Investigation of Functionalized Carbon Nanotubes as a Delivery System for Enhanced Gene Expression with Implications in Developing DNA Vaccines for Hepatitis C Virus

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CANADA

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ABSTRACT

Hepatitis C virus (HCV) causes a significant health problem worldwide due to the lack of effective vaccines. It has been recognized that a rapid, vigorous, and broadly targeted cell-mediated immune response (Th1-like) is often associated with the clearance of HCV infections. DNA vaccines represent a promising means for HCV vaccination because they tend to induce a Th1-biased cell-mediated response in the host cell. Currently, the delivery of DNA vaccine for HCV in large animals as well as in humans is not as effective as in small animals. Nano delivery systems would be a promising approach to overcome this problem. Carbon nanotubes (CNTs) have been extensively studied for delivering drugs, proteins, peptides, and nucleic acids including plasmid DNA to cells and organs with varying degrees of success, but few of them have been applied to DNA vaccine for HCV.

This thesis presents a study of using functionalized CNTs (f-CNTs) to improve the efficacy of plasmid DNA vaccine delivery for HCV. First, CNTs were functionalized via 1,3-dipolar cycloaddition reaction with the appropriate amino acids and aldehydes. NMR and TEM results suggested that the CNTs were successfully functionalized and became soluble in water. Then plasmid DNAs which encode green fluorescence protein reporter gene, luciferase reporter gene, and HCV core protein, respectively, were delivered into human hepatoma cells via calcium phosphate precipitation method, f-CNT delivery system, and a combination of f-CNT and calcium phosphate

method, respectively. The result showed that f-CNTs, in combination with the calcium phosphate method, significantly enhanced the gene expression in human hepatoma cells.

Consequently, this study concludes that the f-CNT can significantly enhance gene expression in liver cells conferred by a plasmid DNA when combined with calcium phosphate precipitation method. Even though the mechanisms of this enhancement await further investigation, the results of this thesis may have important implications in developing DNA vaccines for infectious diseases in general and for hepatitis C in particular.

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Last but not least, a huge thankfulness to my dear Mom and Dad for raising me well, always being there for me and always having faith in me.

Dedication to

My Mom and Dad

Chen Ying & Chen Qihua

For their selfless great love!

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LIST OF ABBREVATIONS

HCV	hepatitis C virus
Th	helper T cells
DNA	deoxyribonucleic acid
CNT	carbon nanotube
NMR	nuclear magnetic resonance
TEM	transmission electron microscopy
f-CNT	functionalized CNT
AIDS	acquired immune deficiency syndrome
RNA	ribonucleic acid
E	envelope
NS	nonstructural
mRNA	messenger ribonucleic acid
SWNT	single-wall carbon nanotube
MWNT	multi-wall carbon nanotube
HVR	hypervariable region
HBV	hepatitis B virus
INF	interferon
HIV	human immunodeficiency virus
RT	room temperature
DMF	dimethylformamide

Huh-7 cell	human hepatoma cell
MeOH	methanol
HCl	hydrochloric acid
GFP	green fluorescent protein
DMEM	Dulbecco’s Modified Eagle’s Medium
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)piperazine-1-erhanesulfonic
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BBS	BES buffered solution
DAPI	4',6-diamidino-2-phenylindole
CaP	calcium phosphate
PEG	poly (ethylene glycol)

Chapter 1

Introduction

1.1 Background and Motivation

Hepatitis C virus (HCV) is the causative agent of non-A, non-B hepatitis. With approximately 170 million infected people in the world, HCV causes a major health problem worldwide. This virus often leads to chronic infection that can lead to chronic liver diseases, including cirrhosis and hepatocellular carcinoma. Unfortunately, there is no effective vaccine available to prevent HCV infections [Lee et al., 1998; Geissler et al., 1997; Tokushige et al., 1996; Li et al., 2006].

For some viral diseases, such as hepatitis C or AIDS, the use of live attenuated virus as vaccines is considered to be too risky, because there are chances that the weakened virus could revert to the virulent form and cause diseases [Azad et al., 2006]. It has been accepted that the clearance of the hepatitis C virus infection is often associated with a rapid, robust, and broadly targeted cell-mediated immune response. DNA vaccine tends to induce a Th1-biased cell-mediated response in the host, and thus becomes a promising approach for generating immunity which can prevent HCV infection [Donnelly et al., 1997].

However, the immune responses induced by DNA vaccines were usually found to be too weak to show sufficient protection against new infection, especially in large animals and humans. One of the reasons could be that the current delivery system of DNA vaccines is not effective in conferring gene expression to a level that is required for inducing sustained immunity.

With the rapid development of nanotechnology, it is becoming feasible that a nanomaterial-based delivery system might be a promising approach to overcome some shortcomings of the conventional delivery system. Nanomaterials, such as biodegradable polymers, carbon nanotubes, and carbon 60 fullerenes, have novel properties that can improve the efficiency of drug delivery [LaVan et al., 2003]. As a novel nanomaterial, carbon nanotubes (CNTs) have attracted considerable attention for their potential biomedical and biotechnological applications [Pantarotto et al., 2003a; Bianco et al., 2005a]. However, pristine CNTs might cause toxicity in mammalian cells or organs [Colvin, 2003]. Their biocompatibility has been made possible by the chemical functionalization of their surface through treatment by peptides, proteins, and other chemicals [Pantarotto et al., 2003b; Karajanagi et al., 2006; Pastorin et al., 2006]. Functionalized CNTs (f-CNTs) could become biocompatible, not immunogenic, and have low toxicity [Bianco et al., 2005b] for interacting with biological components including mammalian cells. Functionalized CNTs have been tested to deliver drugs, proteins, peptides, and nucleic acids including plasmid DNAs to cells, organs and animals. The systems based on the

functionalized CNTS have a high potential to enhance the immune responses induced by DNA vaccines for infectious diseases, e.g., hepatitis C. Currently, functionalized CNTs have been extensively tested for gene delivery with varying degrees of success *in vitro*[Bianco et al., 2005c; Cai et al., 2006]. However, they have not been shown to be effective in HCV vaccination. This thesis work was to study the impact of functionalized CNTs on gene expression with potential implication in developing effective DNA vaccines for infectious diseases such as hepatitis C.

1.2 Hepatitis C Virus

Hepatitis C virus (HCV) is the major etiologic agent of sporadic and transfusion-associated non-A, non-B hepatitis worldwide. Up to 3.0% of the world's population has been infected with HCV. With the diagnostic tests of excluding HCV-contaminated blood products, the incidence of new HCV infections has declined drastically. However, there are still about 170 million people who are persistently infected with this virus and have a high risk of developing severe chronic liver diseases [Lee et al., 1998; Geissler et al., 1997; Tokushige et al., 1996; Li et al., 2006].

HCV is a 33 nm-long positive-strand RNA enveloped virus that belongs to the *Flaviviridae* family and genus *Hepacivirus*. Figure 1.1 illustrates the organization of the virus: it is a microsphere with RNA genome encapsidated by the viral core particle

which is encoded by the HCV Core gene. The viral surface is surrounded by a lipid envelope containing glycoproteins E1 and E2 that are encoded by the HCV E1 and E2 genes, respectively.

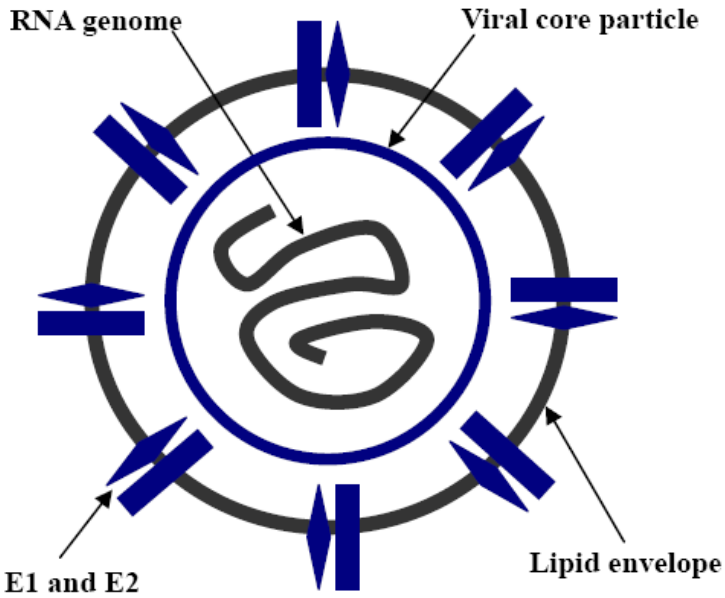


Figure 1.1 The model structure of HCV virion.

In the structure of HCV genome (Figure 1.2), a 5'-untranslated region is followed by an open reading frame which is further followed by another untranslated region at the 3' end. The open reading frame encodes for a single polyprotein which, upon protease digestions, gives rise to several proteins in the order from 5' to 3': C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The C, E1, and E2 proteins are structural and the NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins are nonstructural [Lindenbach et al., 2004].

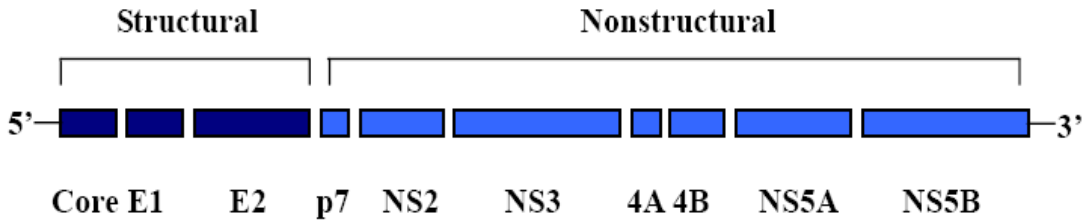


Figure 1.2 HCV Genome.

HCV replicates in the liver, and is detectable in sera during acute and chronic infection. Although the life cycle (Figure 1.3) of HCV is poorly understood, a possible sequence of biological events has been postulated to explain how the virus enters a liver cell, makes copies of itself and then exits to infect other cells.

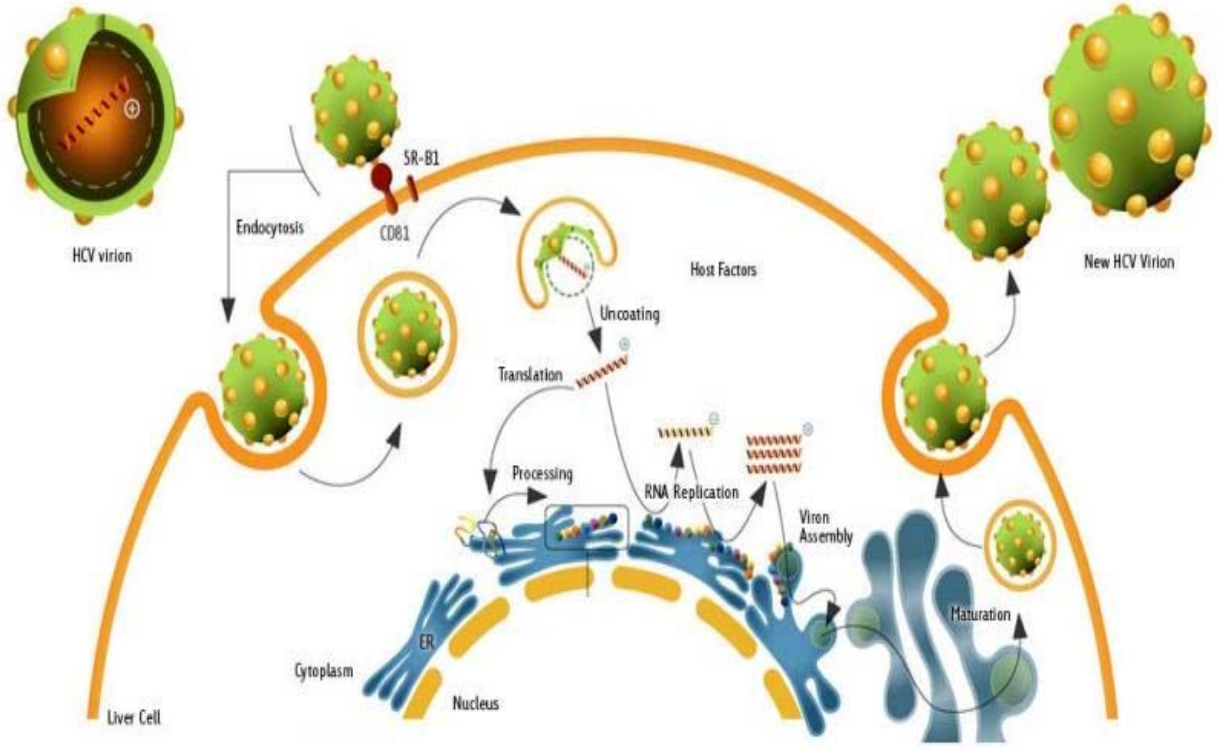


Figure 1.3 Hepatitis C virus life cycle. This figure is cited from:

http://www.tibotec.com/content/backgrounders/www.tibotec.com/hcv_lifecycle.html.

HCV particles, or “virions”, bind with a receptor on the surface of the cell membrane and is endocytosed. Then, the virions somehow fuse themselves with the endocytotic vesicle membrane to deliver a complex of RNA and associated proteins into the cytoplasm of the liver cell. Viral RNAs are translated by ribosomes into a polyprotein, which is then cleaved into structural and non-structural proteins. The same RNAs are also copied into complementary RNAs that serve as templates for making RNA copies. The numerous copies of RNA made through this process then join with the viral proteins to form virions. The virions are secreted from the liver cell via the cell’s secretory pathway, releasing the new virions to infect a large number of new liver cells.

1.3 Immune responses to antigens

Our immune system plays the most important role in vaccination. The immune system works in response to “foreign” molecules, such as bacteria or viruses. Table 1.1 describes common components that are involved in immune responses [Azad et al., 2006] and Figure 1.4 briefly shows the major processes of two general types of immunity: humoral immunity and cell-mediated immunity.

Table 1.1 Components of the Immune system

Component	Description
Ag	Antigen; usually proteins or polysaccharides which are parts of bacteria, viruses, or other microorganisms; can cause the generation of antibodies and an immune response.
DC	Dendritic cells; immune cells; form part of the mammalian immune system; function as antigen-presenting cells.
B cells	B lymphocytes; white blood cells produced in the bone marrow.
T cells	T lymphocytes; white blood cells derived from the thymus gland.
MHC I	Major histocompatibility complex class I molecules; found on almost every nucleated cell of the body.
MHC II	Major histocompatibility complex class II molecules; found only on a few specialized types of cells, including macrophages and B cells.
Th cells	Helper T cells; also known as CD4+ T cells, a sub-group of T cells.
CTL	Cytotoxic T Lymphocytes; also knows as CD8+ T cells, a sub-group of T cells.
Ab	Antibody, also known as immunoglobulin; identify and neutralize antigen.

