# Chapter 3

# Anti-fullerene SCfV Antibodies

## INTRODUCTION

Little research has been done using the recognition properties of proteins to assemble nanotubes into functional architectures, despite the fact that proteins are the same magnitude in size as nanotubes (~20 nm wide) and can be designed to have remarkably high specificity for binding. In the body, proteins routinely distinguish between chiral forms of molecules, a task that is achieved by chemists less efficiently and often at a high cost.

The immune system can be used to generate antibodies that bind to targets with a high specificity, and previous work by Erlanger generated IgG antibodies in mice that bind to C60. We expressed this antibody as a single-chain variable fragment (SCfV) in *E. Coli* by making a gene that encoded the protein sequence of the anti-fullerene antibody binding pocket. It seemed a likely hypothesis that the anti-fullerene SCfV would selectively bind to the ends of single-walled carbon nanotubes because the original antibody was selected against a target that has the $sp^3$ binding character of a fullerene that is found at the ends of nanotubes. Even if the protein does not bind exclusively to the ends, the crystallization of the antibody-C60 complex showed that the antibody has aromatic cavities that can form $\pi$-$\pi$ stacking interactions with other hydrophobes, so it is possible that it could interact with the tubular section of a nanotube as well.

Furthermore, some of the single-chain fragment antibodies employed in this project were designed to form dimers and trimers in order to test whether nanotube

junctions could form upon the binding event. A trijuction of nanotubes bound by a monospecific trivalent protein is shown in **Fig. 3.1**.

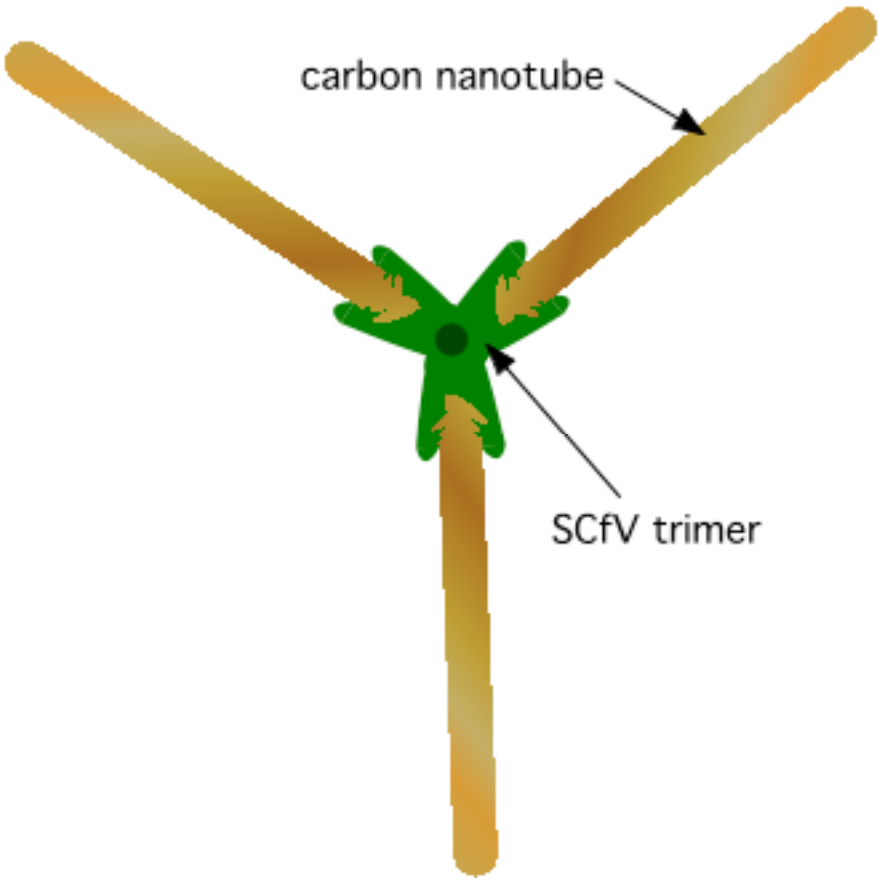


FIGURE 3.1 Hypothetical case where a trijunction of carbon nanotubes is formed by a SCfV antibody.

Binding of nanotubes by protein could change the conductivity of the nanotube or seize it with such affinity that it could be purified from mixtures of different carbon species. We hope to perform single-molecule conductivity measurements on SWNTs to determine any change in electrical character.

# THEORY

## *Molecular recognition by the immune system*

The feat of self-assembly of nucleic acids by Nature has been discussed in the previous chapter. Nature can also engineer molecules to recognize foreign agents, and in this way, a healthy immune system recognizes and destroys invaders. Antibodies can be thought of as a perfectly flattering dress for a beautiful woman who is the antigen fitted by Seamstress Nature. She can make a dress for any woman for every occassion. Researchers who perform monoclonal antibody selections tap into this pool of diverse binding pockets. Nature's powers of molecular recognition are far more effective for identifying high-affinity proteins than any other method. Previous research by Brader and Erlanger (Braden *et al.* 2000) showed that the immune repertoire of mice is diverse enough to recognize and process fullerene-protein conjugates. These anti-fullerene antibodies of type IgG isotype were isolated and their specific response to fullerenes confirmed by ELISA (Chen *et al.* 1998).

