abundant in two

or three individual chiralities were obtained, namely (8, 3) and (6, 5) in fraction f17, (7, 6) and (8, 4) in fraction f25, and (10, 5), (8, 7), and (9, 5) in fraction f37. The high enrichment was demonstrated not only by photoluminescence excitation/emission spectrum, but also by fabrication of field effect transistors^[124]. In addition, they also made use of IEX to fractionate SWCNTs which grew from FeRu at 850°C and were functionalized by d(GT)₂₀ DNA oligomers. (8, 4) SWCNTs were highly enriched and became the most abundant species among semiconducting tubes whereas in the as-grown SWCNTs, (8, 4), (7, 6), and (7, 5) were the dominant species^[97].

Recently Zheng and co-workers reported that single-chirality SWCNT components were purified from ultrasonically dispersed DNA-wrapped HiPco SWCNTs by IEX^[125]. They indicated that the selection was a multifactorial process, which was determined by the DNA recognition sequences, the chemical composition and pH value of the dispersion solution, and the incubation period between the end of sonication and the beginning of the chromatography elution. After optimization of these factors, twelve highly enriched SWCNT species, including (9, 1), (8, 3), (6, 5), (7, 5), (10, 2), (8, 4), (9, 4), (7, 6), (8, 6), (9, 5), (10, 5), and (8, 7), were separated by more than 20 oligo DNA sequences, of which several sequences could selectively elute the same (*n*, *m*) species. They also demonstrated that starting materials with narrow (*n*, *m*) distribution could result in high yield of specific (*n*, *m*) species. Although this IEX sorting process, which is believed to base on electrostatic and electrodynamic interaction between DNA-SWCNT hybrids and ion exchange resin, is of very high selectivity and able to purify up to 12 SWCNT chiral components, difficulty in scaling up and high expense are its Achilles' heels^[126].

Surprisingly, in addition to short, single-stranded oligo DNA, long and double-stranded genomic DNA is also able to enrich SWCNTs of single chirality. Naik *et al.*

showed that salmon genomic DNA specifically attached to (6, 5) SWCNTs, resulting in enrichment of more than 86% of (6, 5) SWCNTs in the supernatants after ultrasonic dispersion and ultracentrifugation by using CoMoCAT SWCNTs as starting materials. This method can provide single chirality purity as high as Zheng's, with simpler separation procedures and much lower cost. However, the drawback is also obvious that salmon genomic DNA can select only (6, 5) species^[127].

Flavin mononucleotide (FMN), a redox cofactor to various oxidoreductases, can selectively wrap around certain (*n*, *m*) SWCNT species to form helical ribbon complexes through concentric π - π interaction between isoalloxazine rings of FMN and graphene sidewalls of SWCNTs. The (8, 6) SWCNTs were enriched from 5% to 17% after HiPCO tube starting materials and FMN underwent a process of sonication and centrifugation. In particular, (8, 6) SWCNT-FMN complexes are sufficiently stable against SDBS/NaCl precipitation, resulting in a dramatic increase of the abundance of (8, 6) from 17% to 85% whereas most of other chiral species were removed via salt precipitation and centrifugation^[128].

Redox molecules, such as bromine and charge-transfer aromatics, are able to preferentially absorb to SWCNTs of certain electronic structure, leading to formation of charge-transfer complex and subsequently density-based metallicity separation^[121, 129]. Nakashima and co-workers adopted the similar strategy to separate (6, 5) SWCNTs by the so-called “nanometal sinker” and density gradient centrifugation. When AuCl_4^- ions were added into SWCNT dispersion, they were reduced by most of (*n*, *m*) SWCNT species to form Au metal nanoparticles attaching to these SWCNTs, leading to the removal of these (*n*, *m*) species by DGU. However, (6, 5) SWCNTs were less sensitive to such oxidation, thereby remaining intact and becoming the lightest species after DGU. Therefore, high purity of (6, 5) SWCNTs could be

obtained by this method^[130].

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Chapter 3

Selection of Peptides for Single-walled Carbon Nanotube Binding and Chiral Separation*

3.1 Introduction and Motivation

Ever since the discovery of single-walled carbon nanotubes^[1, 2], these quasi-one-dimensional nanosized tubes have attracted extensive attention due to their fascinating electronic, mechanical, structural, optical, and thermal properties^[3], which make them extremely promising candidates in a wide range of potential applications, such as molecular electronics^[4], composite materials^[5], and biotechnology^[6]. However, a major obstacle for implementing carbon nanotubes in some applications is that it is difficult to harvest CNTs of uniform chirality, which specifies the roll-up mode of a single graphite layer by the chiral indices, (n, m) , and subsequently determines whether one SWCNT is metallic or semiconducting. To date, various techniques, including selective growth methods and post-synthetic sorting schemes, have been employed for SWCNT separation^[7-9]. The availability of single-chirality SWCNT components is still a formidable challenge, although remarkable progress has been made in obtaining narrow chirality distribution of SWCNTs in the past few years^[8, 10].

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Biological molecules, such as DNA and proteins/peptides, possess inherent molecular recognition capability for various substrates via molecular specificity. It is possible to use biomolecules to solve the SWCNT separation problem because they may selectively bind to certain (n, m) SWCNTs. In contrast to covalent functionalization, biomolecules can interact with SWCNTs with moderate physisorption so as to minimize the perturbation to SWCNTs' atomic structures. Furthermore, for many applications, such as nanocircuitry, it is necessary to directionally immobilize SWCNTs at a particular location on a substrate, resulting in ordered and controlled assembly of addressable conducting and semiconducting CNTs on surfaces. Working with biomolecules also provides a route to immobilize SWCNTs on specific substrates through the interaction between biomolecules and SWCNTs.

Peptides have been shown to selectively bind to SWCNTs of different diameters, depending on the length of the reversible cyclic peptides^[11], however, their chiral selectivity toward SWCNTs has not yet been demonstrated. Considering the fact that currently the knowledge of protein-folding prediction and surface-binding chemistry are unable to support rational design of proteins/peptides which specifically bind SWCNTs of certain chiral structures^[12], random peptide libraries, such as phage display libraries, can be screened for SWCNT chiral structure recognition, as this *a posteriori* method has been extensively utilized to evolutionarily select specific peptide sequences which bind to a wide range of inorganic materials and nanostructures^[13].

Carbon materials, including MWCNTs^[14], SWCNTs^[15-18], SWNHs^[19], fullerene^[20], and graphene^[21], are also target substrates for specific peptide binding. After using surfactant-suspended MWCNTs as targets in phage display screening, some common

features, such as having a motif rich in histidine and tryptophan and showing a quasi-symmetric-surfactant form (hydrophilic on the ends and hydrophobic in the middle), were found in CNT-binding peptide sequences^[14, 15]. For instance, Su *et al.* identified SWCNT-binding peptides having different consensus motifs^[16]. In order to avoid surfactant-SWCNT interaction, other researchers immobilized SWCNTs on certain substrates before performing phage display screening^[17, 18], forging a better interaction between phage-displayed peptides and SWCNT surface. Zheng *et al.* presented an SWCNT-binding motif SXWWXXW (where X is any amino acid) without surfactant form, again emphasizing the importance of tryptophan^[17]. In a recent work of Belcher and co-workers, one peptide, DMPRTTMSPPPR, without any aromatic moieties, still has a tri-block surfactant structure. But the binding affinity is approximately four times as high as its counterpart, HGHPYQHLLRVL, which is rich in histidine and tyrosine^[18]. Additionally, in a cell-surface display experiment, polypeptide having 5 repeats of the sequence PQAQDVELPQELQDQHREVEV was the selected SWCNT binder, which was noticeably different from other SWCNT-binding peptides^[22].

Despite the efforts to find SWCNT-binding peptides and the fact that some sequences have been identified, the differences among these binding sequences lead us to realize that the nature of peptide-SWCNT interaction is largely unveiled. One possible reason for the diversity of the SWCNT-binding peptides is because the SWCNTs used in all the phage-displayed screening experiments had a wide distribution of (n, m) , hence different structures. It is believed that phage-displayed peptides not only have affinity to various inorganic materials, but also can recognize the difference in the crystalline face, i.e., the spatial arrangement of the atoms^[12, 23]. Therefore, phage display technology could be one possible route to recognize

SWCNTs' chiral structures. Using the technique, on one hand, the underlying mechanism of peptide-SWCNT interaction may be unveiled; on the other hand, it shows great promise in separation of SWCNTs with specific chirality, which is the prerequisite for their applications in nanoelectronics.

In this chapter, we describe a combinatorial phage display screening method to select peptides that can bind to different CNT (n, m) species. CoMoCAT (7, 5) and (7, 6) SWCNT samples, with more than 30% single chirality abundance were utilized as the targets of an M13 phage display library expressing linear dodecapeptides on the pIII minor coat protein. Various analyses were performed on the selected peptides to reveal their binding features. Evidence was provided to prove their SWCNT-binding affinity.

3.2 Materials and Methods

All of the chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (USA).

3.2.1 Preparation of SWCNTs

SWCNTs of (7, 5) and (7, 6) configuration, denoted as CNT-75 and CNT-76, respectively, were produced from Co-MCM-41 catalysis by CVD and the single chirality abundance was more than 30%^[24]. The SWCNTs were purified by sonication and ultracentrifugation. As-received SWCNTs were homogeneously dispersed in 0.1% (v/v) Tween-20 at a concentration of 0.0015 w/v% (w/v% = 10 mg/ml) for CNT-75 and a concentration of 0.005 w/v% for CNT-76.

The as-received SWCNTs need to be pretreated as well-dispersed SWCNTs lead to difficulties associated with the separation of bound phages from unbound ones. After 900 μ l 1M NaCl was added into 100 μ l SWCNT dispersion, they were quickly vortexed and allowed to stand overnight to reach equilibrium. The overnight suspension was centrifuged at 16,000 g for 2 h. The SWCNT pellet was rinsed by TBS (tris buffered saline, 50mM Tris-HCl and 150mM NaCl) several times to remove most of the attached surfactants. The SWCNTs were finally suspended in TBS with different concentration of Tween-20 (TBST, Tween-20 concentration of 0.1 - 0.75 v/v%) as very fine particles without precipitation in 3 h. Now the SWCNTs were in a “fine suspension” state in which they neither aggregate into bigger bundles nor precipitate in a short time. However, the SWCNTs could be pelleted by centrifugation so that bound phage-SWCNT complex could be precipitated after centrifugation while the unbound phage were retained in the clear supernatant, resulting in the separation of bound phages from the unbound.

3.2.2 Biopanning

Phage display is initialized by “biopanning”, a directed evolutionary process to retain the desired phage and eliminate the undesired phages in the phage display library. The biopanning process is illustrated schematically in Fig. 3.1. A commercially available 12-mer phage display library (1.0×10^{13} pfu/ml, Ph.D.-12TM Phage Display Peptide Library Kit, New England BioLabs, USA) was added to the pretreated CNT-75 and CNT-76 enriched samples, respectively. The input phage, which was 1.0×10^{11} pfu (plaque forming unit), was consistent in each round. The mixture was subsequently incubated with gentle agitation at room temperature for 1 h,

followed by rinsing the SWCNT pellets 5 times with 0.1% TBST to remove any unbound phages and non-specific bound phages. Bound phages were eluted by incubating with 1 ml of 1 mg/ml bovine serum albumin (BSA) in 0.2 M glycine-HCl (pH 2.2) for 10 min with agitation and immediately neutralized with 150 μ l Tris-HCl (pH 9.1). Longer incubation is believed to reduce the infectivity. The mixture was centrifuged at 16,000g for 15 min and the supernatant containing bound phages were collected. The phage concentration of eluates was determined by titering. This was the “output phage”. In each round, the ratio of output/input phage was calculated to determine whether the binding population was enriched during the biopanning process.

The phage eluate was then amplified through bacterial infection. 500 μ l of the eluate was added into 20 ml 1:100 dilution of an overnight *E.coli* ER2738 culture (a robust F⁺ strain with a rapid growth rate and is particularly well-suited for M13 propagation, New England BioLabs, USA) in a conical flask. The infected bacteria were incubated at 37°C with vigorous shaking for no more than 4.5 h (strictly no more than 4.5 h because longer incubation probably results in deletion mutation of the displayed 12-mer peptides).

Most of the bacteria were pelleted and discarded through centrifugation at 9,000g for 10 min. The phage-rich supernatant was isolated and further purified by overnight polyethylene glycol (PEG)/NaCl precipitation (1/4 volume, 20 w/v% PEG-8000, 2.5 M NaCl) at 4°C. Precipitated phages were centrifuged and resuspended by 1 ml TBS. An additional centrifugation was performed to remove any residual bacteria. The phages were deposited again by PEG/NaCl precipitation and finally resuspended in 200 μ l TBS. This phage solution is the amplified phages, which were titered and subject to the next round of biopanning as a new “library”.

Figure 3.1. Schematic illustration of biopanning experiment against CNT-76-enriched SWCNTs.

In the second and subsequent rounds of screening, consistent quantity (1.0×10^{11} pfu) of amplified phages were exposed to SWCNT samples prepared and incubated

with gentle agitation at room temperature for 1 h. The stringency of washing was increased stepwise in 6 rounds of biopanning by raising the concentration of Tween-20 in TBST in the following order: 0.1, 0.2, 0.3, 0.4, 0.5, and 0.75. Increasing the stringency of binding interaction is necessary to retain the desired peptides and eliminate undesired peptides, and thus an evolutionary process of selection is mimicked. We adopted the stepwise increase method instead of typical method, i.e., 0.1% TBST in the first round and 0.5% TBST in the subsequent rounds^[14, 25], in order to prevent very low eluate titer in early rounds due to unexpectedly high specificity.

A control experiment was also carried out by using wild-type M13 phage (M13KE, 1.0×10^{13} pfu/ml, New England BioLabs, USA) in the first three rounds.

3.2.3 Titering

Titering is a process to quantify phage. A plaque forms on a soft agar from infection of a single bacterium by a single virus particle. Because bacteria infected by M13 phages have a longer generation time than uninfected *E.coli*, plaques appear as areas of slower-growing cells on a faster-growing bacterial lawn (the semisolid medium limits the diffusion of the progeny virus so a plaque has a limited size). Due to superinfection exclusion (i.e., the phenomenon that a virus prevents the subsequent infection of an already infected host cell), each plaque contains offspring from only one phage particle. Therefore, the number of plaques gives a count of the number of phage particles.

In titering, we also used ER2738 as *E. coli* host strain, which is a *lacZα*-absent strain for blue-white screen. Before infection, we inoculated 5 ml of LB medium (10 g Bacto-Tryptone, 5 g yeast extract and 5 g NaCl in 1 L water) with a single colony of ER2738 with shaking at 37°C until mid-log phase ($OD_{600} \sim 0.5$) as monitored with a

BioPhotometer ($\lambda = 600 \text{ nm}$, Eppendorf, Germany). At that time, 200 μl of bacteria was dispensed into individual microcentrifuge tubes. 10 μl of 10-fold serial phage dilutions was used to infect the prepared host strain. The infected bacteria were added into 3 ml melt agarose top (10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 7 g agarose in 1 L water) in a Falcon tube, and then the mixture was vortexed and poured onto LB/IPTG/Xgal plates (LB medium, 15 g/L agar, 1ml IPTG/Xgal [1.25 g IPTG and 1 g Xgal in 25 ml Dimethyl formamide]). These plates contain a combination of IPTG (isopropyl β -D-thiogalactoside) and Xgal (5-Bromo-4-chloro-3-indolyl- β -D-galactoside, Fermentas, Canada) which produce a blue color in the presence of $\Delta(lacZ\alpha)$ bacteria infected by the modified M13 phages which carry the *lacZ α* gene. Therefore, incubation of the plates at 37°C resulted in the appearance of blue plaques, indicating where engineered M13 phage, not the wild-type phage, had successfully infected a colony (Fig 3.2). The plaques on plates having 50 – 200 plaques were counted. Phage concentration is calculated by the following formula in pfu.

$$\text{Phage concentration} = \frac{\text{the number of plaques}}{10 \times \text{dilution factor}} \text{ (pfu}/\mu\text{l)}$$

Figure 3.2. Agar plate containing a bacterial lawn with infected bacteria by M13 phage. The blue plaques represent the bacterial colonies that are infected by engineered M13 phages.