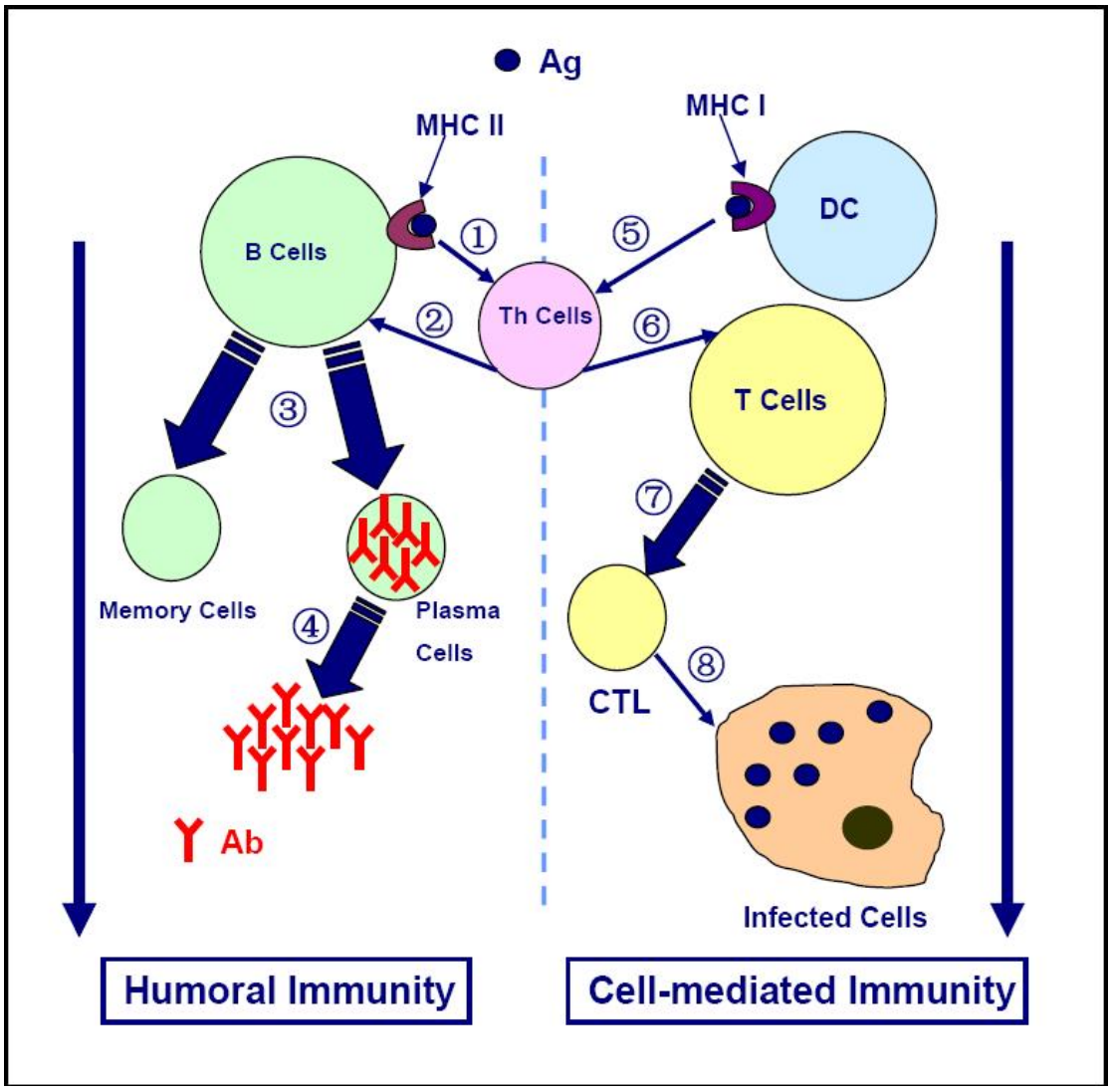


Figure 1.4 Humoral immunity and cell-mediate immunity.

An antigen may induce both humoral immunity and cell-mediated immunity [Davis et al., 1999]. Humoral immunity usually involves B cells. When B cells recognize an antigen via their surface receptors, the antigen will be displayed on the B cell surface bound to MHC II molecules. MHC II is then recognized by the receptors on the Th cell surface (process ①), Th cells release stimulatory molecules that in turn to activate B cells (process ②). In process ③, part of activated B cells become plasma cells, considered as an "antibody factory" which can produce a large amount of antibodies

and secrete them into body fluids (process ④) to destroy the antigen. Other parts of B cells that have not involved in process ③ and process ④ become memory cells that are able to live for a long time and can respond quickly following a second exposure to the same antigen.

T cells are the effectors for cell-mediated immunity. After an antigen is recognized by receptors on DC, the antigen is engulfed and then presented on the DC surface by MHC I molecules that are subsequently recognized by Th cells via process ⑤. Th cells in turn stimulate T cells (process ⑥) to produce CTLs (process ⑦). Once CTLs become activated, they are capable of destroying infected cells, while bypassing normal cells (process ⑧).

The real immune system and immune responses are much more complicated than the aforementioned processes. However, it is certain that humoral immunity and cell-mediate immunity cannot be separated, and they work collaboratively to produce our immune responses.

1.4 DNA vaccine

The DNA vaccines (Fig. 1.5) [Liu, 2003], also known as genetic vaccines or nucleic acid vaccines, are simple rings of DNA, that are bacterially derived plasmids (Fig. 1.6), containing a gene encoding the desired antigen, and a promoter/terminator to

enable gene expression in mammalian cells. A DNA vaccine construction process usually involves (i) isolating one or more genes from a disease-causing agent (e.g., HCV) with known antigenic properties and (ii) cloning these genes into plasmids.

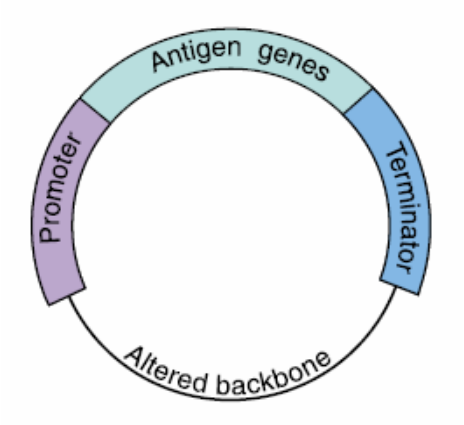


Figure 1.5 A schematic representation of a DNA vaccine.

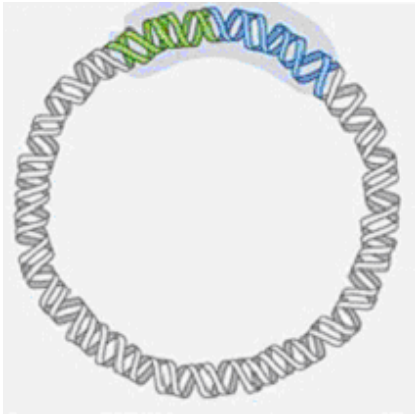


Figure 1.6 A schematic representation of plasmid DNA. This figure is cited from:

http://upload.wikimedia.org/wikipedia/commons/d/da/Making_of_a_DNA_vaccine.jpg

DNA vaccination is a promising approach for generating all types of desired immune responses: cytolytic T lymphocytes (CTL) responses, T helper cells responses, and antibody responses, whilst being a technology that has the potential for global usage in terms of ease of manufacturing, broad population administration and safety [Liu, 2003].

Immunizing the host with a DNA vaccine encoding a viral antigen rather than an antigenic protein of the virus (a subunit vaccine) helps to stimulate the generation of cell-mediated immunity (Fig. 1.7).

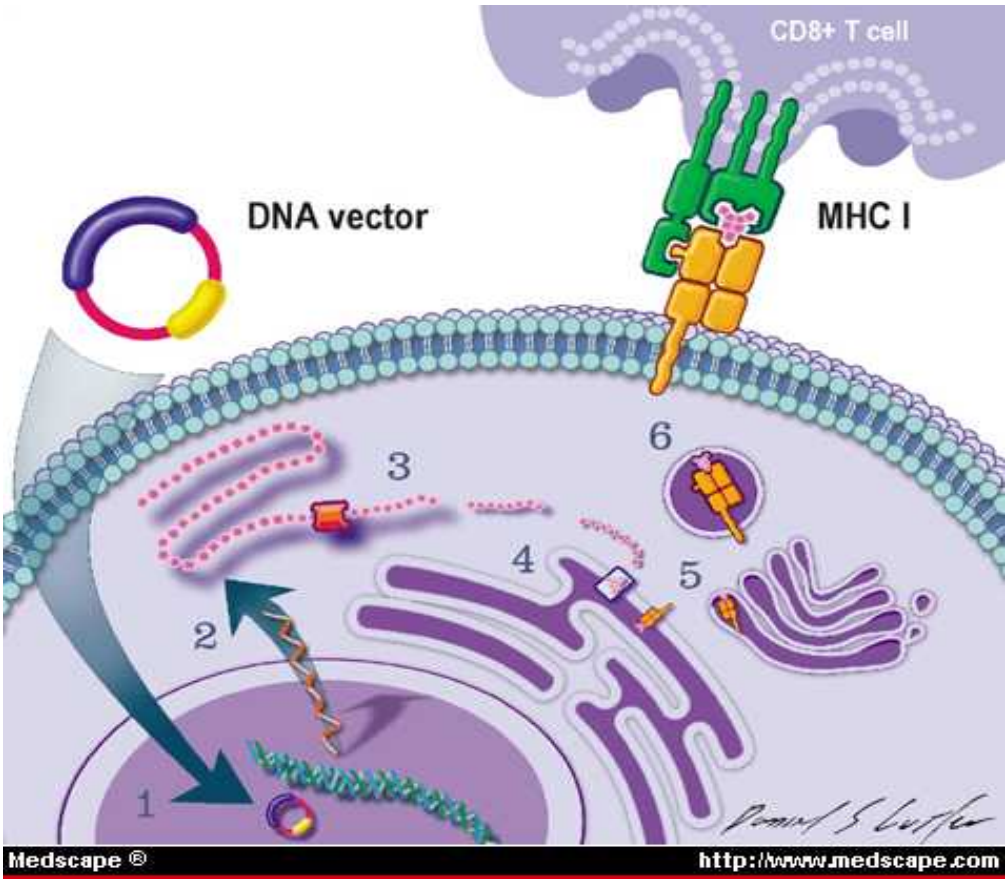


Figure 1.7 How DNA vaccines work. This figure is cited from:

http://www.medscape.com/content/1998/00/40/87/408733/art-mrc4593.fig2.jpg.

DNA vaccines favor a cell-mediated immune response. Plasmid DNA vector vaccines carry the genetic information encoding an antigen to a host cell, allowing the antigen to be produced inside the host cell, leading to a cell-mediated immune response (in particular via the MHC I pathway). The plasmid DNA vaccine carries the genetic code for an antigen. The plasmid vector is taken up into cells and transcribed to RNA in the nucleus (1). The single stranded mRNA (2) is translated into proteins in the cytoplasm. The DNA vaccine-derived protein antigen (3) is then degraded by proteosomes into intracellular peptides (4). The vaccine-derived peptide binds MHC I molecules (5). Peptide antigen/MHC I molecules are presented on the cell surface (6), binding cytotoxic CD8+ lymphocytes, and inducing a cell-mediated immune response. Because DNA vaccines generate cell-mediated immunity, they may be effective against viruses where subunit vaccines have failed.

DNA vaccines may have significant advantages over subunit vaccines. They can express antigenic epitopes which more closely resemble native viral epitopes and could therefore be more effective. With live attenuated vaccines and killed vaccines the manufacturing process can alter the secondary and tertiary structure of the proteins and therefore the antigenicity of the vaccine; with naked DNA vaccines, the host cell is manufacturing the viral epitope. DNA vaccines would be safer than live virus vaccines, especially in immunocompromised patients, such as those infected with

HIV. DNA vaccines may be constructed to include genes against several different pathogens, thus decreasing the number of vaccinations necessary to fully immunize children. Construction of DNA vaccines is relatively simple. Finally, DNA vaccines may hold some promise in treating those already infected with chronic viral infections as therapeutics.

1.5 Carbon Nanotubes

Since carbon nanotubes (CNTs) were first published by Japanese scientist Iijima in 1991 [Iijima, 1991], CNTs became a subject of intensive research interest [Bianco et al., 2005a; 2005b]. Significant attention is being paid to applications of CNTs in mechanics, nanotechnology and engineering, electronics, optics, biomedicines and other fields of materials science due to its unique properties. There are two major types of CNTs, single-wall carbon nanotubes and multi-wall carbon nanotubes.

A single-wall carbon nanotube (SWNT) can be viewed as a rolled-up rectangular strip of hexagonal graphite monolayers (Figure 1.8), while a multi-wall carbon nanotube (MWNT) is rolled-up with many layers of such strips (Figure 1.9)

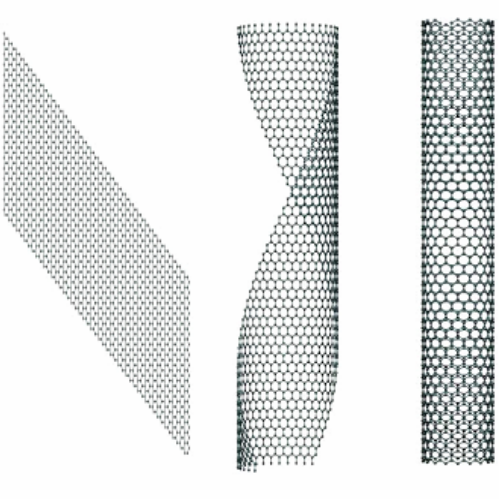


Figure 1.8 Single-walled carbon nanotube. This figure is cited from:

http://www.ewels.info/img/science/nano.html.

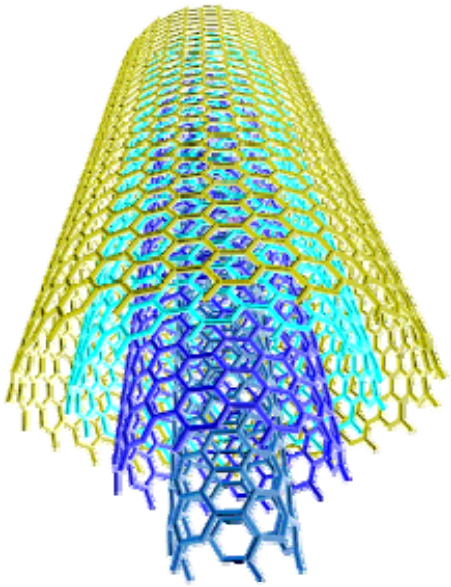


Figure 1.9 Multi-walled carbon nanotube. This figure is cited from:

http://students.chem.tue.nl/ifp03/.

CNTs are usually only a few nanometers in diameter but could be up to micro-scale in length. This structure can have a length-to-diameter ratio greater than 1,000,000 and

gives CNTs a property different from most other nanomaterials which are generally spherical in their shape. CNTs are potentially a good carrier for drug delivery – especially protein drug delivery.

However, pristine CNTs are completely insoluble in almost all solvents [Bianco et al., 2005c] and could cause some health concern because of their potential toxicity [Colvin, 2003]. Fortunately, the development of efficient methodologies for the chemical modification of CNTs, also known as "functionalization", has stimulated the perparation of soluble CNTs that can be utilized in drug delivery and vaccine delivery. A review of CNT functionalization methodologies will be given in Chapter 2.