# MATERIALS AND METHODS

## *Fullerenes and single-walled carbon nanotubes*

Nanotubes and fullerenes are relatively new forms of carbon that can be synthesized by various means. From what is known so far about their physical and electronic properties, these materials are predicted to change the world (Duan *et al.* 2002; Bachtold *et al.* 2001; Huang *et al.* 2001; Cui *et al.* 2001; Kong *et al.* 2000). The Young's modulus (Y) quantifies the resistance that a material opposes against deformation in a particular direction. The Young's modulus of conventional materials

varies from a few GPa to up to 600 GPa for the hardest materials, such as diamond and SiC. Nanotubes demonstrate a young's modulus of 1500-5000 GPa (Harris 1999). Considering their remarkable structural strength, it has been proposed that a rope of nanotubes could be used to raise an elevator to the moon. Since the diameter of nanotubes is less than the wavelength of light, one day they may be used to fabricate high-strength materials that are translucent in the visible range.

## *Characteristics*

The descriptor fullerene is given to any carbon species that has a relatively significant amount of $sp^3$ hybridized carbon bonding to accommodate the geometry of highly curved regions. The most abundantly produced and well-characterized fullerene species is C60, glowing in its beautifully symmetry in **Fig. 3.2**. In contrast, multi-wall carbon nanotubes (MWNTs) and single-walled nanotubes (SWNTs) are composed of nearly exclusively $sp^2$-hybridized carbons, with a few $sp^3$ carbons at the caps of the tube where the curvature is sharper. MWNTs describe a bundle of SWNTs having different diameters and forming shells around each other.

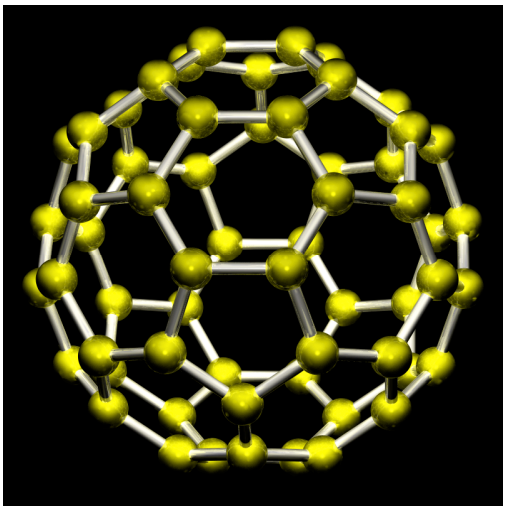

FIGURE 3.2 Buckminster-fullerene is a form of carbon that contains 60 carbon residues in the shape of a truncated icosahedron.

Single-walled carbon nanotubes (SWNTs) may be divided into three different conformers, each of which is a pair of fullerene caps connected by a tube that is a rolled up graphene. Topological defects can occur in the form of pentagons and heptagons along the side walls. SWNT depends on the pattern of hexagons that is observed around the body of the tubule, and **Fig. 3.3** shows the three confomers, zig-zag, armchair, and chiral. The chiral form can be assigned to tubes that twist in either direction. **Fig. 3.4** shows the pattern of hexagons on the tubular part of a chiral nanotube, imaged with a scanning tunneling microscope under ultra-high vacuum conditions.

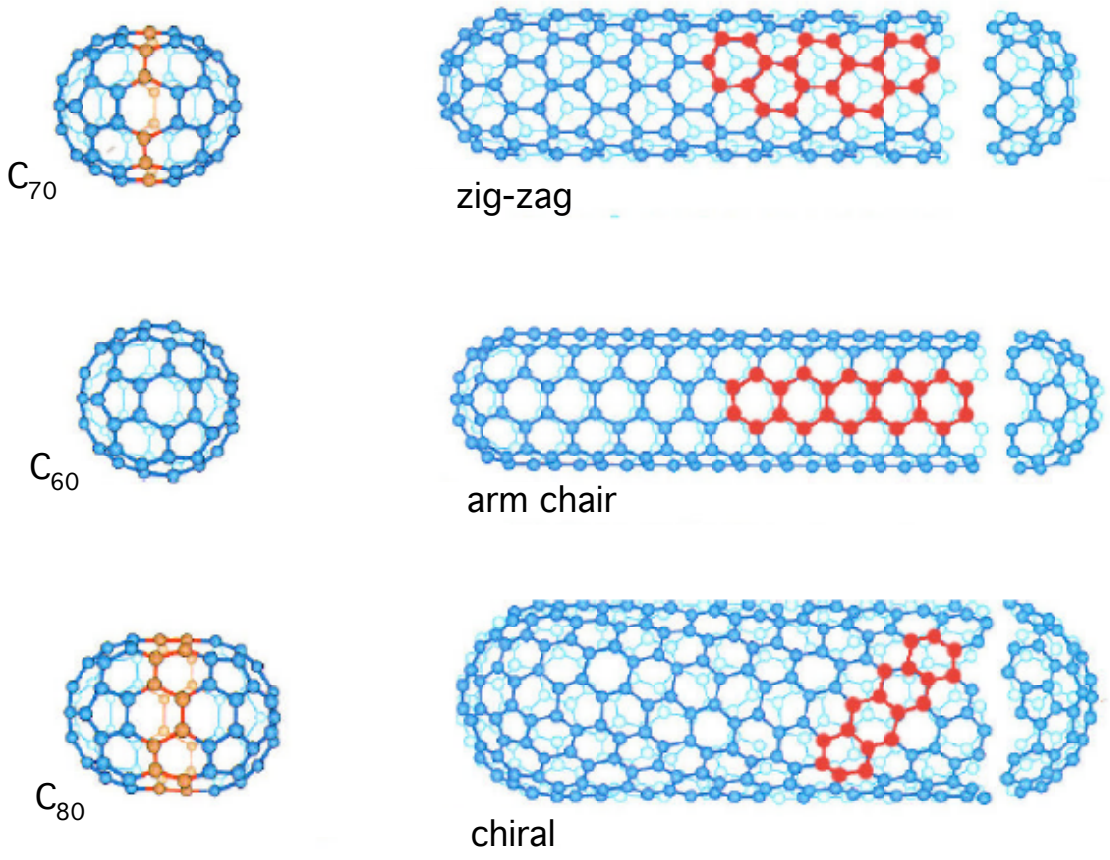


FIGURE 3.3 Three different geometries for a SWNT.