3.2.4 Characterization of Binding Clones

3.2.4.1 Single Phage Clone Amplification

Phages from monoclonal clones were amplified in order to provide sufficient phage DNA for sequencing. 2 ml 1:10 dilution of an overnight *E.coli* ER2738 culture was dispensed into 15 ml centrifuge tubes, one for each clone to be amplified. The eluted phage in the sixth round didn't undergo further amplification and proper phage dilution was prepared to ensure that, after titrating, the plates had no more than 100 plaques (each plaque contains a single DNA sequence). Well-separated blue plaques were isolated by sterile pipet tips and transferred to tubes containing diluted ER2738 culture. The cultures were incubated at 37°C with harsh agitation for 4.5 h (strictly no longer).

3.2.4.2 Phage DNA Extraction

Cultures after shaking for 4.5 h were transferred into 2ml microcentrifuge tubes and bacteria were removed by centrifugation at 10,000g for 15 min. Phage in the top 200 µl supernatant was 1:1 (v/v) diluted with sterile glycerol and stored at -20°C for further applications. The upper 1200 µl supernatant was transferred to a fresh microcentrifuge tube without disturbing the bacterial pellet because any carryover of host bacteria will result in contamination of the M13 precipitation with bacterial chromosomal DNA or double-stranded bacteriophage replicative-form DNA. A second centrifugation step was always performed to ensure a clean supernatant fraction with no bacterial carryover, after which the amplified phage is ready for DNA extraction.

The phage DNA isolation was accomplished by using QIAprep Spin M13 Kit

(QIAGEN, Germany). Precipitation buffer was first used to retain phage on the silica-gel membrane of QIAprep spin column provided by the kit. Then lysis buffer was applied to release M13 single-stranded DNA and keep the DNA adsorbed to the silica-gel membrane. Wash buffer was added to prevent residual salt and ethanol from being carried over into subsequent reactions. To elute DNA, elution buffer (10 mM Tris•Cl, pH 8.5) was dispensed onto the center of the silica-gel membrane. After incubating for 10 min at room temperature, the phage DNA ready for sequencing was collected to a fresh microcentrifuge tube by centrifugation.

3.2.4.3 DNA Sequencing

Phage DNA was quantified on either a BioPhotometer or a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA). 50 - 100 ng of DNA was submitted to sequencing facilities (either Research Biolabs, Singapore, or 1st BASE, Singapore) with -96 gIII sequencing primer (5'- HOCCC TCA TAG TTA GCG TAA CG -3', 10 pmol/μl).

3.2.5 Phage-displayed Peptide Sequence Analysis

The DNA sequences were translated into displayed peptide sequences. All sequences were first analyzed by the overall frequency of amino acid occurrence (i.e., the number of times each amino acid occurs in overall selected sequences), which was compared to the overall library distribution provided by NEB to determine whether the binding sequences were significant or whether they were merely a reflection of the library.

The twenty amino acids were also grouped into several equivalency sets based on both their chemical and structural properties, as defined in Table 3.1. The overall grouping frequency was compared to that of the library values, in order to determine if any homology group contributes to the affinity and to obtain the predominant amino acid groups.

The hydrophobicity of each amino acid in each selected sequence was calculated based on Hopp-Woods scale^[26] with a window size of five amino acids as a function of amino acid position. A tri-glycine (GGG) linker sequence was added to the end of each sequence to involve the influence from the phage body. The mean hydrophobicity was also calculated for all the sequences.

Table 3.1. General amino acid grouping for statistical analysis.

Grouping	Amino Acids
Hydrophobic	A, G, I, L, V, M
(+) Charged	K, R
(-) Charged	D, E
Hydroxyl	S, T
Ring groups	F, Y, W, H
Amide groups	N, Q
Proline	P
Cysteine	C

3.2.6 Binding Affinity Assay

In this assay, the binding affinity is defined as the ratio of output/input phage. The binding affinity of all of the CNT-76 phage monoclones and M13KE to CNT-76 was tested. In the binding affinity assay, phages with the same input pfu, 5×10^8 , were added into 1 ml SWCNTs suspended in 0.5% TBST and gently rocked at room temperature for 1 h. The SWCNT fine suspension was then pelleted by centrifugation and rinsed 5 times with 0.5% TBST to remove unbound phages. Bound phages were eluted by 0.2 M glycine-HCl (pH 2.2) and immediately neutralized by Tris-HCl (pH 9.1). The pfu of eluted phages (output phages) was obtained by titering.

3.2.7 Microsphere Experiments

To obtain direct evidence that selected phages are able to bind to SWCNTs via their pIII protein, microsphere experiments were conducted. Polystyrene latex microspheres with a diameter of 10 μm were dispersed in an aqueous electrolyte, with concentration of 2.0×10^6 particles/ml (Coulter Corporation, USA). 400 μl microsphere dispersion was centrifuged at 16,000 g for 10 min to remove the electrolyte. The pelleted microspheres were resuspended by 700 μl phosphate buffered saline (PBS, pH 7.4) and 100 μl anti-M13 monoclonal antibody (100 $\mu\text{g/ml}$, GE Healthcare, USA), which specifically reacts with the bacteriophage M13 major coat protein pVIII. The antibody was allowed to coat the microspheres by an overnight incubation with gentle agitation at 4°C. The coated microspheres were then washed three times with PBS to remove the free antibody and resuspended by a mixture of 730 μl PBS, 80 μl BSA (2mg/ml) and 8 μl 10 v/v% Tween-20.

4 aliquots of the microsphere suspension were dispensed into 4 wells of a 24-well plate for 4 parallel experiments. Subsequently, different phage samples were added into each well (Well A: 1 μ l selected phage monoclonal [after six rounds of biopanning]; Well B: 1 μ l phage eluate after only one round of biopanning; Well C: no phage added; Well D: 1 μ l wild-type phage). The phages were gently shaken at room temperature for 1 h to allow the interaction with anti-M13 antibody. The unbound phages were removed by centrifugation at 16,000 g for 10 min. After resuspended by 200 μ l CNT-76 dispersion, the precipitated microsphere-antibody-phage complexes were transferred to fresh wells and incubated with gentle agitation at room temperature for 1 h. The unbound SWCNTs were removed by centrifugation at 16,000 g for 10 min. The non-specific bound SWCNTs were removed by washing the microspheres 4 times with 0.1% TBST. To remove the unwanted salt that might affect subsequent imaging, the microspheres were washed by water. The samples were suspended in 10 μ l deionized (DI) water and examined by a field emission scanning electron microscope (FESEM, JSM-6700, JEOL, Japan) at 5 kV. The microspheres were sputter coated with a thin layer of platinum prior to observation.

3.3 Results

3.3.1 Enrichment of Binding Peptides

In each round of biopanning against (7, 5) and (7, 6) enriched SWCNT samples by the 12-mer M13 phage display library, the phages were titered right after they were eluted from SWCNTs. The titer of CNT-76-specific eluate in each round was much higher than that of CNT-75-specific eluate in corresponding round, because the

concentration of CNT-76 was higher than that of CNT- 75. Results are tabulated in Table 3.2.

The ratio of output phage to input phage (output/input ratio) increased gradually because selection stringency was increased by raising the Tween-20 concentration stepwise from 0.1% to 0.75%, indicating that the desired sequences were enriched during the biopanning process (Fig. 3.3). The recovery percentage improved from 0.0156% to 0.2898% for CNT-75 and from 0.0529% to 0.8280% for CNT-76, which is 18.5-fold and 15.7-fold, respectively (Table 3.2).

Table 3.2. Titering results of biopanning experiments for CNT-75-specific and CNT-76-specific phage eluates.

	CNT-75 (pfu/ μ l)	CNT-76 (pfu/ μ l)
1 st round (0.1% TBST)	1.36×10^4	4.60×10^4
2 nd round (0.2% TBST)	3.55×10^4	1.13×10^5
3 rd round (0.3% TBST)	4.30×10^4	1.83×10^5
4 th round (0.4% TBST)	1.33×10^5	2.70×10^5
5 th round (0.5% TBST)	2.30×10^5	3.20×10^5
6 th round (0.75% TBST)	2.52×10^5	7.20×10^5

Figure 3.3. The ratio of output phage / input phage as a function of the concentration of Tween-20 for 6-round biopanning of 12-mer phage library against CNT-75 and CNT-76. No significant increase was observed in the first three rounds when M13KE phage was used as a library in the biopanning against CNT-76.

Wild-type phages can also bind targets through their pVIII major coat protein, thus exhibit non-specific affinity to the targets. Therefore, it cannot always be certain whether bound phage have specific affinity through the displayed peptides or the major coat protein. In the control experiment, M13KE phage was exposed to CNT-76 samples and three rounds of biopanning experiments were conducted. The titer of eluted M13KE was less than 1/12 of CNT-76-specific eluate in the first round and less than 1/45 of CNT-76-specific eluate in the third round. After amplified and exposed to SWCNTs again, M13KE eluate produced the same titer in the second and third rounds because they are genetically identical. This indicates that although background binding (i.e., through pVIII major coat protein) did contribute to the

affinity, the engineered pIII 12-mer peptides played the major role in SWCNT binding.

3.3.2 CNT-75 and CNT-76 Binding Sequences and Statistical Analysis

Phage ssDNA was extracted from amplified monoclonal phage after six rounds of biopanning. Fifteen monoclonal ssDNA samples of both CNT-75-specific and CNT-76-specific phage were identified by automated cycle sequencing (Table 3.3 and Table 3.4). A few sequences appeared as both CNT-75-specific and CNT-76-specific sequences, such as HSSWYIQHFPPL, HESFWYLPHQSY, and HWRQTMPMTSAP. This is not surprising because CNT-75 and CNT-76 are structurally similar and some sequences might not be specific enough to distinguish close SWCNT chiral structures. It also reflects that these sequences have high affinity to SWCNTs because they were obtained from two totally independent selection experiments. The existence of recurrent sequences probably indicates evidence of supreme binding, such as peptides 75-10, 75-11, and 76-6, whose binding energies are among the highest ones in molecular dynamics (MD) simulation results (Table 3.3 and Table 3.4). The MD simulation methods and other results will be presented in next chapter. The sequence HESFWYLPHQSY appeared five times in the CNT-76-specific pool, while for CNT-75, it was isolated only once, implying that this peptide possessed high affinity to both CNT-75 and CNT-76, but higher affinity to CNT-76 than to CNT-75. It is confirmed by MD simulation results that HESFWYLPHQSY has higher binding energy with CNT-76 than CNT-75.

Table 3.3. Binding sequences against CNT-75 isolated from the Ph.D.-12 library

Phage	Peptide sequence	Occurrence	Binding energy (kcal/mol ⁻¹)
75-1	HKLATSPWWPPI	2	-41.2 ± 2.6
75-2	HSTSYQSLRWGA	1	-36.6 ± 3.0
75-3	FPPWWHNSSGAP	1	-50.4 ± 1.0
75-4	HWGNHSHKSHPQR	2	-43.1 ± 1.3
75-5	IYAHSLANSHNS	1	-49.3 ± 4.7
75-6	HSAFWQISPPST	1	-51.9 ± 3.1
75-7	LPPWKHKTSQVA	1	-56.7 ± 2.6
75-8	HWRQTMPMTSAP	1	-62.2 ± 4.4
75-9	SVSVGMKPSRP	1	-48.1 ± 2.8
75-10	HSSQWHPMAVHR	2	-55.2 ± 4.4
75-11	HESFWYLPHQSY	2	-59.6 ± 5.3
75-12	HSSWYIQHFPPL	1	-57.4 ± 3.2

Table 3.4. Binding sequences against CNT-76 isolated from the Ph.D.-12 library

Phage	Peptide sequence	Occurrence	Binding energy (kcal/mol ⁻¹)
76-1	HSSWYIQHFPPL	3	-34.6 ± 5.0
76-2	HLWTYSAPTVPPT	1	-61.4 ± 3.9
76-3	HTQNMRMYEPWF	1	-50.9 ± 4.4
76-4	HSNWRVPSPWQL	1	-54.9 ± 3.9
76-5	HTMSSWWAGHAT	1	-46.0 ± 4.7
76-6	HESFWYLPHQSY	5	-62.5 ± 4.2
76-7	HNRWSSWDEHTP	1	-42.9 ± 4.1
76-8	HWRQTMPMTSAP	2	-52.2 ± 4.1

The frequency of each amino acid in CNT-75-specific pool and CNT-76-specific pool was calculated respectively and compared to the overall amino acid distribution

of the Ph.D.-12 library provided by NEB (Table 3.5 and Table 3.6). The most dramatic differences from the library averages are the increase in histidine, tryptophan, and serine, and the decrease in leucine and threonine. The result confirms that the selected peptides are SWCNT-specific sequences. If there is no distinct difference from the overall library distribution, the binders were probably randomly picked from the library, resulting in merely a reflection of the library. On the contrary, these amino acids, whose frequency is significantly different from that of the library, probably serve as the binding parts in the sequences. Similar analyses were also performed based on the amino acid grouping after the twenty amino acids were grouped into several equivalency sets according to their chemical and structural properties (Table 3.1). Ring group amino acids, including histidine, tryptophan, phenylalanine, and tyrosine, underwent the most significant change (Fig. 3.4), implying their important roles in SWCNT binding. A plausible reason why amino acids with ring groups manifested themselves in SWCNT binding is that they led to strong interactions with the hydrophobic surface of SWCNTs via π -stacking.

Figure 3.4. Amino acid grouping frequency of CNT-75-specific pool, Ph.D.-12 library and CNT-76-specific pool.

Table 3.5. Individual amino acid frequency of CNT-75-specific pool in percent.

Large relative changes (>100.0%) compared with the overall library distribution are highlighted. Negative signs indicate decreasing trend.

Amino Acid	CNT-75	Lib. Avg.	Change	Relative Change
A	5.4	6.0	-0.6	-10.7
C	0	0.5	-0.5	-100.0
D	0	2.8	-2.8	-100.0
E	0.6	3.1	-2.5	-80.8
F	2.4	3.3	-0.9	-27.8
G	3.6	2.6	1.0	37.4
H	13.7	6.3	7.4	117.3
I	2.4	3.4	-1.0	-30.0
K	4.2	2.8	1.4	48.8
L	3.6	9.3	-5.7	-61.6
M	3.0	2.6	0.4	14.5
N	1.8	4.6	-2.8	-61.2
P	14.9	12.2	2.7	22.0
Q	5.4	5.1	0.3	5.0
R	4.2	4.7	-0.5	-11.3
S	16.1	10.0	6.1	60.7
T	4.2	11.1	-6.9	-62.5
V	3.0	3.9	-0.9	-23.7
W	9.5	2.2	7.3	332.9
Y	2.4	3.6	-1.2	-33.9

Table 3.6. Individual amino acid frequency of CNT-76-specific pool in percent.

Large relative changes (>100.0%) compared with the overall library distribution are highlighted. Negative signs indicate decreasing trend.