1.6 Research Objectives

Most experiments have demonstrated that a DNA vaccine for HCV can elicit immune responses in small animals; however, its immune responses in large animals and humans are usually too weak to protect against new infection. One possible reason is that the level of gene expression conferred by DNA vaccines is not high enough to induce sustained immune responses. Functionalized CNTs may be able to enhance the efficacy of DNA vaccine by enhancing gene expression. This study aims to determine whether an f-CNTs-based delivery system increases gene expression by a plasmid DNA.

In particular, this study has the following specific research objectives:

(1) Functionalization of pristine CNTs;

(2) Design and construction of an f-CNT delivery system that can carry plasmid DNAs; and

(3) Evaluation of whether the f-CNT system can enhance gene expression after transfecting plasmid DNAs *in vitro*.

Chapter 2

Background and Related Work

2.1 DNA Vaccines for Hepatitis C Virus

Although approximately, 3% of the world's population is infected with hepatitis C virus, there is no effective vaccine available [Li et al., 2006]. There are many obstacles to the development of a vaccine against HCV [Lechmann et al., 2000]. The fact that HCV can mutate rapidly, allowing it to adapt to new environments is one of them. Most difficult, however, is the finding that HCV infection does not seem to induce a sterile immunity; in other words, reinfection of different or even identical strains can occur after recovery from a previous infection [Bukh et al., 2001]. This finding indicates that inducing high-titer, long-lasting, and cross-reactive antibodies and a cellular immune response that includes both helper and cytotoxic T lymphocytes may be necessary for an effective vaccine. Promising vaccine candidates such as DNA vaccine have been explored and shown to have many advantages.

A DNA-based vaccine usually consists of a plasmid DNA that carries sequences encoding an antigen of interest under the control of a strong promoter. DNA vaccines could be designed to bias the type of T helper cell towards a Th1 or Th2 response [Feltquate et al., 1997]. DNA immunization is able to raise T helper cell responses,

CTL responses, and antibody responses. Many studies have been conducted on the development of DNA-based vaccines against HCV.

HCV core protein represents a valuable target in the development of vaccines, because it is the most conserved viral antigen [Brinster et al., 2001]. Lagging et al. [1995] investigated immune responses to plasmid DNA encoding the HCV core protein. The result from intramuscular inoculation of mice indicated that the DNA vaccine generated HCV core-specific antibody responses, lymphoproliferative responses, and CTL activity. A team from the U.S. [Tokushige et al., 1996] generated a strong CD8+ T lymphocyte activity both *in vivo* and *in vitro* by utilizing DNA vaccine pHCV2-2 (containing the entire HCV core region) and pHCV4-2 (containing both the 5' noncoding region and the core region). Similarly, in another study [Arichi et al., 2000], HLA-A2.1-transgenic mice were inoculated with a plasmid DNA vaccine encoding the HCV core antigen. The immunization elicited CTLs and persisted at least 14 months, and this process has shown to be mediated by CD8+ cells. Further, when challenged, the DNA immunized mice showed a substantial reduction in vaccinia virus titer compared with the mock-immunized controls.

Because of the high degree of genetic heterogeneity exhibited by HCV envelope glycoproteins, it is not at first obvious to include them in a vaccine preparation; in addition, the great capacity of the E2 hypervariable region 1 (HVR1) to mutate appears to be mainly responsible for the appearance of immune escape mutants

[Shimizu et al., 1996]. Forns et al. [2000] used a DNA vaccine encoding HCV surface-expressed envelope 2 (E2) glycoproteins to inoculate chimpanzees. One of the chimpanzees developed antibodies to E2 and HVR1 after the second and third immunization, respectively. A strong CTL response was detected after the HCV challenge in one chimpanzee, and an E2-specific CD4+ response was both detected before and after challenge in two other chimpanzees. Jin et al. [2002] inoculated mice with a plasmid DNA vaccine expressing five fragments of HCV E2 fused to the hepatitis B virus surface antigen HBsAg (HBV S). After one primary and one boosting immunizations, antibodies against both HBV S and HCV E2 were detected in mouse sera and a high-level expression of INF-γ was detected in the cultured splenic cells. The results indicated that these fusion constructs could efficiently elicit humoral and Th1 dominant cellular immune responses against both HBV S and HCV E2 antigens in DNA-immunized mice. In another study [Lee et al., 1998], antibody responses against HCV envelope proteins E1 and E2 were induced in the immunization of DNA vaccine encoding these proteins in rats. O'Hagan et al. [2004] also demonstrated that vaccination of mice with plasmids encoding HCV E1 and E2 antigens induced humoral and cellular immune responses.

HCV nonstructural (NS) proteins are also interesting vaccine candidates because of their crucial functions in the viral life cycle and, consequently, the fact that a number of NS-encoded domains are particularly well conserved among the various existing genotypes and subtypes [Higashi et al., 1993; Kato et al., 1994]. Brinster et al. [2001]

evaluated the immune responses generated by plasmid DNA vaccines, expressing HCV NS3 protein both *in vitro* and *in vivo*. The plasmids were shown to express a protein of expected size and can result in the induction of a stable specific CD8+-mediated response. Two other studies [Encke et al., 1998; Cho et al., 1999] on DNA immunization utilized plasmids encoding NS3, NS4, and NS5 proteins separately or collectively demonstrated that these DNA vaccines successfully induced HCV-specific antibodies against those three nonstructural proteins in both mice and rats. Both studies have shown that the immunization induces the lymphoproliferative responses.

These studies have demonstrated the potency of DNA-based vaccines to induce both humoral and cellular immune responses against various HCV proteins. However, the responses induced by naked plasmid DNAs are usually very weak. Therefore, the delivery of DNA vaccines becomes a crucial step that should be studied in more detail. Factors such as the routes and methods of delivery and the delivery systems should be studied for a more effective DNA vaccine for HCV.

2.2 DNA Vaccine Delivery System

DNA vaccines are stable, heat-resistant, economical and easily constructed by molecular cloning techniques, making them suitable for worldwide application [Davis et al., 1999]. However, the immunization efficiency of the plasmid DNA vaccine is

usually low *in vivo* due to the fast degradation of the plasmid DNA during the course of delivery [Hurk, 2006]. A proper delivery system becomes critical to the development of an effective DNA vaccine.

2.2.1 Routes of administration for DNA vaccine delivery

DNA vaccines have been introduced into animal tissues by a number of different methods. The following are a few of the routes of administration for plasmid DNA vaccines [Fynan et al., 1995]:

- IV: Intravenous – within or administered into a vein,
- IM: Intramuscular –within a muscle,
- ID: Intradermal –within or between the layers of the skin,
- IP: Intraperitoneal – within the peritoneal cavity (body cavity),
- SC: Subcutaneous – within the layer of tissue directly underlying the skin,
- IN: Intranasal –within the nose,
- Oral – within the mouth, and
- Topical – on a body surface.

The most extensively used route for DNA vaccine delivery is the parenteral route, including IM, ID, IV, and SC. However, these routes may bring complications with delivery systems, because they are all needle-based systems [Azad et al., 2006]. Based on the principle that "the delivery methods should be as non-invasive as possible"

[Hurk, 2006], topical administration to the skin [Cui et al., 2006], nasal delivery [Fynan et al., 1993], and oral administration [Chen et al., 2007] have shown their potential by reducing the level of invasiveness while maintaining the efficiency of the delivery.

2.2.2 DNA vaccine delivery systems

The barriers for developing an effective DNA vaccine may be circumvented by improved delivery systems which are constructed using chemical, mechanical, and physical effects or means. Table 2.1 summarizes the delivery systems of DNA vaccines and materials of these systems.

Table 2.1 DNA Vaccine Delivery systems and Materials of the Systems

Delivery System	Materials	Routes	References
Chemical			
Liposomes	Cationic lipid, etc.	Topical, IP, IM, ID, SC	Choi et al., 2006; Lu et al., 2007
Microparticles	Cationic polymer, etc.	ID, oral, IM, IP, SC	Kasturi et al., 2006; He et al., 2005; Singh et al., 2006; O'Hagan et al., 2004; Jilek et al., 2005

Nanomaterials	Biodegradable Polymer, f-CNTs, Silica, CpG ODN, etc.	Oral, SC, ID, IM, Topical, nasal； IP	Chen et al., 2007; Chong et al., 2005; Cohen et al., 2000; Cui et al., 2002, 2003; Mumper et al., 2003; Vila et al., 2002; Panyam et al., 2003 Geissler et al., 1997; Ouyang et al., 2002; Lee et al., 1998; Cui et al., 2003a; Sasaki et al., 2003; Li et al., 2006, Hu et al., 2007, Jeon et al., 2007; Perez et al., 2001; Pan et al., 2007; Bianco et al., 2004, 2005; Rojas-Chapana et al., 2005; Gao et al., 2006; Singh et al., 2005; Pantarotto et al., 2004
Mechanical			
Gene gun	High-velocity bombardment	ID, oral, IM	Chuang et al., 2005; Chen et al., 1999; Wang et al., 2003; Sasaki et al.,2003
Jet injection	High-pressure delivery of liquid	ID, SC, Topical	Cui et al., 2003b; Mumper et al., 2003
Physical			
Electroporation	Short controlled electric pulses	IM, ID	Wideca et al., 2000; Zucchelli et al., 2000
Magnetofection	Paramagnetic particles and magnetic field	IV	Mykhaylyk et al., 2007; Dobson, 2006

Several materials, including genetic, biological and chemical, are being developed as delivery systems for DNA vaccine. Liposomes are synthesized from cholesterol or non-toxic lipids. They are considered to be attractive drug delivery carriers. They can circulate in the blood stream for a long time without causing health concerns [Yih et al., 2006]. Microparticles are small particles ranging from 1 to 100 micrometers in size with different shapes. They could be flexible, biodegradable, and biocompatible. In a microparticle-based drug delivery system, a broad range of polymers are used to encapsulate plasmid DNA. Encapsulating DNA vaccine in polymers makes non-invasive delivery, such as oral or nasal delivery, much more promising because the entrapped plasmid DNA is protected against degradation in the gut during the course of delivery [Azad et al., 2006]. Nanomaterials offer a number of distinct advantages over microparticles due to their nano size. They are able to penetrate through the submucosal layers while the microparticles are mainly localized in the epithelial lining. Nanoparticles have in general a relatively higher intracellular uptake compared to microparticles and this property with the nanoparticles shows its more promise as a plasmid DNA delivery system [Panyam et al., 2003].

The most common method for the delivery of a vaccine is injection with syringes and needles through intramuscular, intravenous, or intradermal routes. The needle-based delivery system could be a painful procedure for animals or humans, especially children. Because needle injection is one of the main routes for infection of some viral

virus, such as HCV or HIV, needle-based delivery system could create certain concerns if they were previously used. Needle-free, less invasive delivery methods for plasmid DNA such as jet injection and delivery by gene gun show their advantage over needle-based delivery systems [Hurk, 2006]. Electroporation is one of the most efficient methods using a high-voltage electrical current to facilitate DNA transfer [Patil et al., 2005]. Unfortunately, this technique results in high cell mortality and is extremely difficult to apply *in vivo* and in clinical trails [Luo et al., 2000]. In the magnetofection delivery system, DNA vaccine can be attached to magnetic nanoparticles and then focused to the target cells, tissues, or organs via high-field magnets. This technique increases the speed and level of transfection but meets major obstacles such as toxicity issue, difficulties in targeting sites in large animals and humans and the complexity in the clinic settings [Dobson et al., 2006].

2.3 Functionalization of Carbon Nanotubes

Carbon nanotubes (CNTs) are considered to be a promising material for several applications, ranging from the mechanical field to biological field. However, the use of CNTs as drug delivery system is limited because the pristine ones are completely insoluble in all solvents and their toxicity could generate some health concerns [Colvin, 2003]. Their compatibility with aqueous environments has been made possible by chemical functionalization of their surface. Functionalized CNTs have

been explored for their potential as delivery vehicles of biologically active molecules, including vaccines [Singh, 2005].

The approaches of functionalization of CNTs can be classified into covalent and noncovalent functionalization [Bianco et al., 2005c]. Covalent functionalization (Table 2.2) is based on the covalent linkage of functional groups onto a nanotube's sidewalls or end parts, where the linkage is usually associated with a change of the property binding between carbon atoms [Campidelli et al., 2006]. Non-covalent functionalization (Table 2.2) is mainly based on supramolecular complexation because of various adsorption forces, such as van der Waals' and π-stacking interactions [Hirsch, 2002].

Table 2.2 Covalent and Noncovalent Functionalization of CNT

Covalent functionalization	References	Noncovalent functionalization	References
1,3-Dipolar cycloaddition	Tagmatarchis et al., 2004; Bianco et al., 2004, 2005; Pantarotto et al., 2003, 2004; Campidelli et al., 2006; Singh et al., 2005; Georgakilas et al., 2001, 2002; Pastorin et al., 2006	Peptides	Witus et al., 2007; Wang et al., 2003

Oxidative treatment	Cai et al., 2007; Rojas-Chapana et al., 2005; Gao et al., 2006; Pan et al., 2007; Campidelli et al., 2006; Hamon et al., 1999; Sun et al., 2002; Yu et al., 2008	Proteins	Karajanagi et al., 2006; Shim et al., 2002
Ammonium	Gao et al., 2006; Pastorin et al., 2006; Georgakilas et al., 2001, 2002; Hamon et al., 1999; Singh et al., 2005	Electrografting	Petrov et al., 2004
Solvent-free	Dyke et al., 2003	Pyrene	Campidelli et al., 2006; Chen et al., 2001
Reduction of diazonium salts	Bahr et al., 2001	Poly (acrylic acid)	Liu et al., 2006

Functionalized CNTs can be applied to not only the drug delivery system but also to other fields such as optical performance in electrical, emission displays, supercapacitors, molecular computers, and ultrahigh-strength materials applications. In these latter applications, the CNTs are required to be separated into individual tubes or bundles of only a few tubes [Qin et al., 2004], while the high molecular weights and strong intertube forces keep the pristine CNTs together in large bundles

[Tagmatarchis et al., 2004]. Covalent functionalization of CNTs using addition reaction has been considered to be very promising for nanotube modification and derivation. One of the most common functionalization techniques is the oxidative treatment of nanotubes by liquid or gas phase oxidation, such as the treatment with strong acid to introduce the carboxylic group and other groups such as hydroxyl, carbonyl, and ester group into the sidewalls of CNTs [Hirsch et al., 2005]. On the other hand, the organic functionalization of CNTs is considered to be a powerful method to improve the solubility and processibility of CNTs [Tagmatarchis et al., 2004]. The 1,3-Dipolar cycloaddition of azomethine ylides was originally developed for modification of C_{60} [Maggini et al., 1993; Prato et al., 1998] and then has been successfully adapted to functionalizing CNTs [Singh et al., 2005; Pantarooto et al., 2004; Capidelli et al., 2006; Georgakilas et al., 2002a]. In this treatment, CNTs undergo the addition reaction when heated in dimethylformamide (DMF) in the presence of a N-substituted amino acid and an aldehyde [Georgakilas et al., 2002b], and a large number of pyrrolidine rings are fused to the carbon-carbon bonds of CNTs. This could allow for a wide number of other functionalities to be immobilized onto the CNTs surface [Banerjee et al., 2005]. Biological moieties can also be introduced onto nanotube surfaces using this chemistry. The favorable antigenicity and immunogenicity of these nanotube-based conjugates make them very promising in vaccine delivery and gene therapy.