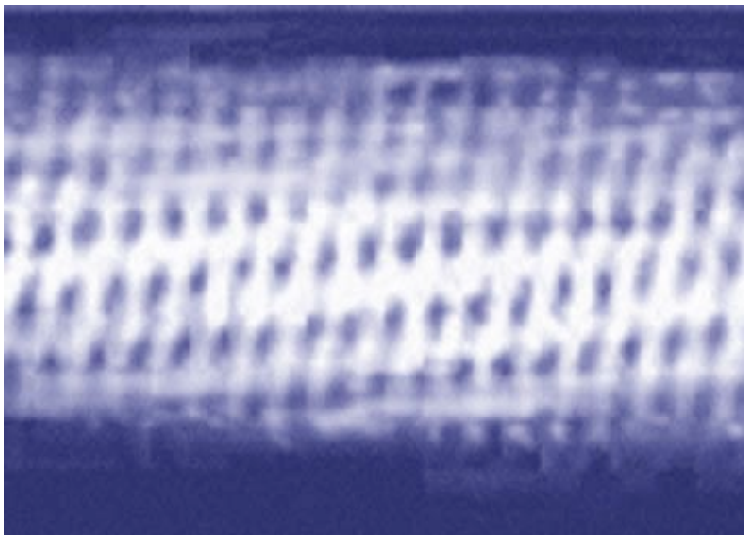

FIGURE 3.4 Ultra-high vacuum scanning tunneling micrograph of a chiral single-walled carbon nanotube (D. Carroll, Clemson University).

Nanotubes exhibit electrical properties that are directly related to their unique quantum mechanical structure. All armchair SWNTs are metallic, and one-third of the possible chiral and zigzag tubes are metallic conductors. The remaining two-thirds of the chiral and zigzag tubes are semi-conductors.

## *Synthesis*

Carbon nanotubes are commonly manufactured by one of three routes. The first of these methods is laser ablation, in which a high power laser is focused into a carbon target such as a rod of graphite. In the plasma plume that is generated by the laser, provided that appropriate gaseous and environmental conditions exist, SWNTs form and are collected downstream from the plasma plume on a "cold finger." In the arc-discharge method, a fairly low voltage power supply is used to strike an electrical arc between two carbon electrodes. Usually the carbon anode is coated with particles of a transition metal, which become the nucleation sites for the growth of nanotubes and other carbon byproducts.

Controlled vapor deposition (CVD) of single-walled carbon nanotubes has gained popularity for the synthesis of SWNT because the majority of the nanotubes produced are SWNT and one nanotube can be grown per catalyst, resulting in isolated SWNTs (Kong *et al.* 1998). First, catalyst islands containing iron, molybdenum, and alumina nanoparticles are deposited in the liquid phase onto a Si/SiO surface. In chemical vapor deposition, the surface is heated to 1,000 C in an argon atmosphere and then the argon flow is replaced by 99.99% pure methane at a flow rate of 1000 to 6000 $cm^3\ min^{-1}$ and this condition is maintained for 10 minutes at 1,000 C. Then the argon atmosphere is restored and the surface is allowed to cool to room temperature. The purpose of the heat in CVD synthesis is to impart energy to the gaseous carbon molecule, cracking it into a reactive radical species. The activated carbon molecule diffuses down the substrate until it encounters the metal catalyst.

The laser ablation technique pioneered by R. Smalley produces the highest quality SWNT material in terms of yield relative to starting material and very few tube defects. Arc-discharge synthesis results in nanotubes that are ropy and multi-walled. These two methods result in a bundled growth of single- and multi-walled nanotubes, carbon dust, and different sizes of carbon fullerenes. Unfortunately, separation techniques for such carbon conglomerates are lacking and therefore laser ablation and arc-discharge were unsuited for the study at hand. One of the goals of the project was to observe the change in conductivity of a single SWNT upon the binding of an anti-fullerene antibody, so controlled vapor deposition (CVD) was chosen as a fabrication method that produces well-isolated single-walled nanotubes on a substrate. The density of islands can be controlled by the initial concentration of metal nanoparticles in the catalyst solution. Ninety percent of the nanotubes produced by CVD are individual SWNTs, having diameters between 1-3 nm. Also, CVD gives control over alignment (Ren *et al.* 1998), position (Ren *et al,* 1999), and grow rate and diameter (Choi *et al.* 2000). Furthermore, it has been shown that the size of the catalyst is correlated to the size of

the nanotubes, giving a choice about the growth of single- or multi-walled nanotubes (Sinnott *et al.* 1999).