Amino Acid	CNT-75	Lib. Avg.	Change	Relative Change
A	2.8	6.0	-3.2	-53.7
C	0	0.5	-0.5	-100.0
D	0.6	2.8	-2.2	-80.2
E	3.9	3.1	0.8	25.4
F	5.0	3.3	1.7	51.5
G	0.6	2.6	-2.0	-78.6
H	13.9	6.3	7.6	120.5
I	1.7	3.4	-1.7	-51.0
K	0	2.8	-2.8	-100.0
L	5.6	9.3	-3.7	-40.3
M	3.9	2.6	1.3	49.6
N	1.7	4.6	-2.9	-63.8
P	11.7	12.2	-0.5	-4.4
Q	6.7	5.1	1.6	30.7
R	2.8	4.7	-1.9	-40.9
S	13.9	10.0	3.9	38.9
T	6.1	11.1	-5.0	-44.9
V	1.1	3.9	-2.8	-71.5
W	10.0	2.2	7.8	354.5
Y	8.3	3.6	4.7	131.5

3.3.4 Binding Affinity Assay

In binding affinity assay, binding affinity is reflected by the output/input ratio (Fig. 3.5). The output/input ratios of 76-2, 76-4, and 76-6 are the highest and in the same trend as binding energies from MD simulations. The highest binding affinity of peptide 76-6 (HESFWYLP HQSY) with CNT-76 is again proved by binding affinity assay. However, the overall trend of output/input ratio does not totally agree with binding energies because in binding affinity assay, SWCNTs with chiralities other than (7, 6) may present in the samples, while in MD simulations, (7, 6) SWCNTs were the sole targets.

Figure 3.5. Binding affinity for CNT-76 of selected CNT-76 specific phages. The ratio of output/input is proportional to the binding affinity. Error bars in this figure represent standard deviation.

3.3.5 Hydrophobicity Analysis

The hydrophobicity of each amino acid and the mean hydrophobicity of all of the selected phage-displayed sequences were calculated based on Hopp-Woods scale (Table 3.7 and Table 3.8). The hydrophobicity of some representative sequences, whose occurrence is more than 1, or that is selected by MD simulations (with the best discernment of binding energy), are plotted in Fig. 3.6 for CNT-75-specific and CNT-76-specific sequences, respectively. Most of the amino acids in this analysis are hydrophobic (with value less than 0). No typical symmetric-surfactant form, which was reported by Wang *et al.*, was found in our results, although some curves (76-6 in Fig. 3.6, bottom) have two hydrophobic flanks and one base with even higher hydrophobicity. It may be resulted from the surfactant-free surface of SWCNTs in our experiment, in contrast to the surfactant-coated MWCNTs in Wang's screening experiments^[14]. We speculate that the hydrophobic property may render the peptides more suitable to interact with the hydrophobic surface of SWCNTs. The symmetric-surfactant form may be necessary for oligopeptides themselves to attach SWCNTs, however, for phage-displayed peptides, because they have already been linked to a hydrophilic phage body, they can be even more hydrophobic to maximize affinity to SWCNTs. Zheng *et al.* reported the similar phenomenon^[17], however, their hydrophobic phage-displayed peptides could not disperse SWCNTs. Contrarily, although our peptides are overall hydrophobic, they are dissolvable in water and capable of dispersing SWCNTs, thus enabling themselves to be utilized as a tool for SWCNT separation. The solubilization of SWCNTs by our selected peptides will be described in next chapter.

Table 3.7. The hydrophobicity of each amino acid and the average hydrophobicity of each CNT-75-specific sequence.

No.*	Hydrophobicity												Avg. Hydrophobicity
	1	2	3	4	5	6	7	8	9	11	11	12	
75-1	-0.5	0.23	-0.04	0.12	-0.48	-0.8	-1.38	-1.3	-1.36	-1.72	-1.04	-0.36	-0.72
75-2	-0.5	-0.2	-0.52	-0.38	-0.38	-0.66	-0.12	-0.34	-0.38	-0.54	-0.18	-0.78	-0.42
75-3	-2.5	-0.83	-1.86	-1.46	-1.42	-1.36	-0.62	0.06	0.06	0.02	-0.04	-0.1	-0.84
75-4	-0.5	-1.3	-0.84	-0.68	0.6	0.66	0.52	0.62	0.6	0.6	0.54	0.64	0.12
75-5	-1.8	-1.53	-0.96	-0.96	-0.6	-0.46	-0.3	-0.46	-0.06	0.1	0.06	0	-0.58
75-6	-0.5	-0.23	-1.32	-1.18	-1.6	-1.44	-0.94	-0.26	-0.24	0.04	-0.02	-0.02	-0.64
75-7	-1.8	-0.6	-0.44	-0.18	0.42	0.34	1.08	0.48	0.28	-0.42	-0.34	-0.4	-0.13
75-8	-0.5	-0.3	-0.22	-0.38	0.3	-0.56	-0.68	-0.54	-0.38	-0.38	-0.12	-0.04	-0.32
75-9	0.3	-0.3	-0.48	-0.8	0.1	0.04	0.4	0.4	1.26	0.66	0.66	0.6	0.24
75-10	-0.5	0.03	-0.62	-0.62	-0.68	-1	-1.14	-0.76	-0.76	-0.16	0.1	0.2	-0.49
75-11	-0.5	0.93	-0.62	-0.98	-1.94	-2	-1.6	-0.88	-0.36	-0.46	-0.46	-0.36	-0.77
75-12	-0.5	0.03	-1.12	-1.38	-1.4	-1.56	-1.38	-0.92	-0.56	-0.96	-0.86	-0.36	-0.91

Table 3.8. The hydrophobicity of each amino acid and the average hydrophobicity of each CNT-76-specific sequence.

No.*	Hydrophobicity												Avg. Hydrophobicity
	1	2	3	4	5	6	7	8	9	11	11	12	
76-1	-0.5	0.03	-1.12	-1.38	-1.4	-1.56	-1.38	-0.92	-0.56	-0.96	-0.86	-0.36	-0.91
76-2	-0.5	-1.9	-1.68	-1.52	-1.26	-0.58	-0.58	-0.42	-0.48	-0.46	-0.46	-0.38	-0.85
76-3	-0.5	-0.23	-0.36	0.34	0.16	-0.34	0.22	0.48	-0.8	-1.04	-0.58	-1.18	-0.32
76-4	-0.5	0	-0.08	-0.28	-0.34	-0.32	0.36	-0.92	-0.58	-0.94	-1	-1	-0.47
76-5	-0.5	-0.73	-0.32	-0.9	-1.5	-1.34	-1.4	-1.56	-0.98	-0.38	-0.28	-0.28	-0.85
76-6	-0.5	0.93	-0.62	-0.98	-1.94	-2	-1.6	-0.88	-0.36	-0.46	-0.46	-0.36	-0.77
76-7	-0.5	0.9	-0.08	0.08	-0.64	-0.64	0.64	0.48	0.34	1.02	0.42	-0.18	0.15
76-8	-0.5	-0.3	-0.22	-0.38	0.3	-0.56	-0.68	-0.54	-0.38	-0.38	-0.12	-0.04	-0.32

Figure 3.6. Amino acid hydrophobicity by position for representative CNT-75-specific (top) and CNT-76-specific (bottom) sequences using Hopp-Woods scale. Positive values represent hydrophobic regions. Connecting lines indicate data trend.

3.3.6 Microsphere Experiments

The association between phages and SWCNTs was demonstrated in the following figures. In Fig. 3.7A, the microspheres were coated with selected phage clone after six rounds of biopanning. This clone is believed to have affinity to CNT-76 so that it captured many SWCNTs. In Fig. 3.7B, phage eluate after the first round of biopanning was used to coat the microspheres. Much fewer SWCNTs were observed because the first round eluate possessed lower affinity to SWCNTs compared with the

selected clone. If no phage were added to the microspheres (Fig. 3.7C), a few tiny SWCNT bundles still remained on microspheres due to the Van der Waals attraction between SWCNTs and antibody encapsulated microspheres. In the last control experiment (Fig. 3.7D), selected phage clone was substituted by phage clone without pIII fusion peptides. Similar to Fig. 3.7C, a few SWCNT bundles appear because of either the Van der Waals force or background binding, or both of the two types of interaction.

Figure 3.7. FESEM images of microsphere experiments showing that SWCNTs bind to phage coated microspheres through different phages. A) Microspheres coated with selected phage clone after 6-round biopanning. The white threads are SWCNT bundles while the white particles are believed to be salt. B) Microspheres coated phage eluate after the first round of biopanning. Tiny SWCNT bundles can be observed. C) No phage coating on microspheres. Very tiny SWCNT bundles can be seen due to Van der Waals force. D) Wild-type phage coating on microspheres. Very tiny SWCNT bundles can be seen due to Van der Waals force or background binding. In all images, the scale bar is 1 μm .

3.4 Summary

In this chapter, phage display technique was used to discover peptides with affinity to different chiral structures of SWCNTs. After 6 rounds of biopanning against (7, 5) and (7, 6)-abundant SWCNT samples, 15 specific binding sequences were identified, respectively. Many of these SWCNT-binding peptides sequences are rich in aromatic amino acids, begin with histidine, and possess hydrophobic property. The SWCNT-binding affinity was demonstrated through the enrichment of binding sequences, statistical analysis, and binding affinity assay. Direct evidence of binding was provided by the attachment of SWCNT to selected phage-coated microspheres via an anti-M13 monoclonal antibody. However, the basis of the selective binding, whether it is surface recognition or diameter selection, is still uncertain. More investigations based on binding energy and discussion with regard to the mechanism of specificity to the SWCNT chirality are covered in next chapter.

3.5 References

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Chapter 4

SWCNT Chirality Sorting by Peptides Selected from Phage Display Library*

4.1 Introduction

The unique properties of single-walled carbon nanotubes make them highly promising in a wide range of applications, however, the coexistence of their heterogeneous structures prevents SWCNT-based applications, especially those requiring their exceptional electronic properties. Although the (n, m) enrichment of SWCNTs has been achieved, single-chirality SWCNT components are still barely available even after post-synthetic sorting^[1].

Biomolecules, such as DNA and proteins/peptides, have been extensively studied for their significant potential in SWCNT chiral separation in the past few years. Both oligo and genomic DNA have been demonstrated to be capable of selectively binding to SWCNTs with certain chiralities^[2-4]. Some proteins/peptides, which are able to bind to and disperse SWCNTs, show no or very weak discernment on chirality in spite of some diameter selectivity^[5-8].

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Generally, there are two ways to find specific peptide binders to certain structures – rational design and screening. Rational design is a conceptually straightforward and efficient method. However, only limited diameter-selective solubilization of SWCNTs has been realized to date by peptides devised from rational design, because of insufficient knowledge about protein-folding prediction and surface-binding chemistry. Screening also has its own drawbacks, as no SWCNT samples with just one chiral structure are available as targets for screening. This is believed to be the main reason that no chirality-specific peptides have been selected from screening as all of the SWCNT screening targets had a wide distribution of chiralities^[9-12]. Another obstacle is that, even if peptides are selected from screening library, the information about their discernment ability on chiralities is unknown. In other words, even though one peptide has the highest binding energy with one specific (n, m) structure, it may also have comparable binding energies with other chiral structures, thus chirality separation is still unachievable because such difference is technically indistinguishable.

In this thesis, we have improved the two aforementioned aspects to obtain peptides that preferentially bind to certain SWCNT chiral structures. First, we used SWCNT samples with more than 30% single-chirality abundance instead of SWCNTs with a wide distribution of chiralities for phage display screening, which was presented in the previous chapter. Second, we performed MD simulations to calculate the binding energies of each selected peptide with several (n, m) structures, which enables us to find peptides having not only high binding energy with certain chirality, but also good distribution of binding energy, i.e., the large energy difference.

In this chapter, the binding energies of all peptides selected from phage display screening to SWCNT (7, 0), (8, 0), (7, 5), (7, 6), (7, 7), (8, 8) were calculated by MD

simulations. Subsequently, the best binders selected by screening experiments and MD simulations were synthesized and used for the dispersion and separation of SWCNTs. SWCNTs can be well dispersed by selected peptides whose conformation is characterized by circular dichroism (CD) spectroscopy. Chiral selectivity was examined by Ultraviolet (UV)-visible (vis)-near-infrared (NIR), Raman, and photoluminescence spectroscopies, showing that the best binder selected by MD simulations favorably dispersed HiPco SWCNTs of large diameter such as (10, 3), (9, 5), (12, 2), (11, 4), and (10, 6). Thus we have demonstrated that peptides are capable of selectively dispersing SWCNTs and paved a new path to SWCNT chirality separation. In addition, one example of conjugation of quantum dots (QDs) to SWCNTs via synthesized peptides is given to provide a possible route to use these SWCNT-binding peptides except chirality separation.

4.2 Materials and Methods

All of the chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (USA).

4.2.1 Molecular Dynamic Simulations

Molecular dynamics simulation is a powerful tool that allows one to examine properties that are not yet accessible to experimental approaches. On one hand, theoretical and computational modeling was adopted in this study to investigate the properties of the interface between viral peptides and SWCNTs so that the mechanisms that govern the interactions between them can be unveiled. On the other hand, binding energy between SWCNTs and peptides was quantified by MD

simulations to verify the experimental results and select binders with the best binding energy discernment on chirality.

Six SWCNT (n, m) models, namely, (7, 0), (8, 0), (7, 5), (7, 6), (7, 7), and (8, 8), were constructed with the same length of 10 nm. Peptide models of extended forms were built in DeepView – Swiss-PdbViewer. The N and C termini were capped with acetyl and N-methylamide, respectively. One peptide and one SWCNT were placed in a simulation box ($12 \times 5 \times 5$ nm) with a minimum distance of 1 nm between the peptide and the SWCNT in order to eliminate interference. Single point charged (SPC) water molecules were added to completely fill up the simulation box and counter ions were added to neutralize the system. Motions of the SWCNT were restrained in the simulations while the peptide was allowed to move freely. All simulations, employing the all-atom AMBER force field, were conducted by GROMACS package version 4.0.3^[13] at 300 K for 20 ns, and the data from the last 10 ns were used for analyzing binding energies. The binding energies were estimated by Generalized Born (GB) implicit solvent model^[14] in sander module of the AMBER 9 package^[15].

4.2.2 Peptide Synthesis and Purification

Selected phage-displayed peptides, including 76-4 (HSNWRVPSPWQL), 76-6 (HESFWYLPHQSY), and 76-4 dimer (HSNWRVPSPWQLHSNWRVPSPWQL), were synthesized by commercial vendor (1st BASE Pte Ltd, Singapore) with purity (HPLC) > 95%.

4.2.3 SWCNT Dispersion by Selected Peptides

In order to study the capability of dispersing SWCNTs, the selected phage-displayed synthetic peptides were first dissolved in 1 ml DI water or deuterium oxide (D_2O) at a concentration of 1000 μM , 500 μM , or 100 μM . The peptide solution was added to HiPco and SG76 (SWeNT $\text{\textcircled{R}}$ SG76, SouthWest NanoTechnologies Inc., USA) SWCNTs (0.25 mg), respectively. The SWCNTs were dispersed by probe sonication (ultrasonicator, VCX-130, SONICS, USA) at 10W for 1 h in an ice bath. The dispersion was centrifuged at 40,000g for 1 h to remove nanotube bundles and the top 75% supernatant was collected for subsequent measurements.

4.2.4 Atomic Force Microscope Characterization

Atomic force microscope (AFM) characterization was conducted on a MFP 3D microscope (Asylum Research, Santa Barbara, USA) in AC mode to confirm whether the selected peptides are able to disperse SWCNTs into small bundles or even individual tubes. SWCNTs dispersed by peptide 76-4 was deposited onto silicon wafer and dried in a desiccator prior to observation.