2.4 Summary

The f-CNTs-based delivery system for plasmid DNA is potentially promising for a further improvement of immune responses induced by DNA vaccines against HCV. However, understanding of the effect of f-CNTs in the delivery process is not quite clear; as such it is necessary to study this system. Although it seems to be promising to coat functional group “ammoniums” on the pyrrolidine rings to CNTs, the testing result was not very good, suggesting research on new functional groups.

Just similar to any artificial system, design and construction are two major parts for the f-CNTs-based delivery system for DNA vaccines of HCV. Here, design refers to the selection of CNTs, especially (i) single-wall or multi-wall, (ii) quantity, and (iii) purity, and (iv) selection of functionalization of CNTs (especially the type, condition of reactions and quantity of chemicals). Construction refers to the actual building of a delivery system. Furthermore, testing and evaluation of the delivery system also need a careful design with a special reference to the transfection procedure.

This thesis, as also outlined in Chapter 1, will describe a study on the design and construction of the f-CNTs-based system for delivering a plasmid DNA encoding HCV protein.

Chapter 3

Materials and Methods

3.1 Functionalization of CNTs

Functionalization of CNTs in the context of this research is to coat chemicals on the surface of CNTs stably. CNTs with few defects would be in favor of such a coating process. For this reason, in this research, single-wall CNTs were chosen. Some other decisions on what chemicals are chosen and what reactions are chosen were described in the following subsections.

3.1.1 General methods and materials

1,3-dipolar cycloaddition reactions are fundamental processes in organic chemistry [Pandey et al., 2006]. This reaction has been successfully applied to the organic modification of fullerene C_{60} [Maggini et al., 1993; Prato, 1998]. CNTs could be functionalized by this reaction [Georgakilas et al., 2002a]. In particular, the 1,3-dipolar cycloaddition reaction of azomethine ylides, the reactive intermediates that could efficiently attack the vast π-π bonds of the CNTs, is a powerful method to produce a large number of pyrrolidines rings fusing to the carbon-carbon bonds [Tagmatarchis et al., 2004; Pandey et al., 2006]. Pyrrolidines rings are an important

building block in the syntheses of many natural products and pharmaceuticals [Pandey et al., 2006]. After being functionalized by this type of chemistry, f-CNTs could be further modified.

Single-Wall Carbon Nanotubes (SWNTs) used in this work were obtained from Carbon Nanotechnologies Inc. (Purified HiPCO Single-Wall Carbon Nanotubes). The detailed specification of the SWNTs was provided by Carbon Nanotechnologies Inc. Prior to the use of the SWNTs, they were also characterized on Raman spectroscopy at Saskatchewan Structural and Science Centre. The result is in consistence with that provided by Carbon Nanotechnologies Inc. Triethylene glycol monomethyl ether, 2-nitrobenzenesulfonyl chloride, and paraformaldehyde were purchased from Sigma-Aldrich. Other reagents and solvents used for functionalization of CNTs were kindly provided by Dr. Palmer (Department of Chemistry, University of Saskatchewan). All these reagents and solvents were purchased reagent grade from Sigma-Aldrich and used without further purification.

3.1.2 Synthesis of the N-substituted amino acid

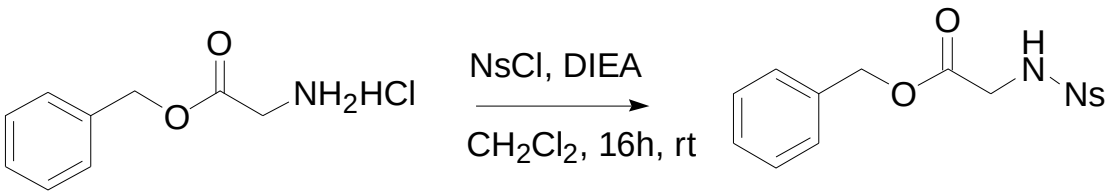
To functionalize CNTs, appropriate amino acids and aldehydes should be applied in the 1,3-dipolar cycloaddition reaction. The functional groups present in the starting materials are critical elements to render CNTs soluble. This explains the reason for choosing the amino acid characterized by the presence of hydrophilic chains – i.e.

poly (ethylene glycol) (PEG) chains. PEG is non-toxic, non-immunogenic and highly flexible. It can be used in surfaces treatment without interfering cellular functions, bioconjugations, or target immunogenicities [Manta et al., 2003; Wood et al., 2005]. Using CNTs as a drug delivery system could be achieved by the f-CNTs that are functionalized with the N-substituted amino acids characterized by PEG and aldehydes via the 1,3-dipolar cycloaddition reaction.

To synthesize this particular N-substituted amino acid, we proposed a method based on the methods proposed by Brough [2006] and Sakaguchi [2007]. The general motivation of the proposed method was that we failed to functionalize the SWNTs based on Brough's method.

3.1.2.1 Synthesis of N-substituted amino acid with Brough's method

Compound A

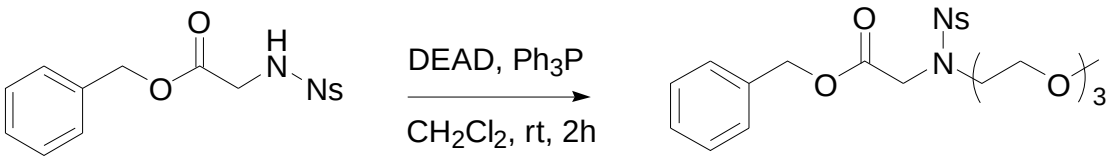


A

To a solution of glycine benzyl ester hydrochloride (0.8475 g, 4.2 mmol) and N, N-Diisopropylethylamine (DIEA, 1.688 mL, 10.7 mmol) in distilled CH_2Cl_2 (35 mL),

a solution of 2-nitrobenzenesulfonul chloride (NsCl, 1.188 g, 5.3 mmol, 10 mL of distilled CH_2Cl_2) was added dropwise over a period of 1 hour. The solution was stirred for 16 hours at room temperature and the reaction mixture was concentrated under reduced pressure. The crude mixture was washed with ethyl acetate (50 mL). The organic phase was washed with saturated $NaHCO_3$ (2X10 mL) and H_2O (2X20 mL), then dried over Na_2SO_4, filtered and evaporated to give **Compound A** as a yellow oil.

Compound B

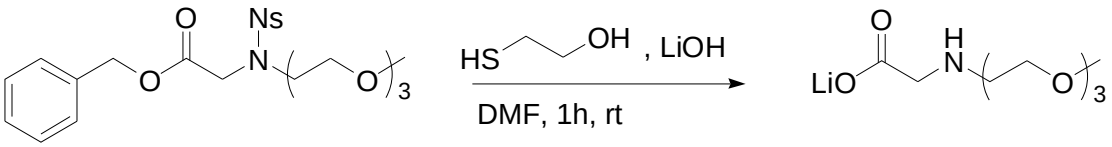


A **B**

To a solution of triethylene glycol monomethyl ether (0.808 g, 4.92 mmol), **Compound A** (0.575 g, 1.64 mmol) and triphenylphosphine (Ph_3P, 0.627g, 2.39mmol) in distilled CH_2Cl_2 (10 mL) were added dropwise into a solution of diethyl azodicarboxylate (DEAD, 40%wt, 1.04 g, 2.39 mmol, 5 mL distilled CH_2Cl_2) over a period of 30 minutes. The solution was stirred for a further 2 hours at room temperature, and then the reaction mixture was concentrated under reduced pressure. The organic phase was extracted with 50 mL of 1M KOH and the aqueous phase was re-extracted with ethyl acetate (50 mL), and then the two organic phases were

evaporated. The crude mixture was purified using flash chromatography on SiO_2 (5% CH_2Cl_2/acetore) giving **Compound B** as a yellow oil.

Compound C



B **C**

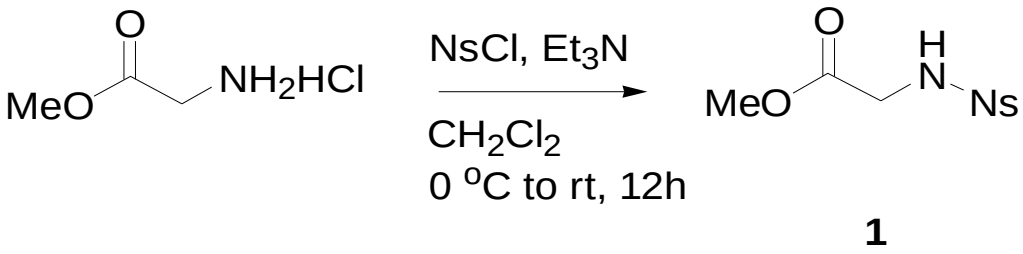
A solution of **Compound B** (0.606 g, 1.43 mmol), 2-mercaptoethanol (201.3 μL, 2.9 mmol) and LiOH (122 mg, 2.9 mmol) in dimethylformamide (DMF, 20 mL) was stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure and the crude mixture was extracted with CH_2Cl_2 (2X20 mL) and H_2O (20 mL). The aqueous phase was lyophylisated to give the target **Compound C** as a yellow solid.

Our experience of using Brough's method concludes: (1) the starting material in Brough's method was very difficult in a separation process, and (2) the process from **Compound B** to **Compound C** involved considerable noises, examined with NMR spectrum. For (1), we chose a different starting material (see discussions in the next section). For (2), we adopted a method proposed by Sakaguchi et al. [2007], which was not used for functionalization of CNTs but general-purpose synthesis of

chemicals. In particular, we replaced the process in Brough's method from **Compound B** to **Compound C** by two processes which were learned from Sakaguchi et al. [2007]. Our new method is presented in the next section.

3.1.2.2 Synthesis of N-substituted amino acid

Compound 1

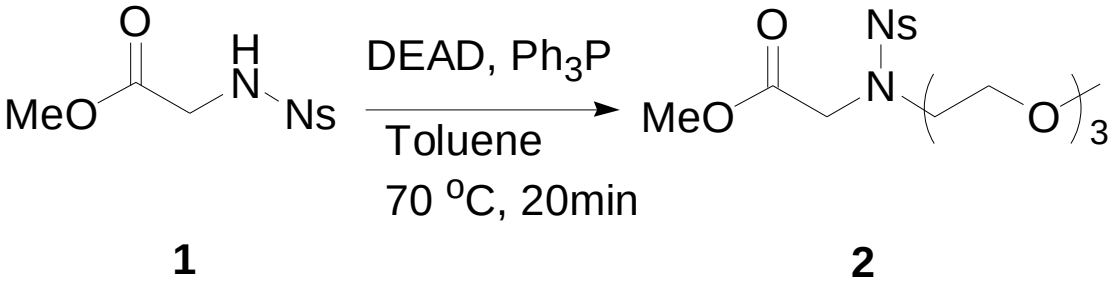


1

To a suspension of Glycine methyl ester hydrochloride (2.45 g, 19.5 mmol) and water (1.5 ml) in CH_2Cl_2 (30 ml), triethylamine (Et_3N, 5.5 ml, 39.5 mmol) and 2-nitrobenzenesulfonyl chloride (NsCl, 4.83 g, 21.8 mmol) were added at 0^oC. The reaction mixture was gradually warmed up to room temperature. After stirring for 12 hours, the mixture was concentrated under reduced pressure. After addition of water, the mixture was extracted with ethyl acetate three times. The combined organic extracts were washed with water and saturated $NaHCO_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was recrystallized from a mixture of ethyl acetate and toluene giving the desired **Compound 1** (3.6 g, 67.3%) as a colorless solid.

^{1}H NMR: δ 8.11-8.09 (m, 1H), 7.96-7.94 (m, 1H), 7.76-7.74 (m, 2H), 6.05 (s, 1H), 4.03-4.02 (d, 2H), 3.61 (s, 3H).

Compound 2

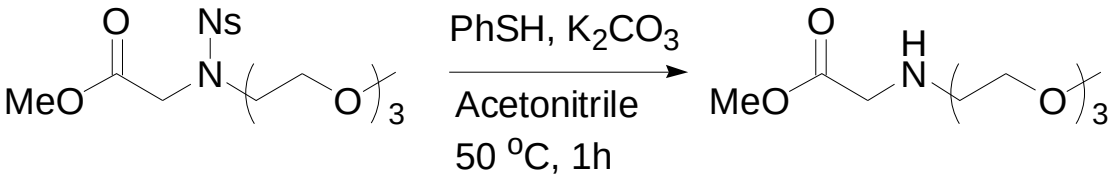


1 **2**

A toluene (25 ml) solution of triethylene glycol monomethyl ether (0.821 g, 5 mmol), **Compound 1** (1.37 g, 5 mmol), and triphenylphosphine (Ph_3P, 1.31 g) were warmed to 70^{o}C. After DEAD (40%wt, 2.28 ml, 5 mmol) in 5-ml toluene solution was added to the mixture above via syringe slowly, the mixture was stirred for 20 minutes at 70^{o}C. After the mixture was cooled down to room temperature, the reaction mixture was concentrated under reduced pressure. The organic phase was extracted with KOH (5M, 30 ml) and the aqueous phase was re-extracted with ethyl acetate (50 ml), and then the two organic phases were evaporated. The crude mixture was purified by flash chromatography on SiO_2 (2% MeOH/CH_2Cl_2), giving **Compound 2** (1.4509 g, 69%) as a yellow oil.

^{1}H NMR: δ 8.09-8.07 (m, 1H), 7.70-7.68 (m, 2H), 7.63-7.62 (m, 1H), 4.37 (s, 2H), 3.67-3.65 (m, 5H), 3.61-3.54 (m, 10H), 3.37 (s, 2H). ^{13}C NMR: δ 169.6, 131.7, 130.9, 124.2, 71.9, 70.6, 70.6, 70.4, 59.1, 52.2, 49.6, 48.2.

Compound 3

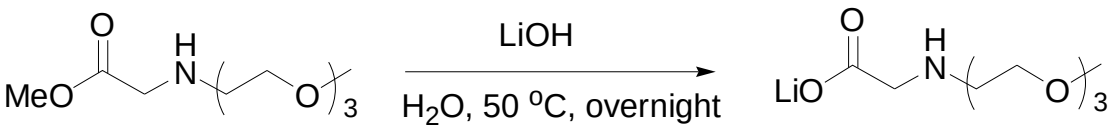


2 **3**

To a solution of **Compound 2** (1.01 g, 2.4 mmol) in acetonitrile (15 ml), benzenethiol (PhSH, 0.74ml) and potassium carbonate (1.33g, 9.6mmol) were added and warmed up to 50°C. After stirring for 1 hour at 50°C, the reaction mixture was cooled down to room temperature. After K_2CO_3 was filtered, the mixture was concentrated under reduced pressure and purified by flash chromatography on SiO_2 with ethyl acetate (100 ml) first and then with 10% MeOH/CH_2Cl_2, giving **Compound 3** (0.39 g, 69%) as a yellow oil.