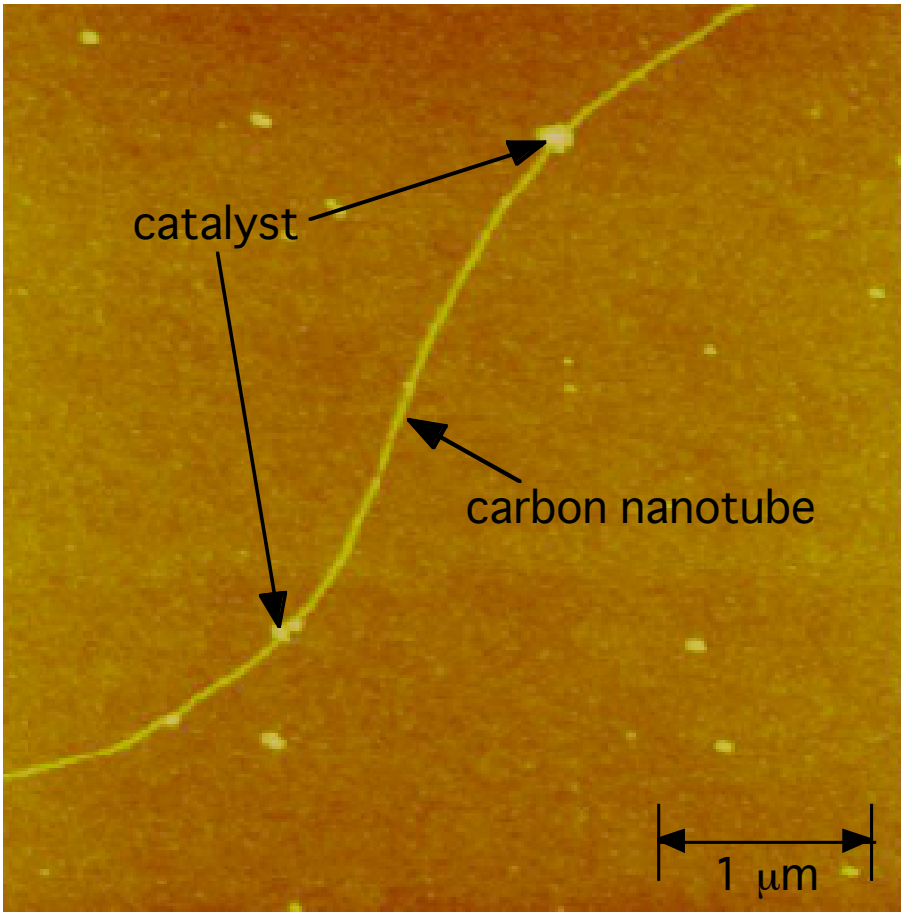


FIGURE 3.5 A SWNT grown by CVD from catalyst islands containing transition metals.

## *IgG and SCfV antibodies*

Naturally occurring antibodies of isotype IgG are produced by B cells, and consist of four polypeptide chains, as shown in **Fig. 3.6**. Two heavy chains, each composed of four immunoglobulin domains, and two light chains, each made up of two

immunoglobulin domains, are held together by disulphide bonds. The bulk of the antibody complex is made up of constant immunoglobulin domains. These have a conserved amino acid sequence, and exhibit low variability. However, tremendous variability exists in the $V_H$ and $V_L$ regions, because this site is where the recognition properties of the antibody are carried. Each variable domain contains three hypervariable regions known as complementarity determining regions, or CDRs. The CDRs come together in the final tertiary structure to form an antigen-binding pocket. The human genome contains multiple fragments encoding portions of the variable domains in regions of the immunoglobulin gene cluster known as V, D and J. During B cell development these regions undergo recombination to generate a broad diversity of antibody affinities. As the B cell populations mature in the presence of a target antigen, the variable region hypermutates, with the B cells producing the most active antibodies being targeted for replication. This process is known as affinity maturation (Goldsby *et al.* 2000).

The binding domain of an IgG antibody is completely conserved in the heavy and light chains. To eliminate the bulk of the constant immunoglobulin regions, it is possible to design a single polypeptide that contains the $V_H$ and $V_L$ regions joined by a glycine-serine linker, which has sufficient flexibility to allow the two domains to assemble a functional antigen-binding pocket. In this way, a 150 kDa protein can be simplified to a 25 kDa protein that still binds antigens as well as the IgG antibody. This type of protein is called a single-chain variable fragment (SCfV). The relationship between IgG and SCfV antibodies is shown in **Fig. 3.6**.