4.2.5 Secondary Structure of Selected Peptides

The secondary structure of a protein/peptide can be experimentally investigated by circular dichroism (CD) spectrometry. CD is defined as the differential absorption of left (ϵ_L) and right (ϵ_R) circularly polarized light. CD is reported either in units of $\Delta\epsilon$, the difference in absorbance of ϵ_L and ϵ_R by an asymmetric molecule, or in degrees of

ellipticity, which is defined as the angle whose tangent is the ratio of the minor to the major axis of the ellipse $[\theta]$, the molar ellipticity in $\text{deg cm}^2 \text{ dmol}^{-1} = 3298 \Delta \epsilon^{[16]}$.

When the chromophores of the amides of the polypeptide backbone of proteins are aligned in arrays, their optical transitions are shifted or split into multiple transitions as a result of “exciton” interactions. The result is that highly asymmetric secondary structural elements, such as α -helices and β -sheets, possess characteristic CD spectra (Fig. 4.1). Therefore, CD can be used to estimate the structure of peptides/proteins^[17]. For example, α -helical proteins have negative bands at 222 nm and 208 nm, and a positive band at 193 nm^[18]. Proteins with extended conformations have a negative band between 190 and 200 nm^[19].

Figure 4.1. CD spectra of polypeptides and proteins with representative secondary structures^[17].

To investigate the conformation before and after binding to SWCNTs, peptides 76-4 and 76-6 were dissolved in both DI water and KH_2PO_4 buffer (100 μM , pH 7.1) at the concentration of 500 μM and 100 μM for CD measurements. CD spectrometry was also employed to study the conformation of HiPco SWCNTs dispersed by sodium dodecyl benzene sulfonate (SDBS, 0.1 w/v%), 76-4, 76-4 dimer, and 76-6. All the CD spectra were measured between 180 nm and 280 nm with quartz cuvettes having a 0.1 mm path length by ChirascanTM Circular Dichroism Spectrometer (Applied Photophysics, Surrey, United Kingdom) with constant N_2 flushing. The final spectra were obtained by subtracting the solvent baseline from the average value of triplicate measurements for each sample. The CD spectra were analyzed by an algorithm called SELCON in order to quantitatively determine the contents of each secondary structure. The secondary structure of selected peptides on SWCNT surface was also analyzed by MD simulations.

4.2.6 Photoluminescence Spectroscopy

Photoluminescence is a process in which an excited electron in a higher energy state is generated (with a paired hole. The electron-hole pair is named exciton) in a substrate by absorbing a photon, and upon relaxation of the excited electron to its ground state, a photon with lower frequency is emitted. In studies with respect to carbon nanotubes, photoluminescence spectroscopy is widely used to identify chirality^[20].

SWCNTs have a set of sharp peaks named van Hove singularities in their density of state (DOS). As shown in Fig. 4.2, van Hove singularities are labeled with v_1 , v_2 , ... v_i in valence band and c_1 , c_2 , ... c_i in conduction band. When an electron absorbs

excitation light, optical transitions occur between v_1 and c_1 as well as v_2 and c_2 , resulting in light emission. The transition energies are represented as E_{11} for transition between v_1 and c_1 and E_{22} for transition between v_2 to c_2 . SWCNT (n, m) structures can be correlated to optical transition energies in a Kataura plot^[21, 22]. Photoluminescence spectroscopy is applicable to only individually dispersed semiconducting SWCNTs. Metallic SWCNTs are not photoluminescent because when an electron in metallic SWCNTs is excited from valence band to conduction band, its paired hole is filled up by another free electron immediately. In other words, no exciton is produced. For bundled SWCNTs, very weak photoluminescence single can be detected because excited electron are transferred to adjacent tubes other than relaxing to its ground state, a process known as intertube quenching.

Figure 4.2. Schematic density of electronic states for a semiconducting SWCNT^[20].

In this study, photoluminescence of peptide-dispersed SWCNTs was measured by a Jobin-Yvon Nanolog-3 spectrofluorometer with an InGaAs detector by scanning the excitation wavelength from 500 to 800 nm and the emission wavelength from 875 to 1450 nm.

4.2.7 UV-vis-NIR Spectroscopy

Different from photoluminescence spectroscopy, which is capable of detecting only semiconducting SWCNTs, UV-vis-NIR absorption spectroscopy is able to detect both semiconducting and metallic SWCNTs. The optical absorption of SWCNTs arises from transition energies E_{11} (both metallic and semiconducting tubes) and E_{22} (only semiconducting). Therefore, UV-vis-NIR absorption spectroscopy is also suitable to identify chirality. However, this characterization method is not so effective compared to photoluminescence spectroscopy on chirality, because SWCNTs with different (n , m) structures may have similar E_{11} and E_{22} , thus significant overlap of absorption peaks may occur. In addition, bundling of SWCNTs may broaden absorption peaks.

In this study, UV-vis-NIR absorption spectra were obtained by a Varian Cary 5000 UV-vis-NIR spectrophotometer in transmission mode and quartz cells with a 1-cm path length. Absorption data were collected every nanometer between 400 and 1600 nm.

4.2.8 Raman Spectroscopy

When a monochromatic lighter encounters a molecule and interacts with the electron cloud of that molecule, a change in the molecular polarization potential or deformation of the electron cloud may occur. An electron will be excited to a virtual

energy state by absorbing an incident photon. The electron will then relax to its ground state by emitting a scattered photon. During this process, if there is no energy exchange between the incident photon and the molecule, the scattering process is named Rayleigh scattering. If energy exchange occurs between the incident photon and the molecule, the scattering process is named Raman scattering. Raman scattering with the resulting photon of lower energy is also named Stokes scattering while the one with the resulting photon of higher energy is also named anti-Stokes scattering (Fig. 4.3). Only a small fraction of photons (approximately 1 in 10^7 incident photons) have energy change in the scattering process, leading to very weak Raman signals. However, when the incident photon possesses nearly the same energy as the energy of an electron transition, the resonance Raman scattering of much stronger intensity happens.

Figure 4.3. Schematic illustration of different types of Raman scattering.

The resonance Raman scattering can be utilized to characterize SWCNTs because (n, m) SWCNTs have unique electronic band structures. When the excitation energy

of a laser source matches the band gap of an (n, m) SWCNT, a strong signal can be obtained in Raman spectrum. Therefore, ideally, each (n, m) SWCNT can be identified with Raman spectroscopy. However, a laser source equipped with continuously tunable energies is almost technically unavailable. The compromise solution is that laser sources with multiple exciting energies that match main chiral species in SWCNT samples are used to characterize SWCNTs with Raman spectroscopy.

A typical Raman spectrum of an SWCNT sample is shown in Fig. 4.4. Raman spectra are informative to identify several properties of SWCNTs from three main features: the Radial Breathing Mode (RBM), the Disorder-induced band (D band), and the Graphite-like band (G band). The RBM results from the vibration of carbon atoms in radial direction, as if the nanotube is breathing. Its position correlates with the SWCNT diameter, which can be calculated by the equation

$$\omega_{\text{RBM}} = A/d + B^{[23, 24]}$$

where ω_{RBM} is the RMB frequency (in cm^{-1}), d is the SWCNT diameter, and A and B are constants. The chirality of SWCNTs can also be determined from the RBM through the correlation between the electronic transition energies and the RBM frequencies^[24, 25]. Contrary to photoluminescence spectroscopy, Raman spectroscopy is able to detect not only semiconducting SWCNTs, but also metallic SWCNTs. It is also less sensitive to SWCNT bundling than photoluminescence spectroscopy. The G band comes from planar vibration of carbon atoms in the sidewall of a nanotube. Unlike a single peak (around 1582 cm^{-1}) of graphite G band, the G band of SWCNTs is split into G^+ peak (around 1590 cm^{-1}) and G^- peak (around 1570 cm^{-1}), corresponding to carbon atom vibrations along the axial and circumferential direction

of an SWCNT, respectively^[26]. The line shape of the G band is sensitive to the metallicity of SWCNTs. The G band of SWCNT samples with more metallic tubes usually fits with Breit-Wigner-Fano (BWF) line shape, while the G band of SWCNT samples with more semiconducting tubes fits with Lorentzian line shape^[26-28]. The D band originates from defects of SWCNTs and impurities, such as amorphous carbon^[29]. The intensity ratio of G-band to D-band is conventionally used to evaluate the quality of SWCNTs. Higher G/D ratios indicate fewer defects in SWCNT samples.

Figure 4.4. A typical Raman spectrum acquired from a HiPco SWCNT sample.

In this thesis, HiPco SWCNTs dispersed by SDBS, 76-4 monomer, 76-4 dimer were filtrated using a membrane filter with a pore diameter of 0.025 μm , respectively. Raman spectra were recorded with a Renishaw Ramanscope in the backscattering configuration over several random spots on samples using laser excitation wavelength of 633 nm (1.96 eV), with laser intensity of 2.5 – 5 mW.

4.2.9 Coupling Quantum Dots to SWCNTs

The selected peptides can be utilized not only for SWCNT chirality selection, but also for SWCNT functionalization. A schematic diagram of coupling quantum dots onto SWCNTs via a selected peptide is shown in Fig. 4.5. 5 mg (7, 6)-enriched SWCNTs (SWeNT ® SG76) were added to H₂SO₄/HNO₃ (3:1, 20 ml) and bath-sonicated for 3 h. The SWCNT dispersion was diluted 10 times, filtrated through 0.22 µm mixed cellulose ester membrane and washed with DI water five times. Dried SWCNTs were suspended in DI water. 0.01 mM highly fluorescent mercaptosuccinic acid (MSA)-capped CdTe QDs reacted with 0.5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 mM N-hydroxysuccinimide (NHS) to form CdTe-NHS ester complex. In brief, QDs, EDC, and NHS were mixed in PBS and vortexed for 1 h. 600 µl ethanol was added to the mixture, vortexed briefly and centrifuged at 8,000 g for 10 min. 900 µl TBS was used to resuspend the QDs and mixed with 100 µl synthetic peptide 76-6 (HESFWYLPHQSY). After reacting at room temperature for 1 h, the QD-peptide complex was incubated at 4°C overnight. The carboxyl-modified SWCNTs were then mixed with QD-peptide complex with gentle agitation at room temperature for 1 h. The unbound QD-peptide complex was removed by centrifugation prior to observation by confocal microscope (Zeiss LSM 510 Meta, Germany).

Figure 4.5. Schematic illustration of conjugation of QDs to SWCNTs via selected peptides.

4.3 Results

4.3.1 MD Simulations

To understand the nature of recognition of SWCNTs and the chiral selectivity of SWCNTs by peptides, MD simulations were performed on all the selected phage-displayed peptides and SWCNTs, whose chiralities are (7, 0), (8, 0), (7, 5), (7, 6), (7, 7), and (8, 8). The initial state of the peptides was set to be extended form according to the results from CD spectroscopy and the minimum distance between peptides and SWCNTs was within larger than 10 Å. During the 20 ns simulations, peptides adsorb onto SWCNTs. All of the peptides exhibit the same binding conformation, and most amino acids are in close contacts with SWCNTs with a distance of 3 ± 0.5 Å. A representative binding conformation of peptide 76-4 on a (7, 6) SWCNT is shown in Fig. 4.6. Most parts of the peptide attaching to and interacting with the SWCNT surface are in a random coil form, which is consistent with peptides' secondary structures obtained by CD spectroscopy after binding to SWCNTs. Ring groups align with the SWCNT surface in a face-to-face manner to form π -stacking, which is the

favorable and dominant interaction between SWCNTs and biomolecules, such as DNA^[30] and peptide^[31].

Figure 4.6. A) Side view and B) front view of binding conformation of peptide 76-4 (HSNWRVPSPWQL) on a (7, 6) SWCNT. Red arrows in A indicate the ring groups parallel to the SWCNT surface.

The binding energy of peptide/SWCNT complex, E_B , can be estimated with the following equation:

$$E_B = E_{\text{peptide/SWCNT}} - (E_{\text{SWCNT}} + E_{\text{peptide}})$$

where $E_{\text{peptide/SWCNT}}$, E_{SWCNT} , and E_{peptide} are the energies of the peptide/SWCNT complex, the SWCNT, and the peptide, respectively. The binding energies of peptides 76-4 and 76-6 with different (n, m) species are listed in Table 4.1. In general, the binding energies increase with larger SWCNT diameter. The binding energies between (7, 5) and (7, 6) SWCNTs are have limited differences owing to their structural similarity.

Peptide 76-6, the best binder selected from the biopanning experiment, has the highest binding energy with (7, 6) in our peptide pool, yet it shows poor discernment on chirality. In contrast, as a result of its best distribution of the binding energy and the favorable energetic interaction with large-diameter tubes, peptide 76-4 is promising to preferentially select SWCNTs with large sizes, thereby narrowing the

diameter distribution and distinguishing certain SWCNT chiralities. Therefore, peptides 76-4 and 76-6 were chosen for the following characterizations to study their capability of dispersing SWCNTs and enriching certain (n, m) species. Dimeric 76-4 was also synthesized to investigate whether longer peptide sequences have better SWCNT dispersibility and chiral selectivity.

Table 4.1. The binding energies of representative peptides with SWCNTs of different chiralities.

Peptide/ (n, m) SWCNT	Diameter (nm)	Binding energy (kcal mol ⁻¹)
76-4/(7, 0)	0.566	-35.0 ± 1.1
76-4/(8, 0)	0.635	-49.6 ± 5.0
76-4/(7, 5)	0.829	-55.3 ± 3.4
76-4/(7, 6)	0.895	-54.9 ± 3.9
76-4/(7, 7)	0.963	-64.0 ± 5.2
76-4/(8, 8)	1.100	-71.4 ± 3.6
76-6/(7, 0)	0.566	-45.3 ± 2.9
76-6/(8, 0)	0.635	-56.1 ± 4.0
76-6/(7, 5)	0.829	-59.6 ± 5.3
76-6/(7, 6)	0.895	-62.5 ± 4.2
76-6/(7, 7)	0.963	-64.0 ± 6.5
76-6/(8, 8)	1.100	-63.4 ± 3.7

4.3.2 SWCNT Solubilization

After probe-sonicated for 1 h followed by centrifugation at 40,000g for 1 h, HiPco SWCNTs were solubilized in DI water as homogenous dark dispersion (Fig. 4.7, left) by peptides 76-4, 76-4 dimer, and 76-6. However, only peptides with high concentration (500 μ M) could disperse SWCNTs. SWCNTs remained as black flocculi after sonication when 100 μ M peptide solution was used (Fig. 4.7, right).

Figure 4.7. Photograph of HiPco SWCNTs dispersed by peptide 76-4 dimer. Left: 500 μ M 76-4 dimer. Supernatant after centrifugation at 40,000 g for 1 h. Right: 100 μ M 76-4 dimer.

The AFM image of SWCNT/peptide dispersion further confirms that our selected peptides can disperse SWCNTs into small nanotube bundles and individual nanotubes (Fig. 4.8). SWCNT dispersions are stable without visible aggregates for weeks in 500 μ M peptide solution and for months in 1000 μ M peptide solution.

Figure 4.8. A) AFM micrograph of well dispersed HiPco SWCNTs by peptide 76-4 dimer at 500 μM . B) An individually dispersed SWCNT by peptide 76-4 dimer. C) AFM height profile along the red line crossing an SWCNT shown in B.