^{1}H NMR: δ 3.72 (s, 3H), 3.65-3.59 (m, 8H), 3.56-3.54 (m, 2H), 3.46 (s, 2H), 3.37 (s, 3H), 2.82-2.80 (m, 2H), 2.21 (s, 1H). ^{13}C NMR: δ 170.0, 74.8, 74.5, 74.3, 69.4, 68.0, 68.0, 67.8, 56.5, 49.2, 48.2, 46.2.

Compound 4



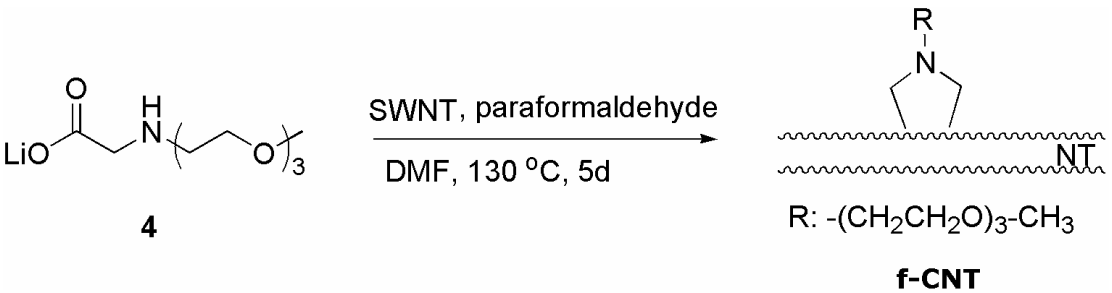
3 **4**

A water (5 ml) solution of **Compound 3** (193 mg, 0.82 mmol) and LiOH (32.7 mg, 0.78 mmol) was warmed to 50°C. The mixture was stirred over night at 50°C. After the mixture was cooled down to room temperature, the reaction mixture was concentrated under a reduced pressure giving the target **Compound 4** (the N-substituted amino acid, 153 mg, 82.1%) as a colorless oil.

^{1}H NMR (D_2O): δ 3.66-3.58 (m, 12H), 3.35 (s, 3H), 3.26 (s, 1H), 2.87-2.84 (m, 2H). ^{13}C NMR (D_2O): δ 177.5, 162.6, 71.0, 69.6, 69.5, 69.4, 69.4, 69.1, 68.6, 61.5, 58.0, 51.6, 51.3, 47.1, 46.9, 34.0, 28.4.

3.1.3 Functionalization of CNTs

Scheme 3.1



SWNT (22.8 mg) and paraformaldeyde (76 mg) were suspended in 45 ml of DMF. The heterogeneous mixture was heated at 130°C, while a solution of **Compound 4** (76 mg) in 5 ml DMF was added in portions (4 X 0.5 ml every 24 hours) and the reaction was continued for 5 days as described [Georgakilas et al., 2002]. The reaction was stopped after 5 days and the organic phase was separated from the unreacted material by centrifugation (2000xg, 10 min, room temperature) and filtration followed

by washing by CH_2Cl_2 for several times. The combined organics were evaporated under a reduced pressure and the remaining oily brown residue was extracted with CH_2Cl_2/H_2O. The organic phase was washed three times with water, dried over Na_2SO_4, and concentrated to dryness giving **functionalized CNTs** (91.7 mg) as a brown solid.

3.1.4 Nuclear Magnetic Resonance (NMR)

NMR refers to a method that exploits the quantum mechanical magnetic properties of an atom's nucleus to study molecules. It is a principal and powerful technique that can provide detailed physical, chemical, electronic and structural information about molecules. Nuclei that contain odd numbers of protons or neutrons could be observed by NMR. The most commonly measured nuclei are hydrogen-1 and carbon-13. In chemical synthesis studies, NMR is very powerful and useful technique that has been widely applied [Muller et al., 1997; Bahr et al., 2001; Kordatos et al., 2001; Brough et al., 2006]. By studying the peaks of NMR spectra, the structure of many compounds can be determined [Gottlieb et al., 1997].

In this research, we used NMR (propertied by Saskatchewan Structural Sciences Centre, University of Saskatchewan) to confirm the structure of the compounds that were synthesized from each step in the whole process of functionalizing CNTs.

Unless stated otherwise, NMR spectra were recorded at room temperature in $CDCl_3$ (^1H at 500MHz, and ^{13}C at 125 MHz).

3.1.5 Transmission Electron Microscopy (TEM)

TEM is a microscopy technique that can reveal an image of an ultra thin specimen through a beam of electrons. It is a very useful instrument that is widely used in material science and biological study [Pantarotto et al., 2003a; Bahr et al., 2001; Yu et al., 2008; Witus et al., 2007]. In this research, we utilized TEM for imaging the structure of pristine SWNT and f-CNTs. The samples were sent to the Department of Materials Engineering at the University of British Columbia. Specimen was prepared and imaged under a Hitachi H-800 electron transmission microscope at 100 kV.

3.2 Biological experiments of plasmid DNA delivery and gene expression *in vitro*

3.2.1 General methods and materials

Plasmid transfection, a process of introducing nucleic acids (DNA or RNA) into cells by non-viral methods, is the most common way of studying gene delivery *in vitro*. We have two objectives in this section: 1) to determine whether f-CNTs generated in this thesis can deliver plasmid DNA into cells; and 2) whether f-CNTs can enhance gene

expression when a plasmid is transfected by a conventional method in the presence of f-CNTs.

All the reagents, chemicals, and equipments needed for the biological experiments for gene expression were supplied by Vaccine and Infectious Disease Organization (VIDO) at the University of Saskatchewan, specifically Dr. Q. Liu's Laboratory. They will be described along with the following description of major individual experiments.

The Huh-7 cells were kindly provided by from Dr. Joyce Wilson (VIDO). Plasmid pLL166 is a plasmid vector containing no transgene, which was used as a negative control. Plasmids pLL122, pLL790 and pLL311 encoding luciferase, green fluorescent protein (GFP), and HCV core protein, respectively, were obtained from Dr. Q. Liu's laboratory.

3.2.2 Cell culture

Huh-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% 4-(2-hydroxyethyl) piperazine-1-erhanesulfonic (HEPES) buffer, and 0.1% gentamicin antibiotic at 37^{o}C in the presence of 5% CO_2.

3.2.3 Calcium phosphate precipitation method

Calcium phosphate precipitation method is based on the principle that spontaneous precipitation occurs in supersaturated solutions. When calcium chloride is added to the solution that contains phosphate at a low concentration, a fine precipitate of the positively charged calcium and the negatively charged phosphate will form, then when plasmid DNA is added to the suspension, it readily co-precipitates with the calcium phosphate and becomes adsorbed onto the cells [Graham et al., 1973; Jordan et al., 2004].

Plasmid DNA (1.0 μg) was dissolved in deionized water (45 μl). $CaCl_2$ (5 μl) was then added to the plasmid DNA solution. Thereafter, 2 × N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffered solution (BBS, 50 μl) was added to the mixture dropwise with vortexing. The mixture was allowed to incubate for 30 min at room temperature prior to use.

3.2.4 Preparation of f-CNTs:DNA complexes

Functionalized CNTs were diluted in an appropriate volume of deionized water at a concentration of 1.0 μg/μl and stored at 4^{o}C until use. Plasmid DNA was hydrated in deionized water at a concentration of 1.0 μg/μl and stored frozen at -20^{o}C until use.

3.2.4.1 Complexes for transfection using f-CNTs only

Plasmid DNA (1.0 μg) was diluted in serum-free DMEM (300 μl). The f-CNTs (15 μg) were also diluted in DMEM (300 μl). Diluted plasmid and f-CNTs were mixed together by rapid pipetting for several times. Complexes were allowed to form for 30 min at room temperature prior to use [Singh et al., 2005].

3.2.4.2 Complexes for transfection using f-CNTs and calcium phosphate

Plasmid DNA (1 μg) was diluted in deionized water (22.5 μl). The f-CNTs (15 μg) were also diluted in deionized water (22.5 μl). Diluted plasmid and f-CNTs were mixed together by rapid pipetting for several times. $CaCl_2$ (5 μl) was added to complexes after they were incubated for 30 min at room temperature and 2 × BBS (50 μl) was added to the mixture dropwise with vortexing. The mixture was allowed to incubate for another 30min at room temperature prior to use.

3.2.5 Transfections

Transfection is the process of introducing genetic materials (such as plasmid DNA) into cells by non-viral methods [http://www.promega.com/guides/transfxn_guide/transfxn.pdf]. In this research, Huh-7 cells, a human hepatoma cell line, were cultured in DMEM supplemented with

10% FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic at 37^{o}C in the presence of 5% CO_2 and incubated overnight. When they reached 80% confluence, the Huh-7 cells were transfected with (1) 1.0 μg plasmid DNA (negative control), (2) 1.0 μg plasmid DNA using calcium phosphate precipitation method, (3) f-CNTs:DNA complexes, (4) f-CNTs:DNA complexes with calcium phosphate precipitation method. Cells were incubated for 48 hours at 37^{o}C with 5% CO_2 in incubator. Two hours after the transfection, the serum-free DMEM used in preparation of f-CNTs:DNA complexes was replaced with DMEM supplemented with 10%FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic.

3.2.6 Determining transfection efficiency using green fluorescent protein (GFP)

GFP, isolated from the sea pansy (*Renilla reniformis*), has a single major excitation peak at 498 nm which is in the lower green portion of the visible spectrum and can be detected by a particular instrument such as a fluorescence microscope. The GFP gene has become a commonly used marker for studies of gene expression in many fields, including cell and molecular biology [Parsons et al., 2006; Philips, 2001; Chu et al., 1999; Gao et al., 2006; Zhang et al., 2004].

3.2.6.1 Fluorescence analysis

4',6-diamidino-2-phenylindole (DAPI) is a blue-fluorescent DNA stain that can be

used to stain the chromosome DNA in both live and fixed cells [Ma et al., 1996; Britton et al., 1998; Bailey et al., 2004]. It can easily enter cells, binds strongly to double-stranded DNA and emits blue emission. Its absorption maximum is at 358nm and its emission maximum is at 461nm [http://omlc.ogi.edu/spectra/PhotochemCAD/html/dapi(H2O).html]. By using spectral unmixing or taking images sequentially, DAPI blue emission is convenient for studying a single sample with the presence of GFP. By calculating GFP fluorescence (green) and DAPI fluorescence (blue) in the same field, the number of transfected cells and the total number of cells could be determined. The transfection efficiency could be evaluated by calculating the percentage of the cells with green fluorescence to total cells.

Huh-7 cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic in 2 well chamber slides (VWR) at 37^{o}C in the presence of 5% CO_2 and incubated overnight. When they reached 80% confluence, the Huh-7 cells were transfected with plasmid DNA pLL790 encoding GFP. Forty-eight hours after transfection, the culture medium was removed. Cells were washed with PBS buffer and were fixed by 4% paraformaldehyde (Sigma-Aldrich) for 20 min. The paraformaldehyde was removed and the nuclei were stained by 300 nM DAPI for 5 min. Prolong Gold Antifade Reagent (Molecular Probes/Invitrogen) was used to mount the coverslips for viewing. The cells were observed and pictured using a Zeiss fluorescence microscope. The transfections were performed in duplicates. Five

fields of each sample were randomly chosen to be observed. Images were taken of both GFP fluorescence and DAPI fluorescence for counting GFP-expressing cells.

The limitation of this technique is that some cells may be expressing very low levels of GFP even though they are transfected. In addition, the GFP has to fold properly to emit a strong fluorescence. Therefore, weak fluorescence may not be readily visible.

3.2.6.2 Western blot analysis

Western blotting is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract [Burnette, 1981]. The sample can be separated by isoelectric charge, molecular weight, electric charge, or a combination of these factors, using gel electrophoresis. The proteins are then transferred to a membrane, where they are detected using antibodies specific to the target protein. This method is used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines [Zhu et al., 2004; Li et al., 2006; Tokushige et al., 1996; Jackel-Cram et al., 2007]. Western blot analysis usually can demonstrate the level of a protein present in a sample.

Huh-7 cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic in a 6-well plate at 37^{o}C in the presence of 5% CO_2 and incubated overnight. When they reached 80% confluence, the Huh-7 cells were

transfected with plasmid DNA pLL790 encoding GFP. Cells were lysed in a lysis buffer (1% SDS, 10mmol/L Tris-HCl, pH 8.0) 48 hours after transfection. The protein concentration of lysed cells was determined by the Bradford assay (Bio-Rad) [http://www.technomedica.com/publikazii/belur/Bio-Rad.pdf] and read at 595 nm on an ELISA reader (SPECTRA max 340PC, Molecular Devices Corporation, Sunnyvale, CA, USA). Proteins separated in 12% polyacrylamide gels (at 120V for 45 min.) were transferred onto PVDF membranes (GE Healthcare) at 25V for 1.5 hours. The blots were blocked using PBS containing 2% non-fat milk for 2-4 hours. Membranes were then incubated at 4^{o}C overnight with a GFP-specific monoclonal antibody (Q-Biogene) or β-actin-specific polyclonal antibody (Cell Signaling Technology). After washing with PBST (PBS with 0.05% Tween 20), membranes were then incubated with IR Dye 800-conjugated goat anti-mouse or IR Dye 680-conjugated goat anti-rabbit IgG antibody (Ly-Cor Biosciences) for 2 hours. After washing with PBS, the membranes were dried overnight in the dark. The protein bands on the membranes were visualized by the Li-Cor Odyssey scanner and subjected to densitometry analysis using the Quantity-One software (Bio-Rad).

3.2.7 Determining gene expression using Luciferase

Luciferase is a generic name for enzymes commonly used for bioluminescence. In a luciferase reaction, light is emitted when luciferase acts on the appropriate luciferin substrate. Photon emission can be detected by light sensitive apparatus such as a

luminometer [Promega Corporation. http://www.promega.com/multimedia/bioLum01.htm]. In biological research, luciferase commonly is used as a reporter to assess the level of gene expression in cells that are transfected with a genetic construct containing the luciferase gene [Jackel-Cram et al., 2007; Oem et al., 2007].

Huh-7 cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic in a 24-well plate at 37^{o}C in the presence of 5% CO_2 and incubated overnight. When they reached 80% confluence, the Huh-7 cells were transfected with plasmid DNA pLL122 encoding luciferase. Forty-eight hours after transfection, the culture medium was removed; the cells were washed with PBS buffer and lysed with 100µl/well Passive Lysis Buffer (Promega, http://www.promega.com/tbs/tm040/tm040.html) for 30 min. Twenty µl of cell lysate was added to 100 µl of Luciferase Assay Reagent (Promega, http://www.promega.com/tbs/tm040/tm040.html), mixed and read by a TD 20/20 Luminometer (Turner Designs) for 10 seconds. The protein concentration of lysed cells was determined by the Bradford assay (Bio-Rad) and read at 595nm on an ELISA reader (SPECTRA max 340PC, Molecular Devices Corporation, Sunnyvale, CA, USA).