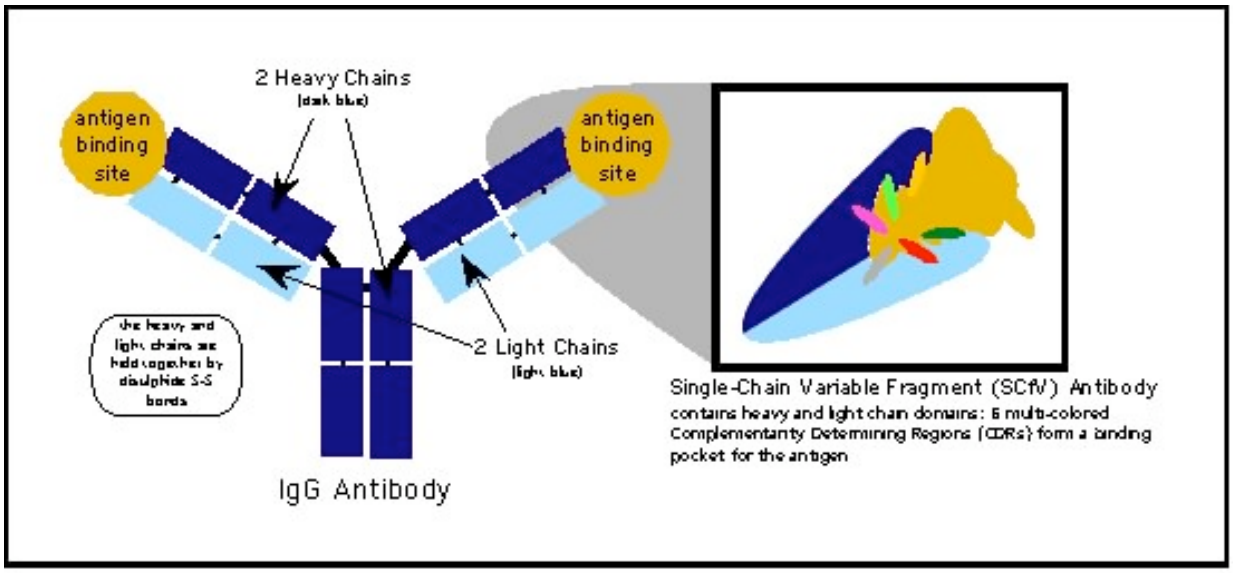


FIGURE 3.6 Relationship between an IgG antibody and single-chain variable fragment antibody. Note that the IgG antibody binds two antigens and has 16 polypeptide chains while the SCfV binds only one antigen and has 2 polypeptide chains.

## *Selection of anti-fullerene antibodies*

A major breakthrough was the generation of monoclonal antibodies, pure populations of antibodies with the same affinity. Monoclonal antibodies are very important research tools, and have been used in some therapies. However, they are very expensive and difficult to produce, and in some therapeutic applications, have elicited an immune response that will destroy the antibody.

Erlanger has selected an anti-buckminsterfullerene monoclonal antibody by immunizing mice with [1-methylsuccinate-4-methyl-cyclohexadiene-2,3]-buckminsterfullerene, a derivative of C60 shown in **Fig. 3.7** (Erlanger, *et al.* 1998). It was necessary to confirm that the antibody binds C60 and not the attached derivatives,

and competitive binding procedures showed that the SCfV binds C60 target with an affinity of 22 nM (Braden *et al.* 2000).

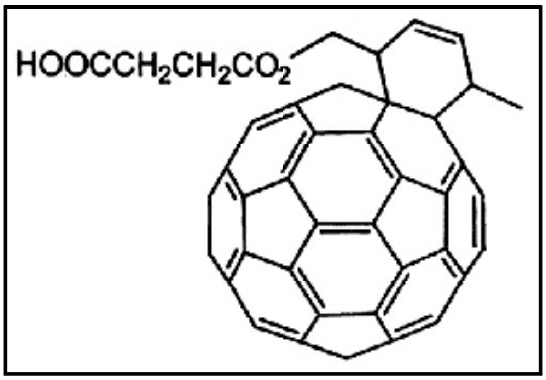


FIGURE 3.7 Mice were immunized with [1-methylsuccinate-4-methyl-cyclohexadiene-2,3]-buckminsterfullerene, a derivative of C60 (Erlanger *et al.* 1998).

The structure of the antibody-C60 complex was determined by X-ray crystallography to a resolution of 2.25 Å (Erlanger *et al.* 2000). Hydrophobic amino acids in the binding pocket of the protein form a spherical cavity, and key aromatic residues participate in π-π stacking interactions with the π-system in C60. Such π-stacking and hydrogen bond forces would be weak interactions, and **Fig. 3.8** shows the amino acids involved in these interactions. By molecular modeling, the fullerene surface area buried by the antibody is 337 $Å^2$, or 90% of the surface area of C60. Dividing the binding affinity, 22 nM, by the surface area of the buried C60, resulted in a force with the value 22 cal/m/$Å^2$, in good correspondence with the weak interactions of hydrogen bonding and π-π stacking. This calculation verified the mode of binding between the antibody and C60. Crystallization and the force calculations confirmed a spherical binding pocket for the antibody-fullerene complex and provided information about the type of interactions that are available to the immune system to recognize this novel carbon form.