4.3.3 CD Spectroscopy

CD spectroscopy was used to study the original conformation of selected peptides and their secondary structure on the SWCNT surface. The explanation of ellipticity and $[\theta]$ is in 4.2.5. Peptides 76-4 and 76-6 were first dissolved in DI water and KH_2PO_4 buffer (500 μM , pH 7.1), respectively. The CD spectra of peptides in DI water and in buffer are almost identical, indicating that salt is unnecessary here. The CD spectra of 100 μM peptide solutions are slightly different from those of 500 μM peptide solutions due to background noise (Fig. 4.9A). Only trivial signals were detected when no peptides were in the sample (SWCNTs dispersed by SDBS), showing that the influence of SWCNTs on the secondary structure of peptides is negligible (Fig. 4.9C).

The CD spectra of free peptides 76-4 monomer, 76-4 dimer, and 76-6 at neutral pH are shown in Fig. 4.9B. Peptide 76-4 monomer exhibits a typical “random coil” conformation, which has been reported as an SWCNT binding conformation^[32, 33]. Peptide 76-6 contains mainly random coil and β structure. Despite some discrepancies, we believe that both 76-4 monomer and 76-6 interact with SWCNTs in an extended form, which is verified by quantitative analysis by an algorithm called SELCON, showing that less than 10% peptides present in α -helical structure for both 76-4 monomer and 76-6. 76-4 dimer presents the same features of CD spectrum as 76-4 monomer except that the signal strength is stronger. The CD spectra of all tested peptides after binding to SWCNTs are almost the same as those of the free peptides (Fig. 4.9C). Therefore, it is highly possible that the peptides just simply attached to the SWCNT surface without any conformational changes. It should be noted that sonication enhanced solubilization of peptides and may cause potential hydrolysis of peptides. However, in this study, probe sonication gives rise to no degradation of the peptides as the spectra are unchanged after sonication. It is reported that there was no adverse effect on the stability of 12-mer phage-displayed peptides even after tip sonication for 3 h at a power level of 20 W^[34].

Figure 4.9. The secondary structure of SWCNT-binding peptides. A) CD spectra of free peptide 76-4 at different concentrations in water at neutral pH. The curves reflect shape, not the actual ellipticity. B) CD spectra of free peptides 76-4, 76-6, and dimeric 76-4 in water at neutral pH. C) CD spectra of peptides 76-4, 76-4 dimer, 76-6 after dispersing SWCNTs in water at neutral pH.

4.3.4 UV-vis-NIR Spectroscopy

HiPco SWCNTs were solubilized in D₂O by peptides for UV-vis-NIR examination to determine whether peptides are capable of selectively dispersing SWCNTs of certain chiralities without losing their intrinsic electronic properties. The background of UV-vis-NIR spectroscopy of SWCNT is covered in 4.2.7. It is shown in Fig. 4.10 that the spectral peaks of peptide-dispersed SWCNTs shift to longer wavelength as compared to those of SDBS-dispersed SWCNTs. We attribute the red shifts to the changes of dielectric properties around the SWCNTs, reflecting a more polarizable local environment^[35-37]. Clear first (E_{11} , 800 – 1600 nm), second (E_{22} , 550 – 900 nm) van Hove transitions, and van Hove transitions of the metallic nanotubes (M_{11} , 400 – 600 nm) are presented in our SWCNT samples, indicating that our peptides non-covalently bind to SWCNTs and their intrinsic electronic properties are preserved. It is also verified by Raman spectra (Fig. 4.13). However, SWCNT chirality separation cannot be discerned from Fig. 4.10 because some peaks are broadened. Photoluminescence spectroscopy was performed to identify (n, m) species in peptide-dispersed SWCNTs.

4.3.5 Photoluminescence Spectroscopy

The chirality distribution of SDBS-dispersed and peptide-dispersed HiPco SWCNT samples was measured by photoluminescence excitation/emission (PLE) spectroscopy. Particular (n, m) species can be assigned on the two-dimensional emission-excitation maps as fluorescence peaks when debundled SWCNT's E_{11} matches E_{22} ^[20]. The spectral intensities are proportional to the corresponding (n, m) species abundance, so the relative abundance of certain (n, m) species can be deduced from the relative intensities of corresponding peaks in the emission-excitation maps.

Figure 4.10. UV-vis-NIR absorption spectra of SDBS/HiPco, 76-4/HiPco, 76-4 dimer/HiPco, and 76-6/HiPco. M_{11} represents E_{11} of metallic SWCNTs.

In Fig. 4.11, contour plots of fluorescence intensity versus excitation and emission wavelengths for different SWCNTs samples are shown. HiPco SWCNTs dispersed in SDBS have a wide distribution of chirality and the most abundant species are (8, 6) and (7, 6), which is indicated by the highest intensity (red color) (Fig. 4.11A). Compared to SDBS dispersion, SWCNTs dispersed in 76-4 monomer and dimer show a significant red shift (to longer wavelength, referring to 4.3.4) on the emission peaks, which is also evident in the UV-vis-NIR spectra. The most abundant chiral species in peptide-dispersed samples are (9, 5), (10, 3), and (7, 6), while (8, 6) is depleted. Some large-diameter tubes, such as (10, 6), (11, 4), and (12, 2) are enriched (Figs. 4.11B and 4.11C); on the contrary, these species are insignificant in SDBS-dispersed

SWCNTs. The relative abundance for each (n, m) species was shown in Table 4.2 and the graphene sheet map (Fig. 4.12). An over 20% increase on total abundance of selected (n, m) species in peptide-dispersed samples was achieved compared to SDBS-dispersed ones, indicating that the peptides preferentially bind to large-diameter tubes (above 0.916 nm), especially those with medium or small chiral angles, as underscored in Table 4.2.

The diameter selection was also demonstrated by MD simulations (Table 4.1), showing that 76-4 monomer has much higher binding energy with (8, 8) than (7, 6). However, the selection of chiral angle is not consistent with our experimental results. 76-4 monomer and dimer show no differences on dispersion preferences, implying that simply doubling or tripling the length of peptide might not be effective on chirality separation. It is noteworthy that the PLE signal of peptide-dispersed SWCNTs has more noise than that of SDBS-dispersed SWCNTs at the same concentration of SWCNT because the concentration of peptide (2 mg/ml) is much lower than that of SDBS (10 mg/ml). No spectrofluorimetric signals could be detected on peptide 76-6-dispersed SWCNT samples for reasons unknown.

Figure 4.11. PLE intensity maps for HiPco SWCNTs dispersed in A) SDBS/D₂O, B) 76-4/D₂O, and C) 76-4 dimer/D₂O.

Figure 4.12. Chirality map of HiPco SWCNTs dispersed by SDBS and peptide 76-4. Red and orange hexagons are dominant (n, m) species of SDBS-dispersed SWCNTs. The thickest blue hexagons are dominant (n, m) species of 76-4-dispersed SWCNTs. The relative abundance is deduced from Fig. 4.11.

Table 4.2. Relative abundance of (n, m) species from PLE intensity of HiPco SWCNTs dispersed by SDBS and peptide 76-4, respectively. The underscored (n, m) species are large-diameter SWCNTs with medium or small chiral angles. “/”: insignificant.

SWCNT Species	Diameter (nm)	Chiral Angle (Deg)	Relative Abundance	
			HiPco/SDBS	HiPco/76-4
(7, 5)	0.829	24.5	7.8%	5.6%
(8, 4)	0.840	19.1	11.5%	7.0%
(10, 2)	0.884	8.9	6.9%	5.6%
(7, 6)	0.895	27.5	13.3%	9.6%
(9, 4)	0.916	17.5	11.9%	8.3%
<u>(10, 3)</u>	0.936	12.7	8.3%	10.0%
(8, 6)	0.966	25.3	15.2%	8.8%
<u>(9, 5)</u>	0.976	20.6	9.2%	9.7%
(8, 7)	1.032	27.8	9.0%	8.6%
<u>(10, 5)</u>	1.050	19.1	6.7%	5.8%
<u>(12, 2)</u>	1.041	7.6	/	7.2%
<u>(11, 4)</u>	1.068	14.9	/	7.2%
<u>(10, 6)</u>	1.111	21.8	/	6.8%
Total abundance of selected (n, m) species			24.2%	46.7%

4.3.6 Raman Spectroscopy

Raman spectroscopy was utilized to determine whether the selected peptides are effective for separating metallic/semiconducting SWCNTs. As shown in Fig. 4.13, the RBM, G-band, and D-band are clearly revealed on the normalized plots, demonstrating a typical behavior of SWCNTs. No difference is observed on G-band

in the SWCNTs dispersed by peptides from the SWCNTs dispersed by SDBS, indicating that the selected peptides are not effective for metallic/semiconducting SWCNT separation. The intensity of D-band of peptide-dispersed SWCNTs is the same as that of SDBS-dispersed SWCNT. It confirms that the selected peptides non-covalently bind to SWCNTs and introduce no defects to their surface when dispersing SWCNTs. The shape of the RBM peak of peptide-dispersed SWCNTs located around 250 cm^{-1} is slightly altered and new minor shoulders appear on that peak. The change can be attributed to the preferential dispersion of large-diameter SWCNTs, such as (9, 5), (10, 3), and (12, 2), and verifies the results from fluorescence spectroscopy.

Figure 4.13. Raman Spectra of SWCNTs dispersed by SDBS, 76-4 monomer, and 76-4 dimer at 633 nm excitation. The G-band signals are normalized to the strongest peak. The inset shows the magnified RBMs and the region of new minor shoulders is highlighted in pink.

4.3.7 QD/SWCNT Conjugation

The application of phage-displayed peptides selected in this study can function more than just chirality separation. They can be used as linkers to conjugate materials of interest to SWCNTs. One example is that QDs can be attached to SWCNTs via peptide 76-6 (HESFWYLPHQSY). Fig. 4.5 illustrates the conjugation of QDs to SWCNTs by a noncovalent chemical approach. SWCNTs were dissolvable in DI water after they were carboxyl-functionalized. The selected phage-displayed peptide was chemically conjugated to CdTe QDs through EDC/NHS coupling. CdTe QDs were attached onto the SWCNTs via the peptide as a linker. As shown in Fig. 4.14, the black clump is composed by SWCNTs and they are luminous due to the conjugated QDs – direct evidence that the selected peptide has relatively high binding affinity to SWCNTs.

Figure 4.14. Optical tracking SWCNTs via a QD-functionalized synthetic phage-displayed peptide. A) Confocal fluorescence image of QDs coupled to SWCNTs due to noncovalent interaction between QD-modified peptides and SWCNTs. B) Transmission image of B. The black clumps are SWCNTs. C) The merged image of B and C. In all images, the scale bar is 10 μm .

The CNT-binding peptides may find practical applications in optically tracking CNTs and linking specific materials onto CNTs via a bi-functional peptide, in which one end is a selected CNT-binding peptide and the other end is an additional

functional domain or epitope. For instance, a bi-functional peptide, containing an SWCNT-binding domain and a trinitrotoluene (TNT)-binding domain, was integrated into a chemical sensor for detecting TNT^[38]. We believe that such CNT-binding peptides can be also utilized for biosensor, enzyme immobilization, etc., particularly in a bi-functional manner, and eventually make CNTs “application tunable” materials.

4.4 Discussion

4.4.1 The Importance of Ring Groups for SWCNT Binding

Amino acids with ring groups were demonstrated to be critical for peptide-SWCNT affinity via the π -stacking interaction from our binding sequence analysis and MD simulations. Such interaction is known to exist between aromatic compounds and graphite. Peptides with ring groups can interact with the sidewalls of carbon nanotubes in a similar manner via π electrons because a monoelectronic π orbital orthogonal to the three σ orbitals (directed along the C-C bonds) is constructed on each carbon atom, although carbon atoms are distributed in a nonplanar system^[39]. It is reported that among the aromatic amino acids based on the conventional categorization (including phenylalanine, tyrosine and tryptophan), tryptophan possesses the highest affinity for CNTs, followed by tyrosine. Phenylalanine has the lowest affinity for CNTs^[40]. The results are consistent with ours that tryptophan is the most frequent aromatic amino acid and phenylalanine appears least often in our selected sequences. The imidazole ring of histidine contains six π electrons and thus can form π -stacking interaction as well. Therefore, it also has high affinity for carbon nanotubes and is one of the most prevalent amino acids in our peptide pool.

4.4.2 Limitation of Phage Display Technique on SWCNT (n, m)

Selection

Although phage display technology is powerful to identify peptides that have affinity to numerous materials^[41] including CNTs, it is noteworthy that the nature of phage-displayed peptides and SWCNTs impedes the ideal interaction between them. The ideal condition for SWCNT recognition by phage-displayed peptides is that phage can interact with individual SWCNTs. However, the high hydrophobicity of SWCNTs drives them to aggregate into bundles in aqueous environment, where the phage display selection occurs. In order to solubilize individual SWCNTs in water, amphiphiles, such as surfactants, polymers, and DNA, must be used to encapsulate the surface of SWCNTs. It is no doubt that SWCNTs can be dispersed in water by this method. Nevertheless, it leads to another two problems. First, the dispersant molecules may interfere the interaction between peptides and SWCNTs. Second, even if phage-displayed peptides can attach to the surface of surfactant encapsulated-SWCNTs, thorough separation of bound phage from unbound phage is very difficult by methods such as centrifugation, filtration, and dialysis.

Some surfactant/co-surfactant^[42], biomolecules^[43] or polymers^[44] can selectively bind to certain SWCNT species because they have dual effects in the selection process, i.e., dispersants and selective encapsulants. However, phage cannot play such dual roles owing to their poor ability to disperse SWCNTs. Since SWCNT dispersion and selection cannot be achieved at the same time by phage, we can realize selection first by using SWCNT bundles suspended in water or immobilized on certain supporting substrates^[12, 45]. The limitation of this method is that in fact the phage-displayed peptides can just recognize different SWCNT surfaces rather than wrap around the SWCNTs because SWCNT bundles are too big for a 12-mer peptide.

In addition, if the SWCNTs cannot totally cover the supporting substrate, i.e., phage can also interact with the substrate, a negative selection is necessary to remove the substrate-binding phage in advance. Another possibility is to use magnetic separation method, which was adopted by Shiba and co-workers in phage display screening experiments on single-walled carbon nanohorns^[46]. The drawbacks of the method are: (1) the covalent sidewall functionalization necessary for magnetic separation may introduce perturbation to electronic optical properties of SWCNTs, and (2) the experimental procedures are complicated.

For (n, m) SWCNT selective binding of phage-displayed peptides, the critical problem is lack of high (n, m) -enriched SWCNT samples. So far, it is uncertain whether such (n, m) selection is based on surface recognition or diameter-related selection. If it is the latter, SWCNT films having individual nanotubes without any substrate may be necessary for the screening experiments. Unfortunately, currently the SWCNT films are not robust enough because they may disassemble in the selection experiments. Thus, an alternative for (n, m) selection by phage display is to use an SWCNT-coated substrate as the target. The substrate should be completely covered by individual SWCNTs and SWCNT bundles as small as possible so that the phage-displayed peptide can wrap around SWCNTs.

4.5 Summary

In this chapter, the interaction between selected peptides and SWCNTs was investigated computationally by MD simulations. The binding energy of SWCNT/peptide complex was calculated also by MD simulations, which confirm that the best binder selected via biopanning experiments has the highest binding energy

with (7, 6) SWCNTs in our peptide pool. Most peptides favorably bind to large-diameter SWCNTs in MD simulations. Selected peptides were synthesized and capable of dispersing SWCNTs into small bundles or individually. They were in an extended form that remained unchanged after they attached to the surface of SWCNTs, as characterized by CD spectrometry and MD simulations. One peptide, HSNWRVPSPWQL, which has the best chiral discernment in MD simulations, preferentially dispersed large-diameter SWCNTs such as (10, 3), (9, 5), (12, 2), (11, 4), and (10, 6). Thus SWCNTs' (n, m)-preferential dispersion has been achieved for the first time via peptides selected by phage display experimentally. The conception and methodology of this work present a new route towards SWCNT chirality separation.