3.2.8 Determining the expression of HCV core protein after transfection

Huh-7 cells were cultured in DMEM supplemented with 10% FBS, 1% HEPES buffer, and 0.1% gentamicin antibiotic in a 6-well plate at 37^{o}C in the presence of 5% CO_2 and incubated overnight. When they reached 80% confluence, the Huh-7 cells were transfected with plasmid DNA pLL311 encoding HCV core protein. Cells were lysed in a lysis buffer (1% SDS, 10mmol/L Tris-HCl, pH 8.0) 48 hours after transfection and examined by Western blot analysis using a core-specific antibody.

3.2.9 Agarose gel electrophoresis assay

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA (or RNA) molecules by size. This is achieved by forcing negatively charged DNA to migrate to the positive pole in an electric field through a sieve of molecular proportions that is made of agarose. After electrophoresis, the gel which is stained with ethidium bromide, is illuminated with an ultraviolet lamp in a light box to view the DNA bands. The gel can then be photographed with a digital or polaroid camera.

In this study, we utilized this assay to analyze the interaction between plasmid DNA and f-CNTs, in particular we examined whether f-CNTs bind with plasmid DNA. Our approach was as this. When plasmid DNA and f-CNTs are mixed, if f-CNTs bind with plasmid DNA and form a stable complex, they will be in the sediment of the mixture, and this also mean that the supernatant will not have free plasmid DNAs.

When a sufficient amount of free plasmid DNAs move into the gel, a DNA band can be observed with the ethidium bromide staining. This helps judge whether there is plasmid DNA in a mixture (supernatant or sediment).

Plasmid DNA (pLL122, 1μg) was incubated with f-CNTs (15μg) for 30min at room temperature. The mixtures were then centrifuged at 6000g for 10min. The supernatants that contain free plasmid DNAs with several different amounts (1μl, 5μl, and 10μl, respectively) were loaded on an agarose gel with 1μg/ml Ethidium Bromide. Furthermore, sediment (5μl and 25μl) was also loaded on the gel with 1μg/ml Ethidium Bromide. Electrophoresis was carried out with a current of 120V for 20min.

3.2.10 Statistical analysis

All the transfections were performed in triplicates and the experiments were repeated at least two times. Results were analyzed for statistical differences using Student's *t* test. A p value of ≤ 0.05 was considered to be statistically significant.

Chapter 4

Results and Discussion

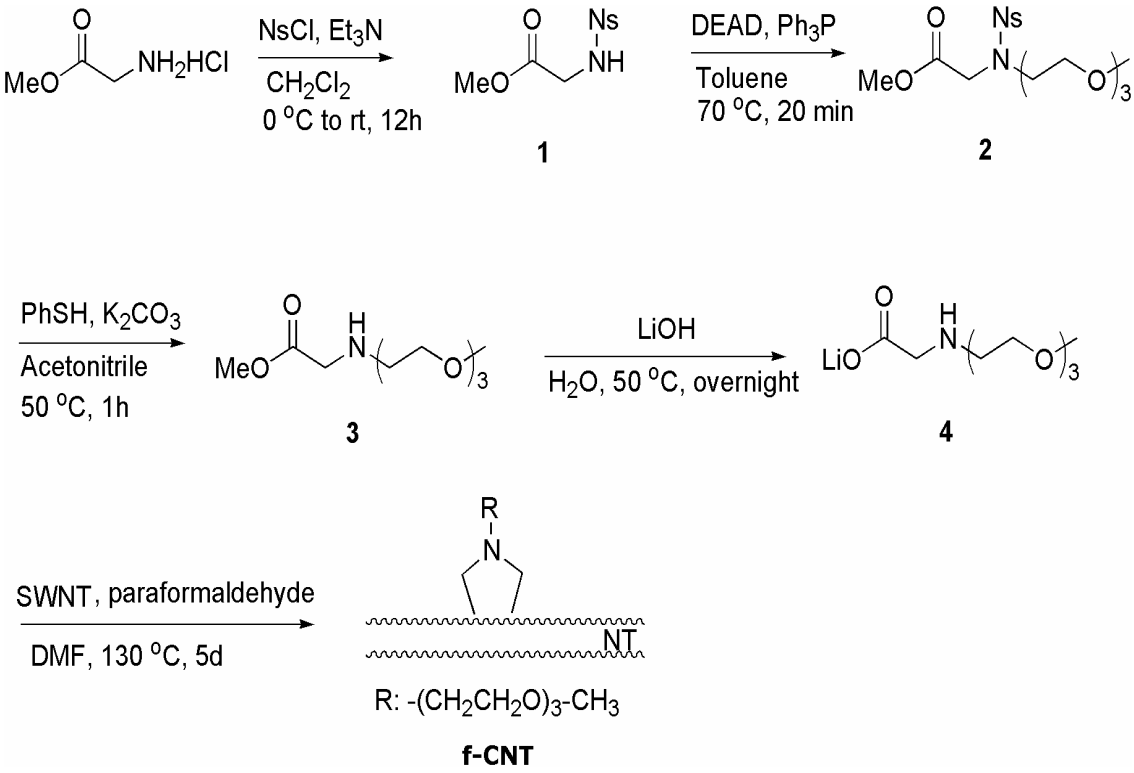
4.1 Results

4.1.1 Characterization of functionalized CNTs

The CNTs were functionalized with the synthesized amino acid via 1,3-dipolar cycloaddition reaction as described in Chapter 3. The products from each step were characterized by nuclear magnetic resonance (NMR) analysis.

Before the CNTs were functionalized, the N-substituted amino acid was first synthesized with a method as described by Brough et al. However, this attempt was successful; see also discussion in Section 3.1.2.1. Therefore, a modified procedure was proposed to make the target compound (**Compound 4**). Unlike the procedure of Brough's method, a pure and well-characterized **Compound 4** was prepared successfully and used for the following functionalization of CNTs (Scheme 4.1).

Scheme 4.1



After 1,3-dipolar cycloaddition reaction, product f-CNTs were first analyzed by NMR. NMR demonstrated the presence of the pyrrolidines rings and the poly ethylene glycol chains. The NMR spectra can be found in the appendix of this thesis.

As well, the pristine CNTs and f-CNTs were both examined by transmission electron microscopy (TEM); see Figure 4.1. In particular, Fig. 4.1 C and D show the TEM image of pristine CNTs, which appear to have coarser surfacs. It is noted that the TEM image of Fig. 4.1 C and D are much similar with those in the SWNT specification provide by Carbon Nanotechnologies Inc. Fig. 4.1 A and B show the TEM image of f-CNT; especially Fig. 4.1 B is an amplification of the area denoted by

"B" in Fig. 4.1 A. The substance on the upper left corner in Fig. 4.1 A was likely chemical impurities. It should be noted that the CNT shown in Fig 4.1 A and B is bulk CNT due to the limitation of resolution of the TEM used and insufficient dispersion of CNT in the sample preparation for TEM imaging. Nevertheless, the TEM images of Fig.4.1 A and B appear to indicate the f-CNT has a relatively smooth surface covered by chemical coatings, which was expected from the functionalization process.

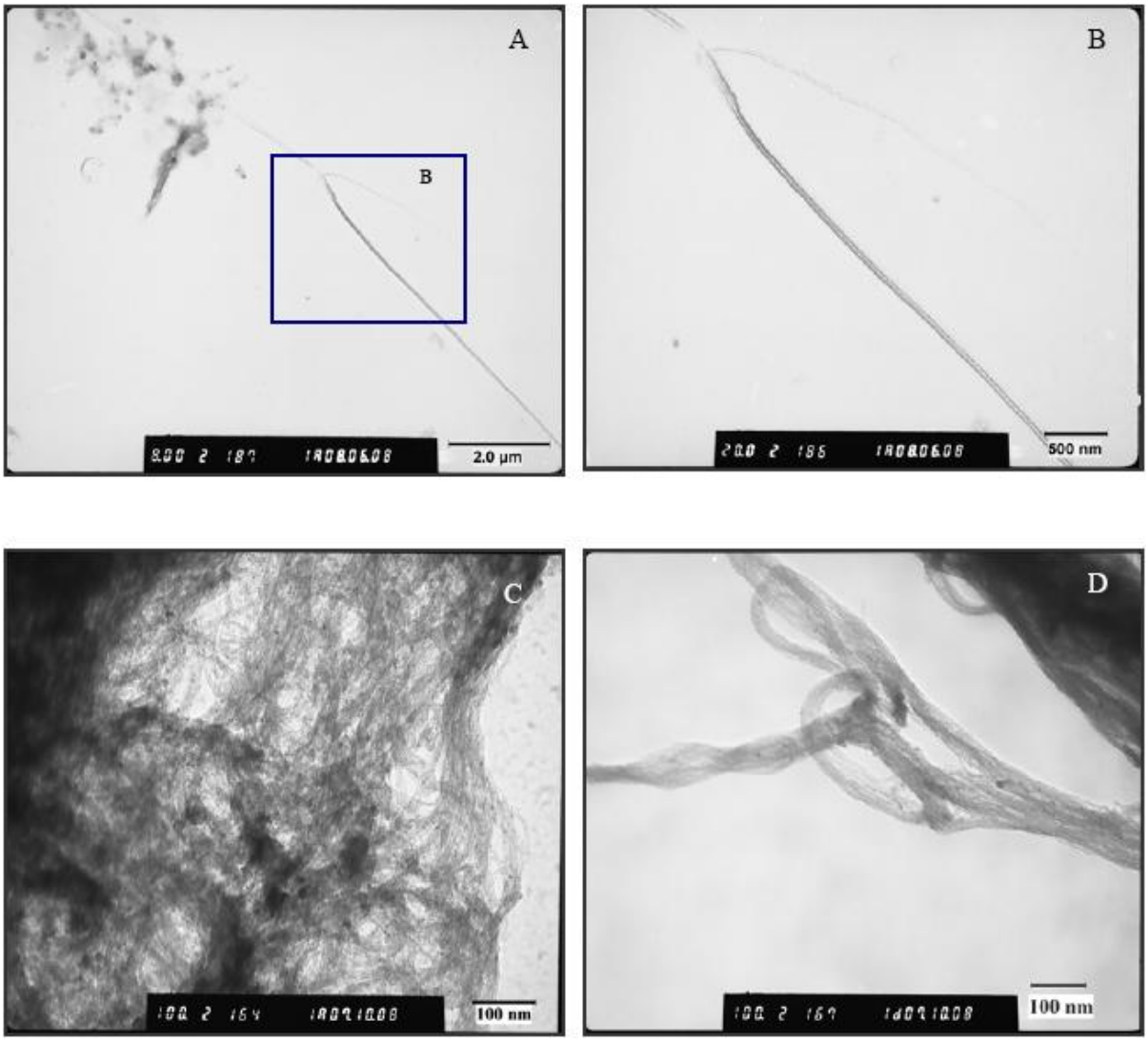


Figure 4.1 TEM image of f-CNT (A, B, C, D) and pristine CNTs (E, F).

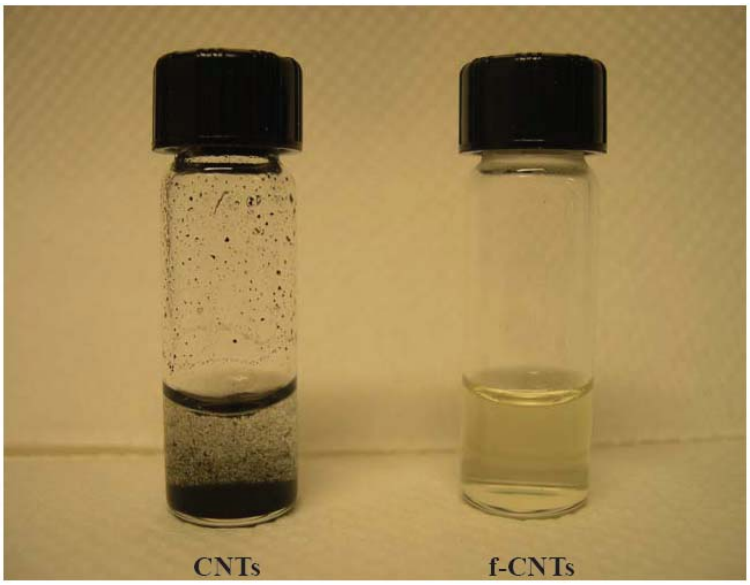


Figure 4.2 Pristine CNTs in water (left); f-CNTs in water (right).

Figure 4.2 shows two bottles with one containing water and pristine CNTs (left) and the other containing water and the f-CNTs that was prepared from N-substituted amino acid; see discussion in section 3.1.2. From this figure, it is clear that the pristine CNTs were not soluble in water, as there are many CNTs suspending in water. The f-CNTs, however, are soluble, as there is nearly no black CNT in water.

In short, the three examinations (NMR, TEM, and solubility test in water) indicated that the expected structure of f-CNTs as shown in **Scheme 4.1** (see pervious discussion in section 4.1.1) has been obtained.

4.1.2 Determining transfection efficiency and gene expression using green fluorescent protein (GFP) as a reporter

To investigate the transfection efficiency of the f-CNTs delivery system, Huh-7 cells were transfected by plasmid DNA pLL790 encoding GFP as described in Chapter 3. Forty-eight hours after transfection, cells were analyzed by fluorescence analysis (Fig. 4.3) and Western blot analysis.

Fluorescent analysis demonstrated that incubating the cells with plasmid DNA alone did not give detectable fluorescence as expected (Fig. 4.3 B). A complex containing f-CNTs and plasmid DNA also did not result in any fluorescence (Fig. 4.3 C and D). In contrast, green fluorescence was detected after transfection with calcium phosphate with or without f-CNTs. The transfection efficiency using calcium phosphate (CaP) alone was 23.7% (Fig. 4.3 E and F, Fig. 4.4), which the transfection efficiency using CaP and f-CNT was 27.9% (Fig. 4.3 G and H, Fig. 4.4). However, there was no statistical difference in transfection efficiency between calcium phosphate precipitation method and its combination with f-CNTs (p=0.18). Interestingly, the f-CNTs alone did not confer any detectable transgene expression in contrast.