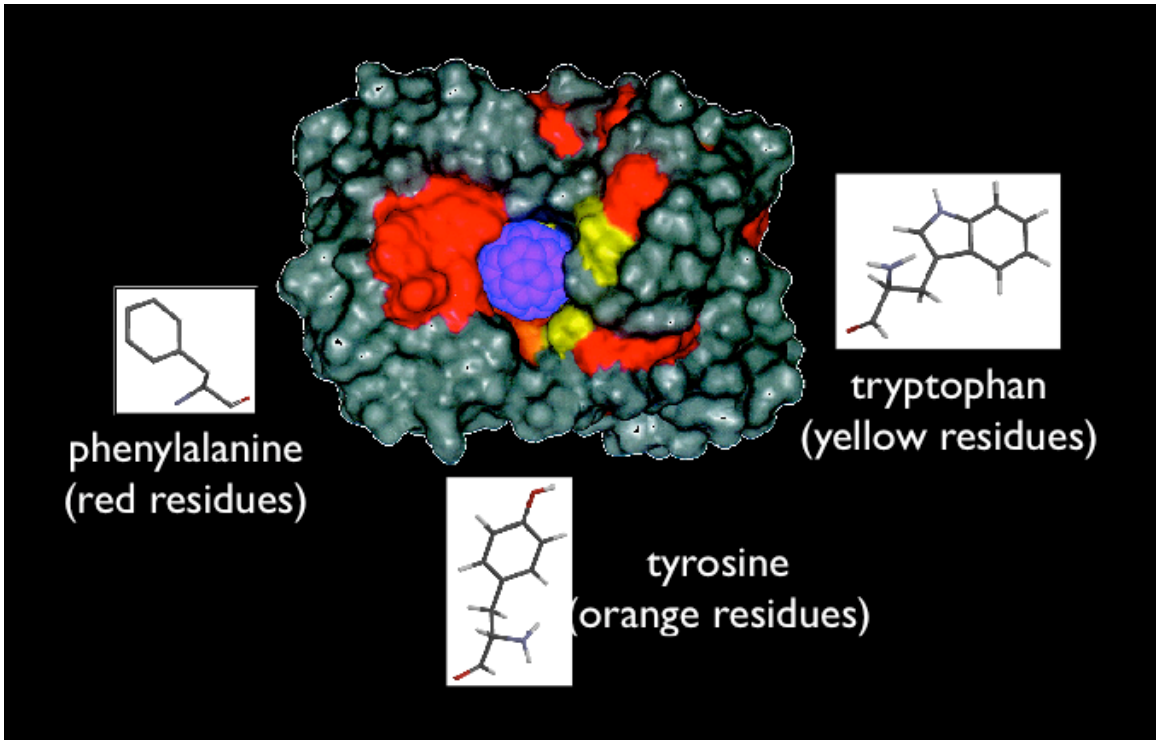


FIGURE 3.8 Van der Waals surface representation of C60 bound to the anti-fullerene antibody. The aromatic portions of phenylalaine, tryptophan, and tyrosine form the binding pocket (Braden *et al.* 2000).

## *Construction of two SCfV antibodies*

The variable regions from the heavy and light chains ($V_H$ and $V_L$) are both approximately 110 amino acids long. Two different genes were designed and they are represented in **Fig. 3.9**. One contained a long linker sequence of 15 amino acids $(glycine_4serine)_3$ and the other one had a short linker of 5 amino acids ($glycine_4serine$). Both genes encode for the heavy and light chains of the anti-fullerene antibody (Braden *et al.* 2000), and differ only by the length of the linker. Shortening the length of the linker has the effect of causing dimer and trimer SCfVs (Atwell *et al.* 1999; Arndt *et al.* 1998; Katja *et al.* 1998; Kortt *et al.* 1997). Due to their smaller size and simpler tertiary

structure, SCvF proteins can be produced in *Escherichia Coli (E. Coli)* using bacterial expression vectors (McGregor *et al.* 1994).

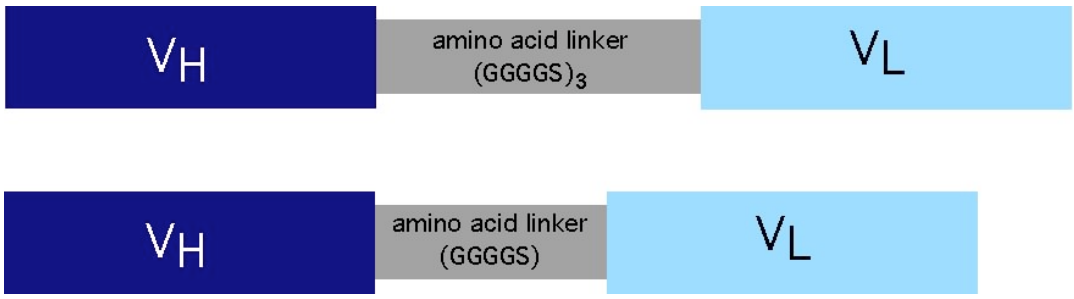


FIGURE 3.9 Design of two genes that differ by the length of the glycine-serine linker. Note that the top gene is designed to produce a monovalent protein and the bottom gene is designed to give monospecific, divalent and trivalent species.

First the protein sequence of the $V_H$ and $V_L$ regions of Erlanger's anti-fullerene antibody was reverse translated into a DNA sequence, using the codons of *E. Coli*. Each gene was constructed from overlapping 38-mer oligonucleotides (Stemmer *et al.* 1995), and recombinant DNA methods using restriction enzymes were used to piece together the correct portions of each gene until the deletions or mutations were eliminated. This gene was sequenced in the forward and reverse direction by an automated ABI 3700 sequencer, and 6 chromatograms for each gene were compared to confirm the presence of the correct sequence. Next the gene was inserted into MOPAC54 vector, a bacterial expression vector for the production of SCfVs, gift of Andrew Hayhurst. This vector has 10-histidine residues to facilitate protein purification from an immobilized affinity column.

## *E-beam lithography*

Electron-beam lithography will be used to construct very small electrodes on top of a single-walled carbon nanotube, trapping it between an insulating substrate and a conducting surface to which larger contact electrodes and data acquisition devices can be attached. The conductivity of the SWNT will be measured before and after SCfV binding to determine any changes in electrical character.