4.6 Reference

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Chapter 5

Fabrication of All-in-one Multifunctional Phage Liquid Crystalline Fibers

5.1 Introduction

Bacteriophages, conventionally considered as infectious agents, have been extensively utilized as nanoscale platforms for material synthesis and patterning^[1-4], biological and chemical sensing^[5], display systems^[6-8], and energy storage^[9, 10], etc. Filamentous phages, such as M13 phage and tobacco mosaic virus (TMV), are attractive nanotemplates for assembly of various materials because of their high aspect ratio, monodispersed viral dimensions, well-defined structure, high-density coupling sites, and inert metabolic activity. Particularly due to their surface tunability via specific binding and chemical modifications, M13 phages are highly promising as building blocks for multifunctional materials.

A promising path for utilizing nano-materials would be through a filamentous structure. Although tremendous progress in fabricating micro- and nanofibers has been demonstrated by assembly of nanoscopic building blocks^[11-21], controllable incorporation of a multiplicity of nano-materials into a fiber to achieve functional fibrous structures remains challenging, especially with a combination of mechanical, electrical, magnetic and optical properties. Recently, multifunctional composite materials, including fibers, have gained increasing attention due to the need for the

development of new hybrids integrating multiple structural and non-structural functions^[22-25]. However, to date, only a few reports dealt with multifunctional microscopic fibers^[15, 26]. In those studies, sophisticated interdisciplinary techniques are required for fiber fabrication and functional versatility is insufficient, imposing limitations on their applications.

When enriched to a high concentration (to our knowledge at least 5 – 10 mg/ml), M13 phage particles are able to self-assemble into films or fibers with anisotropic topography, which is driven by their monodispersity and high aspect ratio. After conjugated with fluorescence protein R-phycoerythrin, M13 phage was first fabricated into micrometer-scale fluorescent fibers by extruding into a crosslinker bath through a capillary tube^[13]. It was demonstrated by Chiang *et al.* that pure virus fibers spun from a genetically modified glutamate-rich phage are mechanically strong (tensile strength of ca. 33MPa and Young's elastic modulus of ca. 3 GPa). These phage microfibers could be fluorescent or capable of gold mineralization, respectively^[15], after conjugated with QDs or exposed to Au³⁺ solution with its reductant. Silverized phage microfibers were also produced by glutamate-rich phage and exhibited antimicrobial activity^[19]. In addition, phage-based microfibers synthesized by cationic polymerization were used as tissue engineering scaffold to control cellular behavior^[21]. Despite that some functionalities have been incorporated, the phage fibers still suffer from brittleness, relatively low strength, and a lack of integration of specific functions without elaborate gene manipulation and surface modification.

Nanoscale materials have attracted extensive attention over the past few decades owing to their fascinating physical, chemical, and biological properties. Carbon nanotube is one outstanding example because the quasi-one-dimensional, nanosized

tube has unique electronic, mechanical, structural, optical, and thermal properties^[27]. Magnetic nanoparticles represent one of the most promising materials that can be utilized in analytical technology owing to their non-destructive detectability. Integrating these materials into composite fibers is expected to bring in additional functions, such as mechanical strength, electrical conductivity, and tailored magnetic property. If fluorescence is enabled, such multifunctional fibers may find applications in diagnostics and therapeutics^[28].

In this chapter, we describe a facile and versatile approach to fabricate multifunctional composite microfibers assembled by M13 phage without complicated genetic manipulations and chemical modifications, and to demonstrate its versatility as building block to produce multifunctional fibers. In brief, highly concentrated wild-type M13 phages in transparent glass capillary tubes were injected into glutaraldehyde crosslinking medium through diameter-controllable orifice to fabricate continuous and anisotropic phage fibers. The minimal diameter of the resulting microfiber could be several micrometers and the length is theoretically without limitation. The tensile strength of these fibers was slightly higher than Chiang's results^[15], indicating that they were also mechanically robust. The pure phage fibers (Type I) have intense and durable fluorescence without any modification. SWCNTs and magnetic iron oxide nanoparticles were added to M13 phage solution to obtain functional fibers with a novel core-shell structure containing conductive and magnetic materials encapsulated by a phage-enriched shell. After incorporation of SWCNTs, the mechanical properties of phage composite fibers, including tensile strength, failure strain, and Young's modulus, were significantly enhanced and the conductivity could be increased by 10 orders of magnitude, compared to $<10^{-6} \text{ S}\cdot\text{m}^{-1}$ for pure phage fibers. The liquid-crystalline phase of phages in SWCNT-blended fibers (Type II)

made the embedded SWCNTs axially align in the phage matrix. The magnetic fibers (Type III) were fabricated from M13 phage non-covalently attached with magnetic iron oxide nanoparticles, which were enclosed inside the fibers, showing that the phage fibers possess compatibility to spheric particles. No SWCNTs or magnetic nanoparticles exist on the outer surface of the phage composite fibers, so further modifications can be made to accommodate more functionalities. One proof is that when graphene was coated to Type II fibers, their electrical conductivity was dramatically enhanced. Moreover, human umbilical vein endothelial cells (HUVECs) were incubated with the composite fibers to demonstrate their biocompatibility. To our best knowledge, it is the first time that conductive, magnetic, and additionally mechanical properties to fluorescent M13 phage microfibers by this simple fabrication method are reported. Together with further surface modifications, the multifunctional phage fiber as an all-in-one toolkit is highly potential in practical applications such as sensing and tissue engineering.

5.2 Materials and Methods

All of the chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (USA).

5.2.1 Large-scale Amplification of M13 Phage

100 μ l of 10^8 pfu/ μ l wild-type M13 bacteriophage (M13KE, N0316S, New England BioLabs, USA) was added to 400 ml 1:100 dilution of an overnight *E.coli* K12 ER2738 (New England BioLabs, USA) culture with tetracycline (Fluka). The infected bacteria were incubated at 37°C with shaking for 4.5 h, followed by

centrifugation to remove bacterial cells. The phages were pelleted by an overnight PEG/NaCl precipitation (1/6 volume, 20 w/v% PEG-8000, 2.5M NaCl) at 4°C. Deposited phages were resuspended by ~10 ml DI water and underwent another PEG/NaCl precipitation process to remove any residual bacteria. After the phages were resuspended in ~1 ml DI water, PEG/NaCl was applied to obtain concentrated phages. The concentration of the resulting phages was measured by a spectrophotometer (Shimadzu UV-2450, Japan) at 270 nm with an extinction coefficient of $3.84 \text{ cm}^2 / \text{mg}$, i.e., an absorbance of 0.38 equivalent to 0.1 mg/ml of phages^[29].

5.2.2 Preparation of Phage Composite Fibers

Borosilicate glass capillary tubes (1.0 mm outer diameter, 0.5 mm inner diameter, Sutter Instrument Co., USA) were pulled into tips with a range of inner diameter from 7 to 11 μm by a Flaming/Brown micropipette puller (P – 97, Sutter Instrument Co., USA) and a microforge (Narishige, Japan). The tips were cut into a range of inner diameter from 80 to 100 μm , which was examined under an optical microscope (Leica DM IL, Germany). Liquid crystalline phage suspension (20 mg/ml) was injected into a crosslinker bath of glutaraldehyde manually at a speed of approximately 1 $\mu\text{l/s}$ through the tip of the aforementioned capillary tubes. 1, 2, 4, 6, and 8% (v/v) of glutaraldehyde were used to determine the optimal concentration for fabrication of pure phage fibers with the best mechanical properties. As-spun fibers (Type I) were incubated in glutaraldehyde for 20 min. After rinsed in DI water, phage fibers were stretched and air-dried, resulting in microfibers with a diameter of 10 – 30 μm .

Figure 5.1 illustrates the fabrication process of phage fibers and phage composite fibers.

Figure 5.1. Schematic illustration of fabrication of phage-based functional microfibers. A) A schematic presentation of a filamentous wild-type M13 phage. The crosslinking reaction occurs mainly on its major coat protein (pVIII, blue). B) Schematic designs for preparation of fluorescent, conductive and magnetic composite phage fibers with anisotropic structures. C) A schematic diagram depicting the fabrication process of an SWCNT-blended phage fiber from SWCNT-phage dispersion into a crosslinker bath.

Single-walled carbon nanotubes synthesized by CVD were purchased from Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences. As-received SWCNTs were dispersed at a concentration of 5 mg/ml in 1 v/v% Tween-20 by

sonication (ultrasonicator, VCX-130, SONICS, USA) at 20W for 30 min in an ice bath. Different amounts of SWCNTs in aqueous dispersion were then added into concentrated phage suspension to obtain a constant concentration of phage at 20 mg/ml and various SWCNT loadings of 0.25, 0.5, 1, 2, 5, 10, 15, 25, and 45 wt% (percent by weight). The mixture was vortexed, bath-sonicated briefly and allowed to stand for 1 h to reach equilibrium. The SWCNT-blended phage fibers (Type II) were fabricated by the same method described earlier using a micropipette. Magnetic iron oxide nanoparticle clusters (Bangs Laboratories, Inc., USA), which are covalently coated with goat anti-Mouse IgG (Fc) antibody, were first mixed with anti-M13 antibody and incubated with very gentle agitation at room temperature for 1.5 h. The particles were magnetically separated from the solution, resuspended by concentrated phage suspension (20 mg/ml), and incubated at room temperature for 1 h. The mixture was briefly resuspended each time before extruded into glutaraldehyde to form magnetic fibers (Type III) in the same way as described earlier.

5.2.3 Morphological Characterization

The fluorescence micrographs of phage fibers were obtained with a fluorescence microscope (Zeiss Axiovert S100, Germany) equipped with a SPOT CCD camera (Diagnostic Instruments, USA). SWCNT-blended phage fibers were immersed and fractured in liquid nitrogen to obtain cryogenic cross-sectional surfaces. The surface and cross-sectional morphology of various types of phage fibers was examined by using a field-emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Japan) at 5 kV. The fibers were sputter coated with a thin layer of platinum prior to

observation. The anisotropic structure of fibers was examined by a polarized optical microscope (POM, XJP400T, KOZO, China).

5.2.4 Mechanical Characterization

Uniaxial tensile testing of pure phage fibers and SWCNT-blended phage fibers was carried out by using a microforce tester (model 8848, Instron, USA) equipped with a 10 N static load cell. Before mounting on the testing machine, the fibers were fixed on two small pieces of paper (5 mm × 5 mm) by super glue, with a gauge length of approximately 3 mm. 10 ~ 15 samples were tested for Type I fibers under each glutaraldehyde concentration and for Type II fibers under each SWCNT loading at a loading rate of 0.06 mm/min at ambient temperature.

5.2.5 Electrical Conductivity Measurements

Two electrodes were prepared across the phage fibers (~5 mm) using conductive silver paint as the electrical contact. Electrical measurements were carried out (sweep from -3V to 3V) by using a semiconductor device analyzer (Agilent, B1500A, USA) for Type I fibers and Type II fibers under each SWCNT loading. For each SWCNT loading, 5 samples were tested to obtain an average value.

5.2.6 Cell Culture and Scaffold Preparation

HUVECs were used to investigate the cytotoxicity and biocompatibility of phage composite fibers. HUVECs were cultured in Dulbecco's modified eagle medium (DMEM, HyClone) supplemented with 4.5 g/L glucose, 4 mM glutamine and 10

v/v% fetal bovine serum (FBS, GIBCO). All the HUVECs used in this study contain a fusion gene called CFP-DEVD-FYP, which enables the cells to emit green fluorescence.

The as-spun composite fibers (1 wt% SWCNT loading and 1 mg/ml magnetic nanoparticle loading) were rinsed with DI water to remove residual glutaraldehyde and mounted onto 24-well polystyrene cell culture plates. After they were dried, the fibers were sterilized by exposing to UV light for 30 min. Confluent HUVECs were harvested by trypsin digestion and 2×10^4 cells were seeded to each well containing fibrous scaffold. All the cells were incubated under humidified condition with 5% CO₂ at 37°C. Hoechst 33342 (Invitrogen) was added to culture medium at a final concentration of 2 µg/ml to stain the nuclei of living HUVECs and incubated at 24°C for 15 min prior to observation. After 48 h of incubation, fluorescence images of cells on phage scaffolds were captured by the same fluorescence microscope mentioned earlier.

5.3 Results

5.3.1 Design and Fabrication of Multifunctional Phage Fibers

Continuous phage microfibers, 10 to 30 µm in diameter, were fabricated by wet-spinning through glass capillary tubes (Fig. 5.2A). Wild-type filamentous M13 phages were concentrated to 20 mg/ml to form a liquid-crystalline phase and injected into a glutaraldehyde bath, where the main crosslinking reaction occurred between aldehyde and amine groups on phage coat proteins^[30]. The extrudate self-assembled into a transparent fiber as the phages were being injected into the glutaraldehyde

solution, in which a period of incubation was allowed for the crosslinking reaction to complete, resulting in a Type I fiber.

To study the capability of M13 phage as a scaffold to fabricate functional composite fibers, SWCNTs and magnetic iron oxide nanoparticles were added to phage suspension. Type II fibers with the same dimensions as Type I fibers were prepared by the same wet-spinning technique discussed earlier. Prior to blending with phage, SWCNTs were dispersed in Tween-20 by sonication to obtain SWCNT suspension containing well-dispersed SWCNTs in small bundles, which were essential to improve the mechanical and electrical properties of phage composite fibers. When they were mixed, phages and SWCNTs merged into a homogenous phase from two separate phases by vortex and brief bath-sonication, without visible SWCNT precipitation. Before fabricating Type III fibers in the same way, magnetic iron oxide nanoparticles were attached to M13 phage via the specific interaction between pVIII phage major coat protein and anti-M13 antibody, which was coupled with the IgG (Fc) antibody on the magnetic particles. The SWCNT loading could be 45 wt% or higher, and the magnetic particle loading could be as high as 70 wt%, indicating that the phage fibers have good compatibility for nanofibrils and nanoparticles so that other materials with similar dimensions can also be spun into the phage fibers to achieve new functionalities.

5.3.2 Fluorescent Phage Fibers

The as-produced viral microfibers were highly fluorescent without any modification (Fig. 5.2B and 5.2C). It is believed that the bright fluorescence comes from several particular amino acids on the phage coat proteins, such as tryptophan.

The fibers become less fluorescent after blending with SWCNTs or magnetic nanoparticles, as shown in Fig. 5.2D and 5.2E. The dark regions on the images are SWCNTs or magnetic nanoparticles, respectively. However, the fluorescence intensity in Fig. 5.2D is still comparable to that of green fluorescent protein in cells. The fluorescence can be fine-tuned by blending with quantum dots or modified by surface coating with extrinsic fluorescent substances. Additionally, it is found that the fluorescence was durable, as after a one-hour constant exposure to a 70W mercury lamp (HBO 50, Carl Zeiss), only limited photobleaching had occurred; fluorescence was still sufficiently bright after a 2-second exposure time, which is routinely applicable to cell imaging (Fig. 5.3).

Figure 5.2. Macroscopic and microscopic characterizations of phage microfibers. A) Photographs of pure phage fiber (left), SWCNT-blended phage fiber (middle) and magnetic phage fiber (right). The fibers are flexible to be different shapes. B) Fluorescence image of pure phage fibers placed in an “NTU” logo. C) Fluorescence micrograph of pure phage fiber. D) Fluorescence micrograph of phage fiber with 1 wt% SWCNT loading. E) Fluorescence micrograph of magnetic phage fiber. In all images, the scale bar is 50 μm .