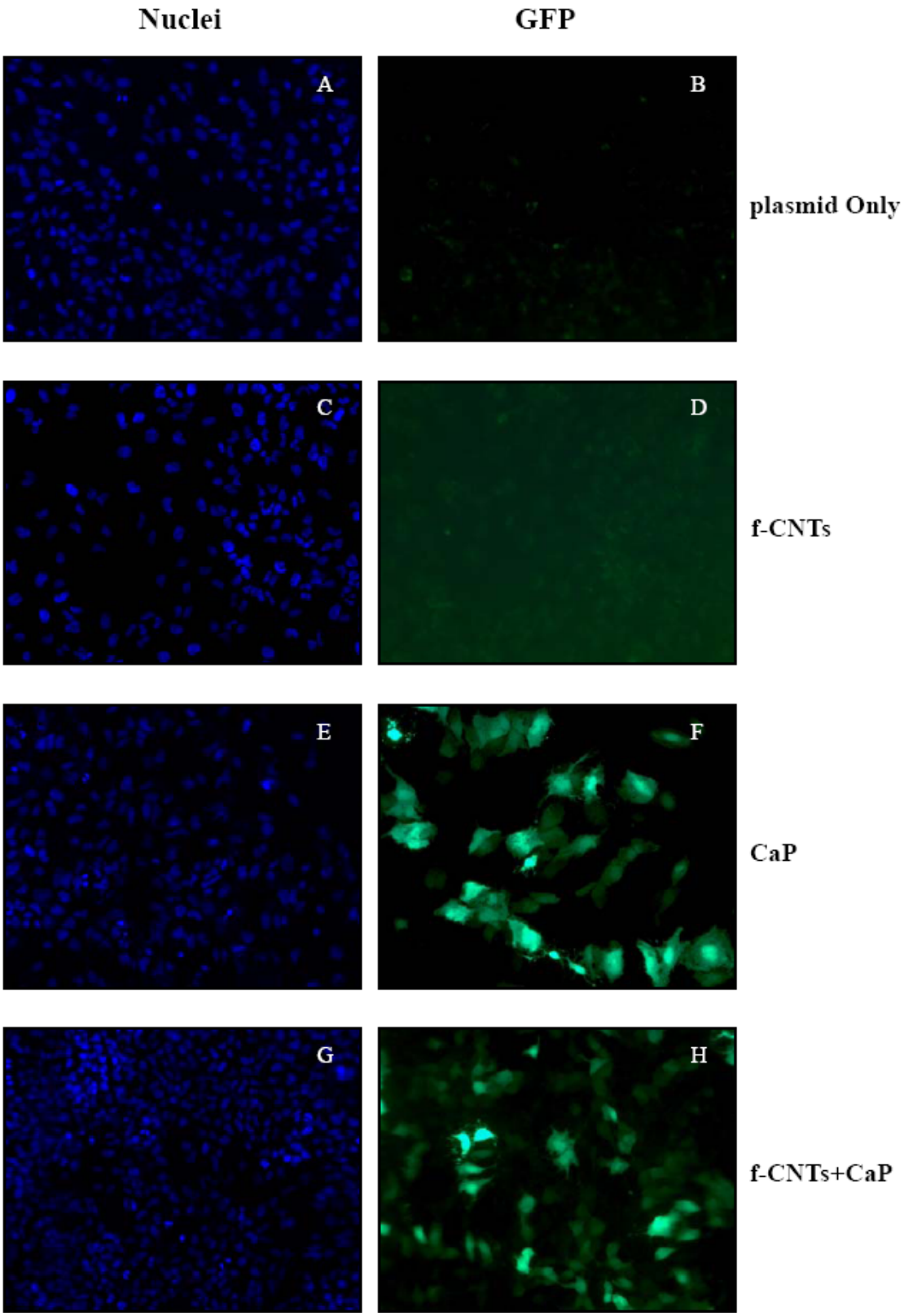


Figure 4.3 DAPI (blue) and GFP (green) fluorescent images of Huh-7 cells transfected with a plasmid encoding GFP via various delivery systems.

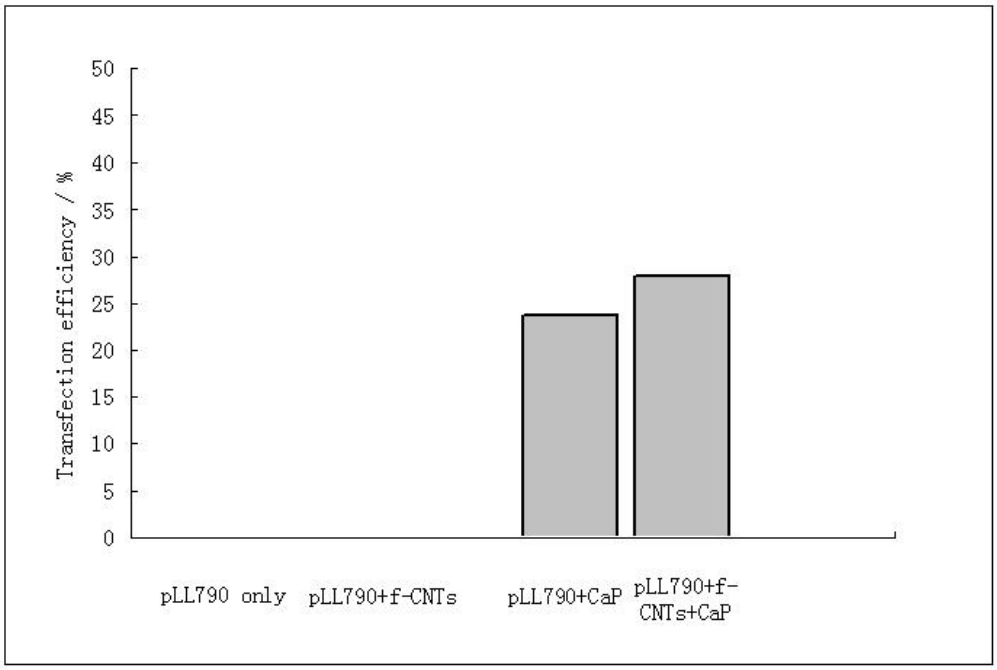


Figure 4.4 Evaluation of the transfection efficiency of a plasmid encoding GFP (pLL790) by using various delivery systems.

Consistent with the results of fluorescent microscopy, Western blot analysis showed that there was no GFP expression in the cells which have been exposed to f-CNTs complexed with plasmid DNA (Fig. 4.5a). The cells transfected by either calcium phosphate method or its combination with f-CNTs were all expressing GFP. When the levels of GFP were normalized against those of a housekeeping gene β-actin, f-CNTs were shown to enhance GFP expression when it was combined with calcium phosphate (see Fig. 4.5b, p=0.38). However, such an enhancement was not statistically significant.

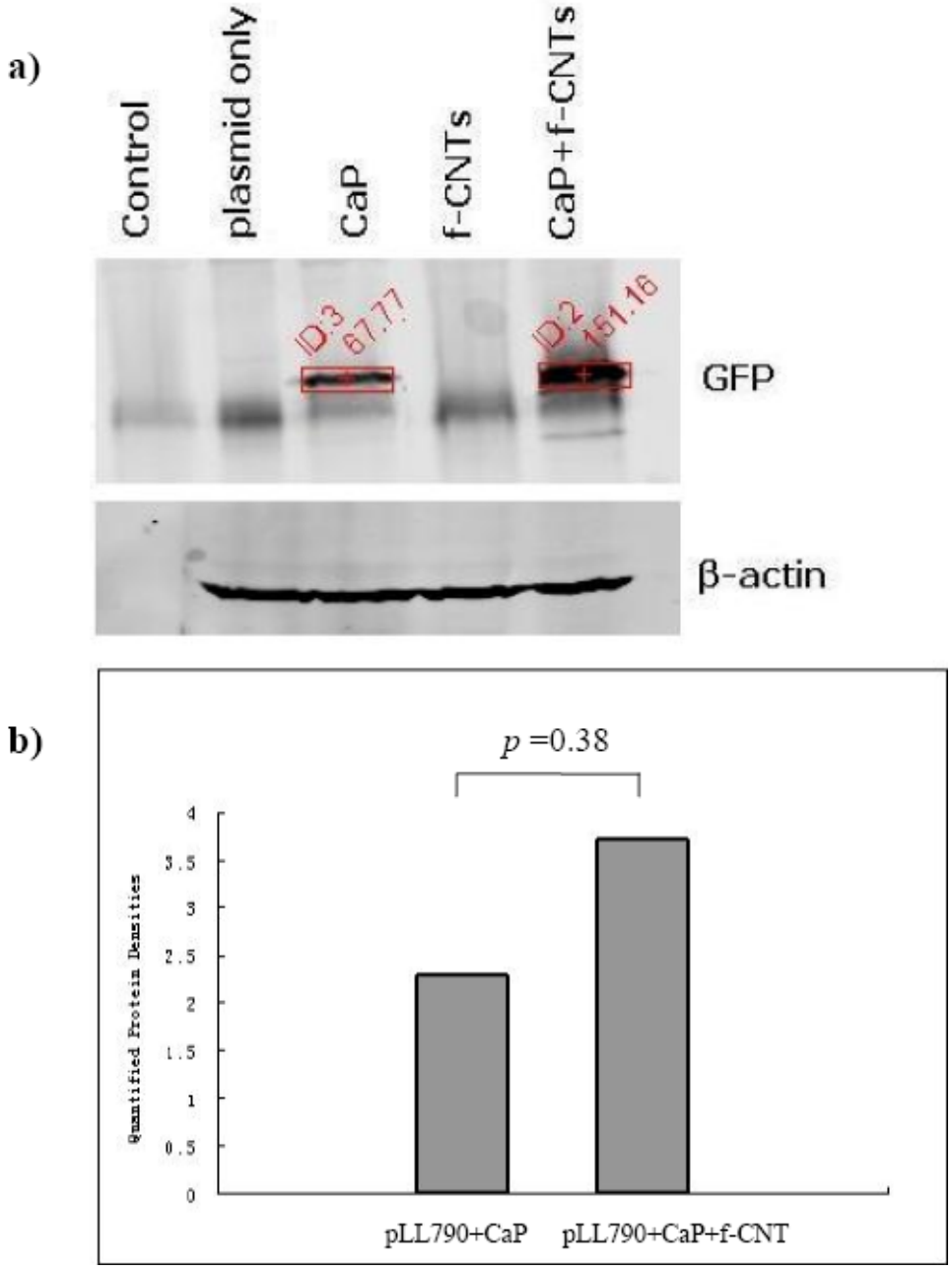


Figure 4.5 GFP western blotting of Huh-7 transfected via various delivery systems.

4.1.3 Determining gene expression using luciferase as a reporter

Plasmid pLL122 encoding luciferase was transfected to Huh-7 cells as described in Chapter 3. Luciferase assay was carried out 48 hours after transfection. Luciferase

activity of cells that is transfected via calcium phosphate precipitation method and the combined method of f-CNTs and calcium phosphate resulted in significantly higher luciferase activity than plasmid only transfection (Fig. 4.6).

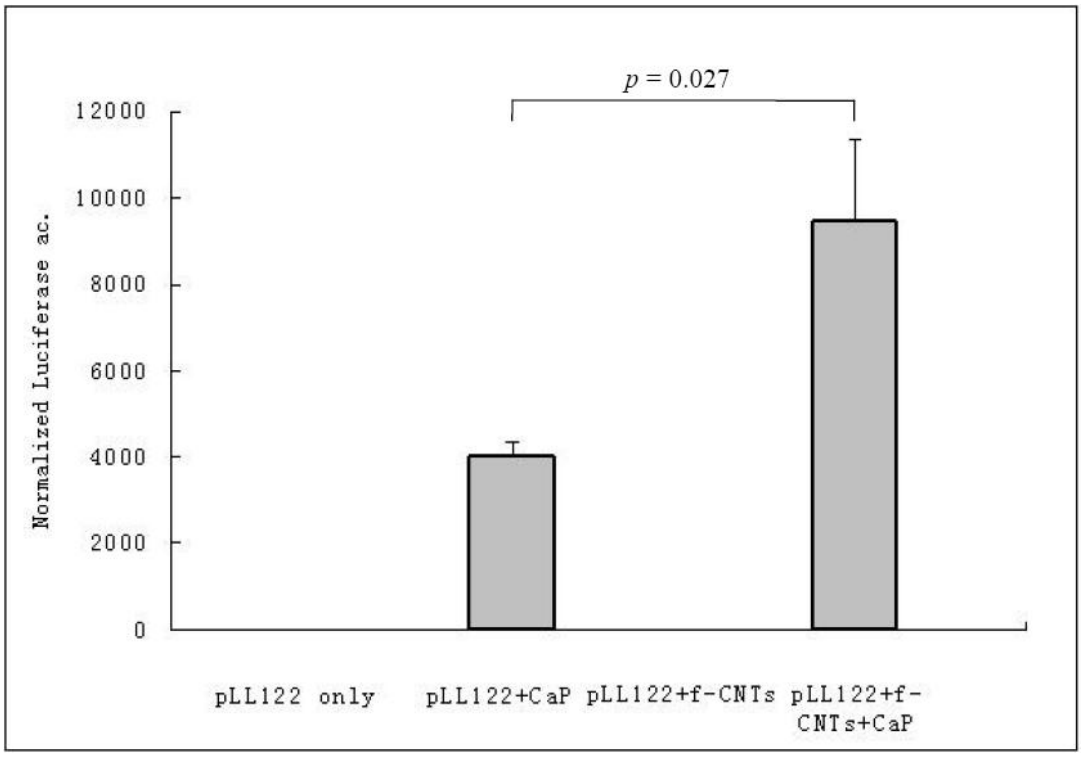


Figure 4.6 Normalized luciferase activity of Huh-7 cells transfected with a plasmid enconding luciferase gene (pLL122) via various delivery systems

Importantly, cells transfected via the combined method exhibited significantly higher luciferase activity than calcium phosphate method only (Fig.4.6, CaP method vs. f-CNTs and CaP combined method, p=0.027). After transfection via f-CNT delivery system, luciferase activity was barely detectable. There was only a minor difference in luciferase activity between control (plasmid only) transfection and f-CNTs transfection (Fig. 4.7). The result of luciferase assay indicated that f-CNTs played a

role of enhancing luciferase gene expression of calcium phosphate method in Huh-7 cells.

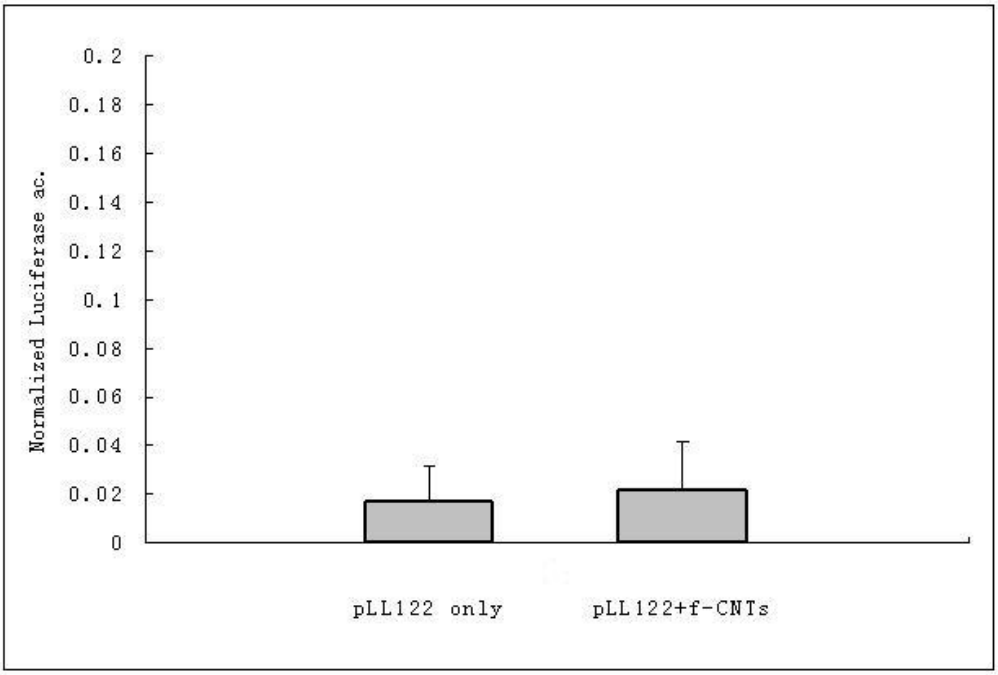


Figure 4.7 Normalized luciferase activity of Huh-7 cells transfected by plasmid pLL122 or via f-CNTs delivery system.