A flow diagram for e-beam lithography is pictured in **Fig. 3.10**. After the carbon nanotubes are grown onto a substrate by CVD, the entire surface is coated with a polymer polymethyl methacrylate (PMMA), and then baked for one hour to solidify the polymer. Then the sample is scanned by AFM to locate and precisely mark the sites on the sample that will be targeted for the electron beam in the subsequent step. The electron-beam of a Raith-50 system (**Fig. 3.11**) is focused onto the substrate in the locations where metal electrodes will eventually be desired. Considering the width of the electron-beam and limits of diffraction, it is possible to "write" lines with a width no less than 100 nm. When a beam of electrons contacts PMMA, the polymer is weakened so than the exposure to a developer (MIKB and isopropyl alcohol) will solubilize the regions of PMMA that were weakened by the electron beam. Next, the surface is placed in a vacuum chamber ($1 \times 10^{-6}$ Torr) and chromium and gold are thermally evaporated onto every exposed surface, giving a layer of about 5 nm and about 50 nm, respectively. Although gold is a better conductor than chromium, it is necessary to use a thin layer of chromiun as "glue" to stick gold to the PMMA surface since gold does not bind a to PMMA. In the last step, termed "lift-off", the surface is exposed to boiling acetone (100 degrees C), which has the effect of removing all excess PMMA and the gold and chromium that had been deposited on top of PMMA. The resulting surface has chromium and gold electrodes deposited directly on top of nanotubes, trapping the carbon nanotube between an insulating silicon oxide substrate and conducting electrodes.

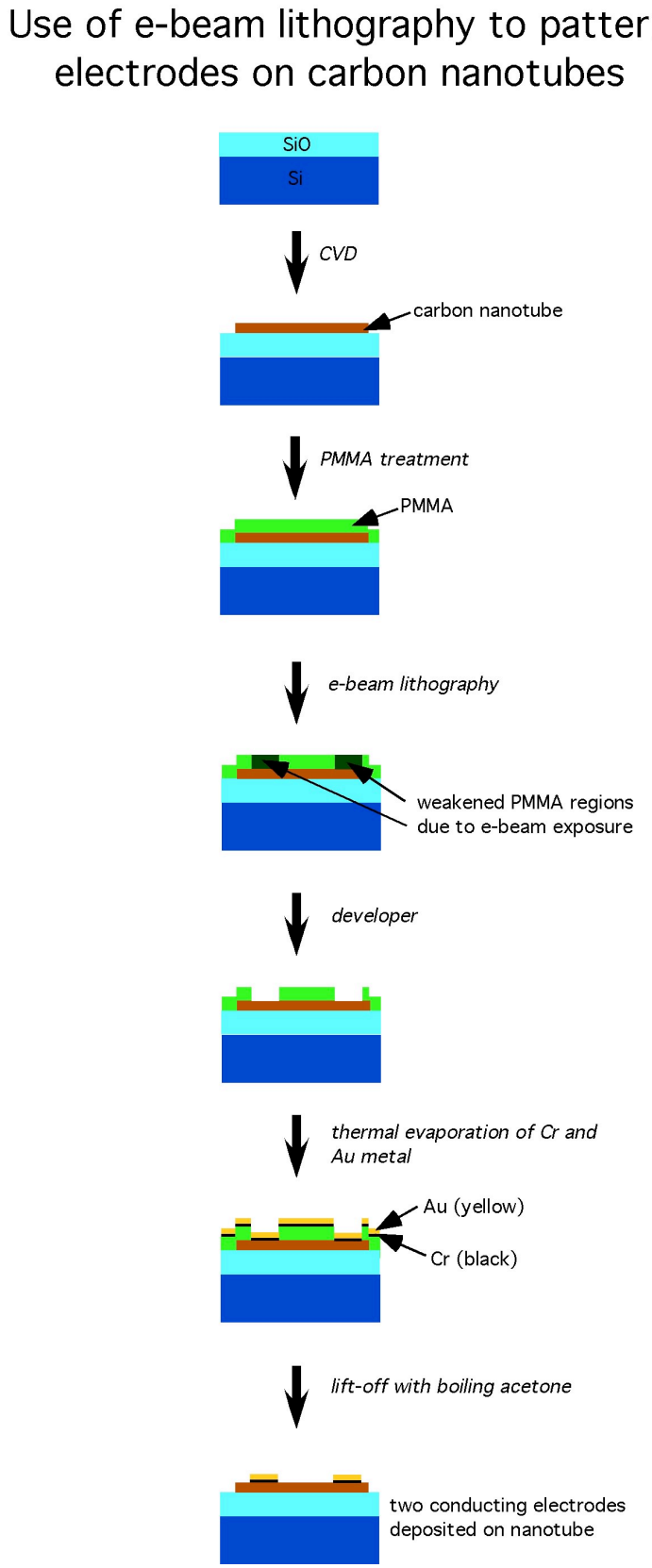


FIGURE 3.10 Flow diagram describing e-beam lithography, *see text for details*.


FIGURE 3.11 Raith-50 Electron beam lithography system, picture from the Center for Nano- and Molecular Science and Technology at the University of Texas at Austin.

## RESULTS

Results for the antibody-nanotube binding event were limited by the construction of the two genes that encode for the anti-fullerene SCfV antibody. The gene assembly method based on Stemmer incorporates the use of an editing polymerase Vent, which reduces the amount of errors that are incorporated in the gene product. However, mutations and point-deletions arose throughout several generations of gene construction efforts, which included:

1. 1 and 2 megaprimer PCRs followed by DpnI digestion
2. Vent-only assembly techniques based on overlap PCR
3. de novo gene reconstruction with Vent-only and Pfu-only
4. restriction enzyme digestion of correct gene portions followed by ligation

Of these techniques, only restriction enzyme digestion and ligation has resulted in the correct assembly of the short gene with a 5 amino acid linker, the species that is expected to form dimers and trimers. Protein expression and purification with this gene followed by analysis with a denaturing SDS-Page protein gel showed species with molecular weights of ~25kDa and ~50kDa by comparison to known markers. As determined by a biopolymer calculator, the SCfV is expected to have a molecular weight of 21.5 kDa, so the protein gel results imply that the SCfV and its dimer are being successfully expressed and purified. Blank nanotube surfaces such as the ones in **Fig. 3.12** have been scanned as a control.