Figure 5.3. Photobleaching of pure phage fibers exposed to a 70W mercury lamp over a period of A) 0 min; B) 15 min; C) 30 min; D) 45min; E) 60 min at an exposure time of 400 ms. F) Fiber in E with an exposure time of 2 s, which is usually used for cell imaging. In all of the fluorescence images, the scale bar is 50 μm .

5.3.3 Anisotropic Structure and CNT Alignment

Phages in Type I fibers were aligned parallel to the long axis of the fibers. The anisotropic structure of the air-dried fibers was evidenced by the POM images (Fig. 5.4A). The fibers are bright under POM due to birefringence, showing that the fibers are anisotropic materials. The mechanism of the alignment was reported elsewhere^[31].

Figure 5.4. POM images of phage microfibers with various SWCNT loadings. A) Pure phage fiber. B) Fiber with 1% SWCNT loading, showing no visible difference to pure phage fiber. C) Fiber with 2% SWCNT loading. The dark regions are supposed to be big SWCNT aggregations. D) Fiber with 10% SWCNT loading. The fiber is black except the two flanks, indicating that no SWCNTs are in the sheath of this fiber. The two flanks in faint yellow are reflected in the inset of D at a higher magnification by the arrows. In all images, the scale bar is 50 μm .

It is interesting to see that SWCNTs embedded in the phage matrix were also oriented parallel to the long axis of the fibers. The orientation could be attributed to steric repulsion and flow/capillary forces. Here carbon nanotubes are considered as long, rigid nanorods, which have similar geometry as the M13 phage. Before they were added to phage suspension, SWCNT dispersion had an isotropic topography despite some unidirectional alignment (Fig. 5.5). The liquid-crystalline structure of phage forced SWCNTs to adapt the parallel conformation in order to reach a

favorable spatial arrangement due to the limited free space, and hindered the aggregation of SWCNTs themselves by steric repulsive interaction. On the other hand, the shear force from injection further oriented the SWCNTs when phage-SWCNT suspension was extruded through the orifice of the capillary tube. SWCNTs of either low or high contents can be aligned in the phage composite fibers (Fig. 5.6D and 5.6F), while the phages are still in liquid-crystalline phase as shown in POM images (Fig. 5.4B). The alignment of SWCNTs in phage composite fibers can be also observed on the cross-sectional surfaces after cryogenic fracture (Fig. 5.7), excluding the alignment achieved by tensile test. When SWCNT loading was lower than 1 wt%, SWCNTs in phage matrix were also in lyotropic liquid-crystalline phase, as no dark area can be seen on the POM image, yet dark areas can be found on the fluorescence image (Fig. 5.2D). In contrast, aggregation of SWCNTs arose when the SWCNT loading was increased to 2 wt% (Fig. 5.4C). Severe SWCNT aggregation blackened almost the entire fiber in the POM image, but the superficial phages were still in liquid-crystalline phase (Fig. 5.4D). No particular alignment of magnetic particles was observed in Type III fibers.

Figure 5.5. The isotropic topography of SWNTs prior to fabrication of phage composite fibers despite some unidirectional alignment. Left: SWNTs well dispersed in Tween-20. Right: SWNTs after filtration and washing by DI water. The scale bars in both the FESEM images are 100 nm.

5.3.4 Surface Morphology

The surface morphology and fractured end of phage fibers was revealed by FESEM images. As shown in Fig. 5.6A, the as-produced fibers are in uniform diameter and have relatively smooth surfaces with slight corrugation at the magnification shown. These wrinkles can provide more area for specific coupling and non-specific binding. One example is that SWCNTs can still attach to phage fibers even after several iterations of washing (Fig. 5.8). No phages or phage bundles, i.e., objects with rod-like shape, can be observed on the fracture surface, indicating that the phages were polymerized and condensed (Fig. 5.6B). The surface morphology of Type II fibers is almost identical to that of Type I fibers except that larger corrugations appear on Type II fibers (Fig. 5.6C). The incorporation of SWCNTs into these fibers is verified by the cross-sectional images (Fig. 5.6D and 5.6F). No SWCNTs are observed on the surface of Type II fibers, i.e., the bright flanks of the black fiber in Fig. 5.4D, showing a core-shell structure with a phage-enriched shell and an SWCNT-doped core. The SWCNT-free surface is also revealed at a higher magnification in Fig. 5.10A. Type III fibers have similar surface morphology to Type I and Type II fibers. No magnetic particles are on the outer surface of the fibers (Fig. 12A).

Figure 5.6. FESEM micrographs of phage composite fibers. A) Surface morphology of pure phage fibers. B) Fracture morphology of pure phage fibers. C) Phage composite fibers with 1 wt% SWCNT loading failed in uniaxial tensile testing, showing the evidence of necking. D) Enlarged image of the fracture surface in C. The embedded SWCNTs are unidirectional. E) Phage composite fibers with 15 wt% SWCNT loading failed in uniaxial tensile testing. F) Enlarged image of the fracture surface in E, showing highly oriented SWCNTs. The insets of A, B, C, and E are the high-magnified FESEM images of either surface or cross-sectional morphology.

Figure 5.7. FESEM micrographs of the cross-sectional morphology after cryogenic fracture. Left: Phage composite fiber with 1 wt% SWCNT loading. Right: Phage composite fiber with 10 wt% SWCNT loading. The scale bar is 100 nm in both images.

Figure 5.8. FESEM micrograph of SWCNTs non-specifically binding to pure phage fibers after several iterations of washing. The scale bar is 100 nm.

5.3.5 Glutaraldehyde Concentration vs. Mechanical Strength

Liquid-crystalline phages were extruded into crosslinking baths with various glutaraldehyde concentrations of 1, 2, 4, 6, and 8 wt%, in order to investigate the influence of glutaraldehyde concentration on the mechanical properties of the resulting fibers. It is shown that fibers spun under 4% glutaraldehyde possess the highest average tensile strength (37.4 MPa) and Young's modulus (2.3 GPa), with a failure strain of 2.6% (Table 5.1). The discrepancy of mechanical properties between our results and Chiang's results^[15] (tensile strength of 33 MPa, Young's modulus of 3 GPa, and failure strain of 1.3%) can be partially attributed to different phages used for fiber spinning (Ours: wild-type M13 phage; Chiang's: genetically modified M13 phage). Although we have chosen 4% glutaraldehyde for subsequent fabrication of phage composite fibers, in fact it has little effect on the tensile strength of phage fibers and only slight influence on the Young's modulus and failure strain.

Table 5.1. Mechanical properties of pure phage fibers with respect to the concentration of glutaraldehyde.

[Glutaraldehyde] (%)	Tensile Strength (MPa)	Young's Modulus (GPa)	Failure strain (%)
1	36.7 ± 17.8	2.1 ± 0.7	2.9 ± 1.0
2	37.3 ± 15.3	1.8 ± 0.5	2.8 ± 1.3
4	37.4 ± 19.0	2.3 ± 1.9	2.6 ± 2.0
6	36.6 ± 20.0	1.5 ± 0.9	2.0 ± 0.9
8	36.7 ± 16.6	1.5 ± 1.0	2.0 ± 1.7

5.3.6 Improved Mechanical Properties by Integrating SWCNTs

Carbon nanotube is an ideal reinforcement for composite materials^[32]. It is anticipated that incorporating SWCNTs into phage matrix will enhance mechanical properties of the fibers. Uniaxial tensile testing was carried out on an Instron microtester in order to investigate the mechanical properties of phage fibers with respect to SWCNT loading. Typical stress-strain curves of Type I fibers and Type II fibers with 0.5 and 1 wt% SWCNT loadings are shown in Fig. 5.9. The stress-strain curve for Type I fibers presents no plastic deformation, displaying a brittle failure behavior. A moderate SWCNT loading renders the brittle phage fibers more ductile, as indicated by the stress-strain curves and the necking phenomenon shown in Fig. 5.6C. As shown in Table 5.2, mechanical properties, which encompass tensile strength, Young's modulus, and failure strain, are significantly improved by incorporating SWCNTs into phage fibers as expected. At 1 wt% SWCNTs, the tensile strength increased by 94%, Young's modulus increased by 65%, and failure strain increased by 115%, compared with those of Type I fibers. At low SWCNT loadings, SWCNTs were dispersed in the phage matrix in a relatively homogeneous manner, providing structural integrity and carrying tensile load through interfacial shear stress transmitted from the phage matrix^[33], so that the fibers were strengthened. Crack bridging^[34] is observed on fibers with low SWCNT loadings (Fig. 5.10A), implying that SWCNTs aligning perpendicular to the crack and contribute primarily to the enhanced toughness. On the contrary, at high SWCNT loadings, SWCNTs aggregated into big bundles because of Van der Waals interaction, resulting in structural inhomogeneity and reduction of mechanical properties. It is speculated that stress concentration and severe phase segregation occur in Type II fibers under

uniaxial tension. Similar phenomenon has been observed in earlier studies, describing CNT aggregates as micron-sized flaws^[35].

Table 5.2. Mechanical properties of phage-SWCNT composite fibers

SWCNT loading (wt %)	Tensile strength (MPa)	Young's modulus (GPa)	Failure strain (%)
0	37.4 ± 19.0	2.3 ± 1.9	2.6 ± 2.0
0.25	42.8 ± 12.8	2.1 ± 0.6	3.0 ± 0.6
0.5	69.0 ± 8.9	2.8 ± 0.6	5.8 ± 1.7
1	72.6 ± 11.7	3.8 ± 1.5	5.6 ± 2.0
2	46.7 ± 13.4	2.4 ± 0.7	4.9 ± 1.1
5	43.7 ± 14.1	2.3 ± 0.9	4.1 ± 1.3
10	32.8 ± 5.9	2.1 ± 0.7	3.4 ± 0.7
15	32.1 ± 5.2	2.0 ± 0.7	2.9 ± 0.7

Figure 5.9. Stress-strain behavior of phage fibers with different SWCNT loadings.

On the fracture surfaces, protruding SWCNTs are longer in the Type II fibers with high SWCNT loadings than those in fibers with low SWCNT loadings (Fig. 5.6D and 5.6F), indicating that the interface strength between SWCNTs and phage matrix is stronger than that between SWCNTs, which is higher than the interfacial interaction between phages. In other words, larger frictional resistance makes SWCNTs embedded in phage matrix less likely to be pulled out. Although the friction between SWCNTs and phage matrix is difficult to experimentally measure or computationally calculate, we can conclude that the frictional energy between SWCNTs and phage matrix is larger than 0.22 Nm^{-1} , which is the frictional energy between SWCNTs and has been determined in our previous studies^[36].

The enhancement of Young's modulus of Type II fibers can be explained by a simple rule of mixture relation

$$Y_f = (\eta_0 \eta_1 Y_c - Y_m) V_c + Y_m \quad (1)$$

where Y_f , Y_c , and Y_m are the Young's moduli of fiber, carbon nanotube, and viral matrix, respectively, V_c is the volume fraction of nanotubes, η_0 is the orientation efficiency factor, and η_1 is the length efficiency factor^[37]. From Table 5.1, the Young's modulus of phage fiber without addition of SWCNTs is 2.3 GPa, and here we take the Young's modulus of SWCNTs as 1 TPa^[36]. Consider the case of SWCNT loading is 1 wt%, which gives $V_c = 0.24\%$ (the density of M13 phage and SWCNTs is about 0.48 g/ml^[29] and 2 g/ml, respectively), with orientation factor $\eta_0 = 1$ when SWCNTs are fully aligned along the axis of the fibers fabricated. Substituting these values into Equation 1, Y_f can be calculated as 4.6 GPa. This value falls into the variation range of the experimental result of $3.8 \pm 1.5 \text{ GPa}$, indicating that SWCNTs are fully dispersed and well aligned in most of the samples tested, which further

verifies our observation from FESEM images. Such agreement also suggests that the external load can be effectively transferred to the SWCNTs within the phage matrix, so that SWCNTs are fully utilized as reinforcement materials due to the strong SWCNT/phage interfacial interaction in our composite fibers. It is noteworthy that the variation of measured Young's modulus is attributed to both the resolution of the uniaxial tensile apparatus and the imprecise elongation of phage fibers during the drying process.

The failure strain is increased under each SWCNT loading as a result of the SWCNTs, which align along the fiber axis and bridged the cracks, thus increasing the failure strain. At high SWCNT loadings, the bridging effect is not obvious due to the aggregation of SWCNTs, leading to “pullout” and a quasi-“sword-in-sheath” type of failure, where some SWCNTs are pulled out from the aggregates while pores remained on the phage matrix (Fig. 5.10B and 5.10C). Here if SWCNTs are not dispersed well, the randomly and unevenly distributed SWCNTs in Type II fibers would cause similar failure strain and failure type (brittlelike, flat and normal to the loading axis, Fig. 5.10D) as those of Type I fibers under a mechanism proposed somewhere else^[38].

Figure 5.10. FESEM images of fracture morphology of SWCNT-blended phage fibers after stretching. A) Deformation of fiber with 1 wt % SWCNT loading. The inset is an enlarged image of the crack bridging of SWCNTs. B) Fracture surface of fiber with 5 wt % SWCNT loading, showing “pulled-out” SWCNTs and resulting pores. C) Fracture surface of fiber with 10 wt % SWCNT loading at a higher magnification, showing the “sword-in-sheath” type of failure. D) Brittlelike fracture surface of fiber with poorly dispersed SWCNTs. Scale bars are 100 nm in A and C, and 10 μm in B and D.

Therefore, we successfully introduced much better mechanical properties, i.e., more robust and more ductile, to phage fibers by adding SWCNTs with little perturbation to their pristine structure, thus remaining their role of functional template and simultaneously broadening their potential applications. We believe that incorporating longer carbon nanotubes with fewer defects to the fibers would further improve their mechanical properties.

5.3.7 Electrical Conductivity from Embedded SWCNTs

Another functionality we have achieved on the phage fibers is electrical conduction. Adding SWCNTs into phage fibers can significantly improve the electrical conductivity of the phage fibers. The conductivity of the fibers with various SWCNT concentrations was evaluated by a two-probe technique using a semiconductor analyzer and plotted against SWCNT loading. Both Type I and Type II fibers exhibited linear current-voltage (I - V) feature, represented by the ohmic behavior in resistance (Fig. 5.11, inset). The pristine phage fibers were non-conductive owing to the insulating nature of M13 phage. After SWCNTs were integrated, an interconnected network consisting of numerous SWCNT pathways formed within a phage fiber. The network is like a conductive core in a porous phage scaffold, which provides a platform for the formation of the percolative network, thereby rendering the fiber a new functionality.

Addition of 0.25 wt% SWCNTs into phage fibers resulted in over three-order-of-magnitude improvement in electrical conductivity. With increasing SWCNT contents, more connecting pathways are formed so that the electrical conductivity increases with higher SWCNT loadings (Fig. 5.11). The maximum electrical conductivity obtained is $1790 \text{ S}\cdot\text{m}^{-1}$ at 45 wt % SWCNTs, which is ten orders of magnitude increase than that of pure phage fibers and comparable to most of other as-produced microfibers spun by biomolecules/biopolymers^[39-42]. Good dispersibility of SWCNTs is crucial for high conductivity, because aggregated SWCNTs in big bundles lead to a less effective network, i.e., less tunneling pathways and more bulky SWCNTs confined within non-conductive phage matrix. At 15 wt% SWCNTs, the conductivity of Type II fibers from carboxyl-SWCNTs suspended in DI water after sonication without surfactants was three-order of magnitude lower than those spun from well

dispersed SWCNTs. As such, we have introduced electrical conductivity to phage fibers as high as $12.9 \text{ S}\cdot\text{m}^{-1}$ (15 wt% SWCNT loading) with almost no adverse effect on tensile strength compared with Chiang's results^[15]. This value is also over 200 times that of phage fibers fabricated from water-soluble conductive polymer^[43]. It offers a new feature to phage fibers, which may be put in applications where an electrical field is favorable, such as muscle and nerve repair.

Figure 5.11. Electrical conductivity of Type II fibers as function of SWCNT mass fraction. Error bars in this figure represent standard deviation. Inset: typical current-voltage characteristic plot of SWCNT-blended phage fibers.

5.3.8 Magnetic Properties and All-in-one Tool

Type III fibers (Fig. 5.12A) gain magnetic property from magnetic particles and fluorescence from phages at the same time. It is shown in Fig. 5.12C that Type III fibers suspended in water (left) and could be collected by an external magnet (right), thereby demonstrating their magnetic property.

Figure 5.12. A) FESEM micrograph of a magnetic fiber and its fractured surface. The surface morphology is similar to that of Type I and Type II fiber. B) FESEM micrograph of fracture surface of phage composite fibers with both SWCNTs and magnetic particles. C) Magnetic fibers suspended in water without external magnetic field (left) and attracted by an external magnet (right). The blue parts in C are background objects. The inset of A is the high-magnified FESEM image of the fractured surface of magnetic fibers. Small particles can be observed on the fracture surface.

We have also fabricated phage fibers with both SWCNTs and magnetic nanoparticles. SWCNTs can be observed on the fracture surface with the same morphology as that of magnetic fibers from the cross-sectional image (Fig. 5.12B). These fibers had fluorescence, magnetism, improved electrical conductivity, and moderate mechanical properties. Other functional materials can also be added to the composite fibers to achieve new functions. It is also noteworthy that phage fibers can be further decorated by coating new materials because there are no doping materials on the outer surface of the fibers. Recently we attached graphene to Type II fibers with 2 wt% SWCNT loading and the electrical conductivity was increased by 3 orders of magnitude. Additionally, more specific binding sites through gene modification can be integrated to the phage fibers, thereby capable of involving more functions. It is believed that one single hybrid integrating several different functions can serve as an all-in-one tool, a more efficient and versatile system for carrying out different tasks in parallel. For example, highly fluorescent phage fibers may be produced, which can be visualized and sufficiently robust to be manipulated by using an external magnetic field.

5.3.9 Biocompatibility

The cytotoxicity of phage composite fibers was evaluated by cultivating HUVECs on phage fibrous scaffolds (Fig. 5.13), in order to obtain information on the biocompatibility of this material. The fibers were still wet when fixed to the surface of 24-well plates, so they were much thicker than those air-dried. HUVECs consistently emit green fluorescence due to the C3 sensor protein encoded by the CFP-DEVD-YFP fusion gene^[44]. Hoechst 33342, a fluorescent dye specifically

labeling DNA of living or fixed cells, were used to counterstain the nuclei of living HUVECs. As shown in Fig. 5.14, HUVECs adhered to, spread on and grew on both SWCNT-blended fibers and magnetic fibers without visible difference to those growing on polystyrene cell culture plates, indicating that phage scaffold is a suitable physical support for cell adhesion and growth. Cells exhibited bright green fluorescence and displayed the typical morphology of endothelial cells, which are flat and have a central nucleus. Presence of strong fluorescence further confirms that HUVECs were alive and metabolically normal. Therefore, the phage composite fibers are demonstrated to be non-toxic for cell adhesion and proliferation as no apparent adverse toxicity effects were seen on cells growing on the fibers. Moreover, the fluorescence from phage fibers may help to track and locate the cells. Combined with their electrical, magnetic, and mechanical properties, the non-cytotoxicity provides new chances to the robust phage composite fibers in tissue engineering and biomedical devices.

Figure 5.13. Photograph of the fibrous scaffold for cell culture. SWCNT-blended fibers are placed in parallel and dried in a well of a 24-well plate.

Figure 5.14. Adhesion and growth of HUVECs after 48 hours of proliferation on phage composite fibers with 1 wt % SWCNT loading and magnetic phage fibers, showing the fibers are non-toxic. The green fluorescence from CFP-DEVD-YFP4 fusion protein and the blue fluorescence from Hoechst 33342 were recorded from the same observation field in the green and blue channel, respectively. The blue fluorescence from nuclei of cells on fibers was not obvious in the merged images owing to the intense background fluorescence from phage fibers. The inset is the fluorescence image of one HUVEC at a higher magnification. In all of the fluorescence images, the scale bar is 100 μm .

5.4 Summary

In this chapter, we have successfully fabricated multifunctional liquid-crystalline phage microfibers that incorporate SWCNTs and magnetic nanoparticles by a facile wet-spinning method. Our results show that fluorescence is evident and durable for all phage fibers except those with high SWCNT loadings. The mechanical strength can be enhanced by almost 100% and the electrical conductivity can reach up to $12.9 \text{ S}\cdot\text{m}^{-1}$ with retention of the mechanical properties by using 1 wt% and 15 wt% SWCNT loadings, respectively. SWCNTs embedded in the phage matrix aligned parallel to the long axis of the fibers. No additive materials appeared on the exterior shell of phage composite fibers, indicating that the superficial phages were intact and capable of providing specific binding sites for surface modification. The phage composite fibers are also demonstrated to be cytotoxicity-free and good physical support for cell adhesion and growth. These fibers may be utilized in the fields of tissue engineering and biomedical devices. In spite of some scaling-up limitations, it is believed that the phage fibers can be developed into versatile templates to integrate different functions into an all-in-one hybrid.

5.5 References

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Chapter 6

Conclusions and Future Research

In this thesis, we focus on a filamentous virus – M13 bacteriophage and a novel quasi-one-dimensional material – SWCNT. The M13 phage was utilized in two aspects with SWCNTs. From the viewpoint of life sciences, it was used as a display system to select desired peptides with (n, m) specific binding affinity to SWCNTs. From the viewpoint of materials sciences, it was regarded as a proteinaceous nanoparticle for the fabrication of multifunctional composite materials, of which SWCNT was an important constituent.

Combinatorial M13 phage display library expressing variable dodecapeptides on pIII minor coat protein was adopted for selecting peptides capable of SWCNT chirality separation, based on the assumption that binding affinity between SWCNTs and phage peptides are sensitive to amino acid sequences. Fifteen specific binding sequences were identified after six rounds of an evolutionary phage display screening process against (7, 5) and (7, 6) enriched SWCNT “fine” suspension, respectively. Most of the binding sequences are rich in aromatic amino acids, begin with histidine, and they are overall hydrophobic in nature. The SWCNT-binding affinity was demonstrated by the enrichment of binding sequences, statistical analysis, and binding affinity assay. Direct evidence of binding was provided by the attachment of SWCNTs to selected bacteriophage-coated microspheres via an anti-M13 monoclonal

antibody and by the attachment of CdTe QDs coupled to SWCNTs by the selected peptides.

MD simulations were performed on all of the selected phage-displayed peptides with SWCNTs of chirality (7, 0), (8, 0), (7, 5), (7, 6), (7, 7), and (8, 8) to calculate the binding energies and reveal the binding conformation. The binding energies of SWCNT/peptide complex confirmed that peptide 76-6 (HESFWYLP HQSY), the best binder from (7, 6)-specific peptide pool selected by the biopanning experiment, has the highest binding energy with (7, 6) SWCNTs. The binding energies of selected peptides have limited differences between (7, 5) and (7, 6) because they are structurally similar. Most selected peptides favorably bind to large-diameter SWCNTs. Peptide 76-4 (HSNWRVPSPWQL) was selected by MD simulations for chirality separation owing to its best distribution of binding energy and favorable energetic interaction with large-diameter tubes. All of the selected peptides bind to SWCNTs in an extended conformation with ring groups attaching to the SWCNT surface in a π -stacking form. The peptides' secondary structures were also experimentally measured by CD spectroscopy, showing that both before and after binding to SWCNT, the peptides were in an extended form without conformational changes, which is consistent with the results of MD simulations.

Peptides 76-6, 76-4 monomer (12-mer), and 76-4 dimer (24-mer) were synthesized to disperse SWCNTs and separate chiral structures. The peptides are able to disperse SWCNTs into small bundles and individual tubes without losing their intrinsic electronic properties. Photoluminescence results show that both monomeric and dimeric 76-4 preferentially disperse large-diameter SWCNTs such as those with chirality of (10, 3), (9, 5), (12, 2), (11, 4), and (10, 6). Therefore, (n , m)-preferential

dispersion of SWCNTs has been experimentally achieved for the first time via peptides selected by phage display.

When M13 phages are enriched into high concentrations (>5 mg/ml in this study), they can form a liquid crystalline phase, i.e., phages aligning along their long axis. We have made use of this behavior to fabricate diameter-controllable multifunctional M13 composite microfibers via incorporating SWCNTs and magnetic iron oxide nanoparticles into these nanoscale building blocks because the geometry of nanotubes and nanoparticles is compatible with such a liquid-crystalline molecular structure. The fibers were produced by injecting highly concentrated phage with/without SWCNTs and magnetic nanoparticles into a glutaraldehyde crosslinking bath. The diameter of the phage fibers is $10 - 30\ \mu\text{m}$ and the length is theoretically without limitation. The as-produced fibers are in uniform diameter and have relatively smooth surfaces with slight corrugation. Pure phage fibers and composite phage fibers possess high and durable natural fluorescence except those with SWCNT loadings higher than 10 wt%.

Pure phage fibers exhibit no plastic deformation under uniaxial tension, displaying a brittle failure behavior. SWCNTs are able to align themselves parallel to the long axis of the fibers in the phage matrix rather than the prior isotropic topography. Moderate SWCNT loadings of 0.5 and 1.0 wt%, render the brittle phage fibers more ductile, as indicated by the larger failure strain and necking phenomenon. For these cases, SWCNTs are relatively homogeneously distributed in the phage matrix. At 1 wt% SWCNT loading, the mechanical properties of the phage composite fibers are optimized: 94% increase in tensile strength, 65% increase in Young's modulus, and 115% increase in failure strain, as compared with those of pure phage fibers. At high

SWCNT loadings, such as 10 wt% and above, the mechanical properties are deteriorated due to the severe aggregation of SWCNTs.

Pure phage fibers are insulating. Addition of 0.25 wt% SWCNTs into phage fibers results in an over three-order-of-magnitude improvement in electrical conductivity. At 45 wt% SWCNT loading, the electrical conductivity is $1790 \text{ S}\cdot\text{m}^{-1}$, which is 10 orders of magnitude higher than that of pure phage fibers and comparable to most of other as-produced microfibers spun by biopolymers.

Magnetic phage fibers were fabricated after magnetic nanoparticles were attached to M13 phages via conjugated anti-M13 antibody. When these fibers are suspended in water, they can be collected by an external magnet, thereby demonstrating their magnetic property. Moreover, the phage composite fibers are non-cytotoxic and suitable physical supports for cell adhesion and growth, as HUVECs growing on the fibers were in a normal morphology and no apparent adverse toxicity effects were seen on the cells.

The phage composite fibers possess a novel core-shell structure, i.e., no SWCNTs or magnetic nanoparticles exist on the outer surface of the fibers, so further modifications can be incorporated for more functionalities. Therefore, the phage-based multifunctional composite fibers, integrating fluorescence, electrical conductivity, magnetism, improved mechanical properties, biocompatibility, and surface functionalization sites, have been produced by a facile fabrication method for the first time, and it offers a new prospect of an all-in-one tool, as well as potential applications ranging from tissue engineering to biomedical devices.

In summary, on one hand, a methodology to attain peptides with chirality selectivity for SWCNTs is presented in this thesis. Phage display technique was

adopted to selected peptide candidates for MD simulations, which were performed to calculate binding energies and advise peptides with good discernment on binding energy. Subsequently, the selected peptides were synthesized and employed for SWCNTs dispersion and chirality separation. On the other hand, as a nanoscale building block, M13 phage was fabricated into naturally fluorescent microfibers with well-ordered molecular structure. Rod-like materials, such as SWCNTs, and small spheres, such as nanoparticles, are compatible with such a structure, thereby integrated into phage microfibers to provide new functionalities. Consequently, the M13 phage composite fibers are built into all-in-one tools, possessing several different functions and promising for carrying out different task in parallel.

Despite these accomplishments so far, the following studies are proposed for future research:

(1) Improving the biopanning process

SWCNTs' hydrophobic nature impedes effective phage display screening. If no surfactants attach to the surface of SWCNTs, SWCNTs aggregate into bundles, which is too large to be wrapped around by a phage-displayed peptide. Thus the only way for peptides to bind to SWCNTs is simply attaching on the surface in an extended conformation. If SWCNTs are dispersed in aqueous solutions by surfactants, the peptide-SWCNT interactions are interfered by the surfactants. One resolution for this dilemma is to fabricate an SWCNT nanomesh with individual tubes as the screening target, which is free-stand in aqueous solutions and robust enough not to disassemble during the screening experiments.

Phage display technique also has its own shortcomings on SWCNT chirality selection currently. Some researchers claim that the pH-driven elution method in customary phage display screening is not suitable for selecting inorganic materials and nanostructure binders. PCR-driven approach or rolling circle amplification (RCA) technique is an alternative for this issue, based on the premise that the strongest binders are never eluted from the inorganic targets. In these two techniques, after interacting with phage display library, SWCNTs will be intensively washed and eluted by conventional elution buffer to remove even the strong binders. The DNA of strongest binders will be released by proteases or heat, and then amplified by PCR or RCA to obtain the genetic information of the binding peptides. The strongest binding phages can even be regenerated by transforming the RCA products (the whole phage genome) into competent M13 phage host bacterial strains.

(2) Using SWCNTs with higher single-chirality abundance for phage display screening experiments.

(3) Using density gradient ultracentrifugation and chromatography to further separate single chirality enriched fractions from peptide-dispersed SWCNTs.

(4) Mass production of selected peptides

Currently the full experimental investigation on chirality selection by peptides is hampered because of the high cost of synthetic peptides. It is also the bottleneck for scaling-up the separation. Mass production of peptides in recombinant *E.coli* is the best way to solve this problem.

(5) Upscaling the fabrication of phage composite fibers

The fabrication of phage composite fibers is currently in a small scale because large quantities of M13 phages are required to spin numerous fibers or long fibers. Thus, producing sufficient M13 phage is the basis for upscaling the fabrication. Efforts are suggested to make to the following aspects. First, the volume of M13 phage culture can be increased. Second, the culture duration can be extended (only applicable to wild-type M13 phage). Third, the recovery percentage of M13 phage from culture solution is less than 40% by the conventional amplification and purification method. A more efficient technique or improved conventional method is necessary to replace the current one. Fourth, the fabrication process should be conducted automatically.

(6) Fabricating nanometer-scale phage composite fibers

The phage composite fibers can be fabricated into nanometer scale to broaden its applications. One facile method is electrospinning.

(7) Integrating more functionalities to phage composite fibers

Materials, which are compatible with the well-ordered liquid-crystalline molecular structure, can be integrated as fillers for new functionalities. Functional materials can also be attached to the surface of phage fibers by various methods, such as covalent modification and nanoparticle nucleation.

Appendix

List of Publications

1. Ting Yu, Yingxue Gong, Tingting Lu, Li Wei, Yuanqing Li, Yuguang Mu, Yuan Chen, Kin Liao, Recognition of carbon nanotube chirality by phage display. *RSC Advances* **2012**, 2, 1466 - 1476.
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