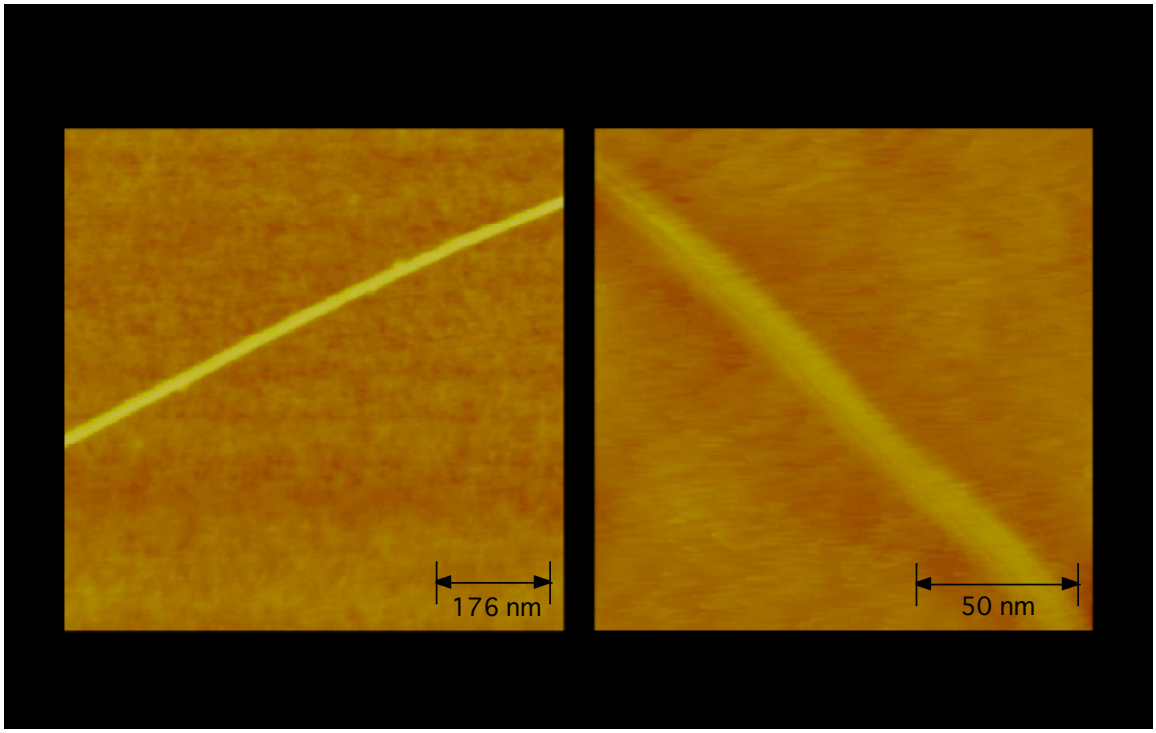


FIGURE 3.12 Controls. AFM micrographs showing bare single-walled carbon nanotubes in the absence of protein.

Experiments in the immediate future will deposit this purified protein onto a surface with SWNTs, followed by AFM scanning to determine if any proteins are bound to the ends or tubular portions of the nanotubes. If so, then single-molecule conductivity measurements will analyze changes in nanotube electrical character before and after protein binding. Additionally, further work with restriction enzymes is planned to obtain the correct sequence for the long gene product.

## DISCUSSION

The research to develop the anti-fullerene antibodies and determine whether they bind to nanotubes is still in progress. However, the advantages of using biorecognition to manipulate nanotubes should be stressed.

Anti-nanotube proteins could be added to the raw carbon products made from arc-discharge or laser ablation, and thus used for purification of SWNTs. In this sense, the SCfV is like a molecular handle, limited only by the chemistry of protein modification, which is extremely diverse and versatile. Many biotech companies e.g. Pierce, manufacture hundreds of reagents that couple amino acids in proteins to linkers. Nanotubes could be easily linked to quantum dots, biotin-avidin conjugates, M-DNA, other proteins, conducting polymers, fluorescent dyes, and other interesting substituents.

Protein binding to nanotubes may change the conductive properties of nanotubes so that one dimensional physics can be studied. Also, measuring the changes in conductivity of the nanotube gives information about the dynamics of antibody/antigen binding.

If the SCfV system discussed fails to produce protein that can bind with a high specificity to nanotubes, then the project could advance by other approaches. For example, monoclonal antibody selections using short pieces of carbon nanotubes might generate proteins that are more specific than the anti-fullerene antibodies. An effort to perform selections with a pure population of conducting nanotubes may result in an antibody that would be tremendously useful as a commercial product for making affinity columns that purify conducting nanotubes from semiconducting and nonconducting species. Protein binding could be further enhanced with organic total synthesis methods, where chemical groups that can $\pi$-stack with the tubular graphene sheet of nanotubes are attached to anti-fullerene or anti-nanotube antibodies.