4.1.4 Determining the level of HCV core protein expression

Huh-7 cells were transfected by plasmid DNA pLL311 encoding HCV core protein as described in Chapter 3. The level of HCV core was examined by Western blot analysis 48 hours after transfection.

Western blot analysis demonstrated that there was no expression of HCV core protein in cells with plasmid DNA complexed with f-CNT (Fig. 4.8a). The cells were transfected by calcium phosphate method and its combination with f-CNTs delivery system were all expressing HCV core protein. The cells transfected by the combined method exhibited much higher core protein expression than the ones transfected by calcium phosphate method when they were normalized against β-actin levels; however statistically such a difference is not significant – see Fig. 4.8b (p=0.41).

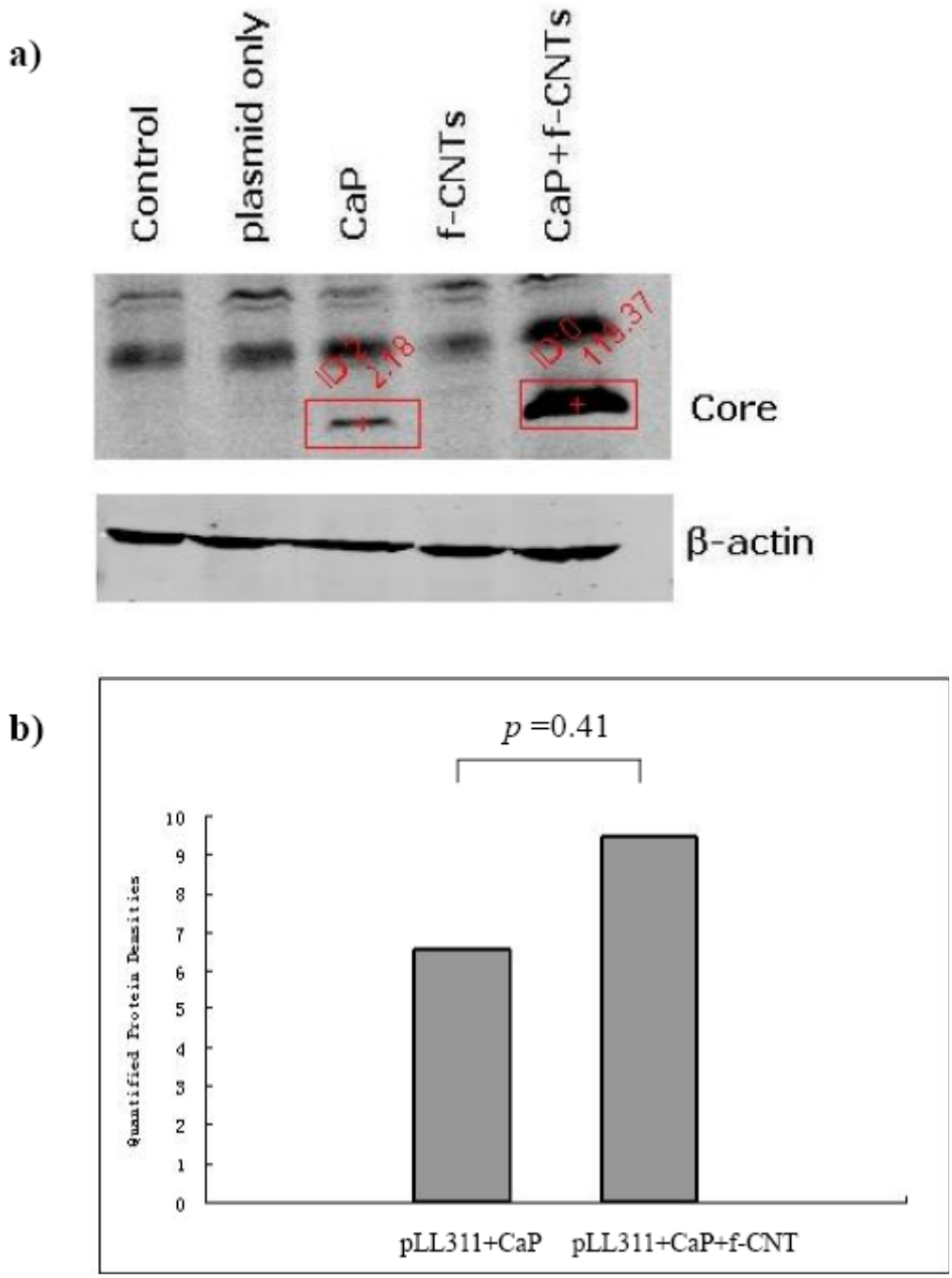


Figure 4.8 HCV core protein expression analyzed by Western blotting of Huh-7 cells transfected with a plasmid encoding HCV core protein (pLL311) via various delivery systems.

4.1.5 Agarose gel electrophoresis assay for plasmid DNA binding with f-CNTs

To investigate the interaction between plasmid DNA and f-CNTs, agarose gel electrophoresis assay was carried out as described in Chapter 3. As shown in Fig. 4.9, the DNA bands of free DNAs in the supernate of the mixture of plasmid DNA and f-CNT can be observed; but no DNA band can be observed of the sediment of the mixture.

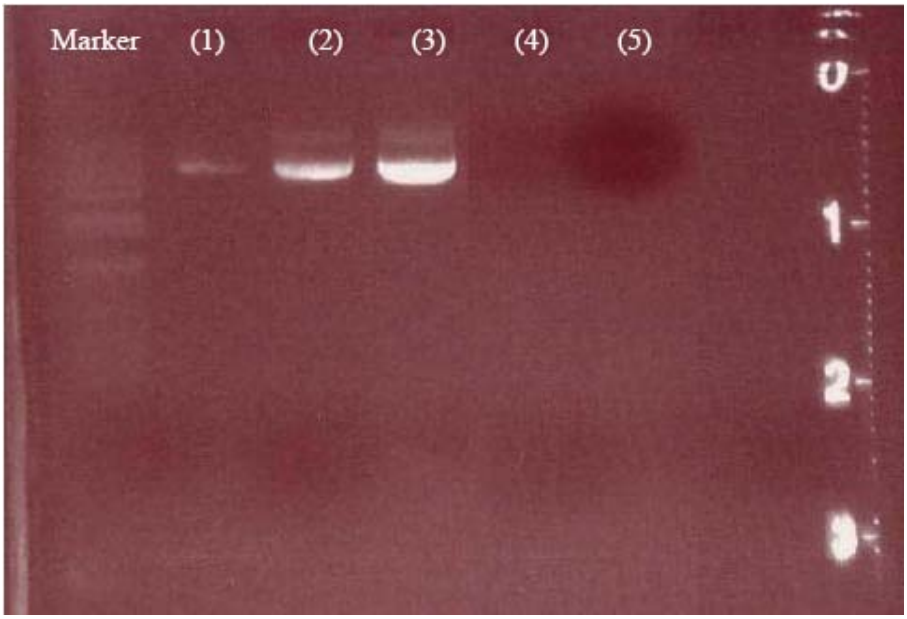


Figure 4.9 The interaction between plasmid DNA and f-CNTs analyzed by agarose gel electrophoresis. (1) 1μl surpernate, (2) 5μl supernate, (3) 10μl supernate, (4) 5μl sediment, and (5) 25μl sediment

The above result suggests that f-CNTs did not bond with plasmid DNA (pLL122) effectively; otherwise there should be plasmid DNAs in the sediments of the mixture of f-CNTs and plasmid DNAs water solution.

4.2 Discussion

We initially encountered problems in synthesizing N-substituted amino acid by following Brough's method [2006]. Therefore, a glycine methyl ester hydrochloride was chosen as the starting material, which was readily prepared from a commercially available glycine. Further, Brough's method, the N-substituted amino acid (**Compound C**, section 3.1.2.1) was not purified from the reaction mixture and used directly for their subsequent reactions. The impurities would bring the complexity to the following step because in our case, it was difficult to isolate f-CNTs with appreciable purity.

We further modified Brough'slast step based on Sakaguchi's method [2007] with some changes (see later discussions). The change was that we divided Brough's last step (see section 3.1.2.1, **Compound B** to **Compound C**) into two individual steps (see section 3.1.2.2, **Compound 2** to **Compound 3**, **Compound 3** to **Compound 4**). In Brough's procedure, solvent DMF and excess LiOH (2 equivalent of **Compound B**) were used without isolation of intermediate compound; the disadvantage with that was that it was not easy to remove residual DMF and unreacted LiOH from lithium salt of **Compound C**. The NMR spectrum of their final **Compound C** did not seem to be consistent with the structure they proposed and the purification procedure in their supplementary information was not complete. Therefore, we chose to prepare pure intermediate **Compound 3** by removing the protecting group from **Compound 2**

first. With pure **Compound 3** in hand, we used less LiOH (0.9 equivalent of **Compound 3**). Thus in the resulting reaction mixture, there was no LiOH and the only impurity was the unreacted **Compound 3** which was removed readily by extraction. After evaporation of the aqueous layer from extraction, **Compound 4** was obtained as a pure compound.

The main purpose of functionalization in this research was to introduce particular functional groups onto CNT sidewalls so that it could be dissolved in water. The successful synthesis of N-substituted amino acid (**Compound 4**) was critical for this functionalization because our N-substituted amino acid contained poly (ethylene glycol) (PEG) chain which was the source of the particular functional group. The PEG chain which is highly hydrophilic and therefore can increase the hydrophilicity of CNTs, and the pyrrolidine ring can form ammonium salt to enhance their solubility in water. The solubility of CNTs in water opens the gate for its biological and biomedical applications. It allows us to explore its function as a drug/gene delivery system which could apply to biological, immunological, and pharmacological studies.

Several research groups demonstrated that CNTs, including single-wall CNTs and multi-wall CNTs, can be functionalized and utilized in gene delivery applications [Bianco et al., 2005a; Rojas-Chapana et al. 2005; Singh et al., 2005, Pantarotto et al., 2004; Gao et al., 2006; Pan et al., 2007]. In this study, a series of biological experiments was carried out to investigate the ability and efficiency of our f-CNTs as

a gene delivery system. Human hepatoma cells were transfected with three plasmid DNAs encoding three different genes, respectively, which was delivered by: (1) f-CNTs, (2) calcium phosphate precipitation method, and (3) a combination of f-CNTs and calcium phosphate method. Different assays and analyses were carried out to determine the levels of protein expression.

Negatively charged plasmid DNA could well bind to the functionalized CNTs which have positively charged groups (such as ammonium group) on their surface [Pan et al., 2007; Singh et al., 2005; Pantartto et al., 2004; Gao et al., 2006]. In this study, the functional group PEG chains on f-CNTs surface was neutral, which explained the lack of success of interaction between f-CNTs and plasmid DNA. The biological experimental results in this thesis showed that the level of gene expression conferred by plasmid DNA complexed with our f-CNTs had only a minor difference with the negative control (i.e., when the cells were incubated with plasmid DNA only) in Huh-7 cells.

Interestingly, when we combined the f-CNTs with a conventional transfection method (i.e., calcium phosphate precipitation method), the gene expression levels were enhanced. It is noted that although only one measurement showed that this enhancement was statistically significant; all the measurements did show the trend of this enhancement. In fact, these measurements, e.g. western blot analysis, was done only three times, which we believed is not sufficient to perform *t*-test. We expected

that more replicates of these measurements would show this enhancement statistically significant.

Nevertheless, sufficient evidences have shown that our f-CNT enhance the calcium phosphate method in gene expression. This result may be explained by the following conjecture. The calcium phosphate transfection method relies on the coprecipitation of plasmid DNAs formed with calcium phosphate. The complex (coprecipitated compound) may further be presented to the cell surface, and the plasmid DNA is taken by the cell via endocytosis [Jordan et al., 2004]. The PEG side chains on f-CNTs or f-CNTs themselves may cross-link to form a network structure [Correa-Duarte et al., 2004; Xie et al., 2004] via electrostatic interaction probably. This network may enhance the complex of calcium phosphate and plasmid DNAs in transportation to cells. However, the actual interaction among f-CNTs, plasmid DNA, and calcium phosphate need to be fully investigated.

Chapter 5

Conclusion and Future Work

5.1 Conclusions

This study was motivated by an observation that the delivery of plasmid DNA for HCV vaccine in large animals is not effective, and nanotechnology may be a promising approach to improve this situation. The research was aimed to develop a nano delivery system for plasmid DNA to human hepatoma cells (Huh-7 cells). In particular, two major experiments were conducted in this study: functionalization of carbon nanotubes and comparative biological experiments of the f-CNTs delivery system and conventional delivery method *in vitro*.

The study concludes:

(1) The single-wall carbon nanotubes can be functionalized to form a large number of PEG side chains stably through a proposed method which revises Brough's method so that the functionalized single-wall carbon nanotubes are water soluble.

(2) The functionalized CNTs significantly enhanced gene expression when combined with calcium phosphate for plasmid DNA transfection.

(3) The functionalized CNTs complexed with plasmid DNA did not result in detectable gene expression in human hepatoma Huh-7 cells. This is likely because the f-CNTs (in particular N-substituted amino acid) do not bind with plasmid DNAs in water solution.

5.2 Limitation and Future Work

Gene expression in Huh-7 cells after transfection with a plasmid DNA complexed with f-CNTs alone was at an undetectable level. However, it was found that f-CNTs can enhance the level of gene expression of calcium phosphate precipitation method in delivering plasmid DNA into Huh-7 cells. The mechanism of how this new approach works is to be unfolded. Furthermore, this study has only investigated single-wall CNTs and one functional group to CNTs (i.e., PEG side chain). With an overview of the contemporary literature on f-CNTs-based nano drug delivery systems (see Chapter 2 of this thesis) and the limitation of the knowledge generated from this thesis work, a couple of thoughts for future work on the drug/gene delivery system for the HCV vaccination development are discussed in the following.

First, it should be interesting to study the effect of multi-walled carbon nanotubes for plasmid DNA delivery systems. There have been ample evidences to show different properties in many aspects (e.g., absorption of H_2S in sera [Zhan, 2008]) between single-wall CNTs and multi-wall CNTs.

Second, it is warranted that different functional groups to CNTs with different functionalization processes should be examined. Different functional groups may have different binding capabilities with plasmid DNA and possibly different binding affinities with Huh-7 cells. These interactions are primarily responsible for different drug delivery performances.

Third, there is a need to study the mechanism of integration of f-CNTs and calcium phosphate given the finding that their combination can significantly improve the gene expression. The goal of this study is to optimize the design of such a combined system for the best delivery performance. This goal seems to be achievable. Our preliminary experiments have shown that the amount of f-CNTs may play a role in the delivery performance and an optimal amount of f-CNTs makes sense.

Finally, in all the studies including both the present and future work, there is a need to study the selectivity of the delivery. To make the delivery system work for large animals and humans *in vivo*, not only the delivery efficiency is important but also the drug selectivity (or in engineering terminology, the robustness of a process) should be a concern.

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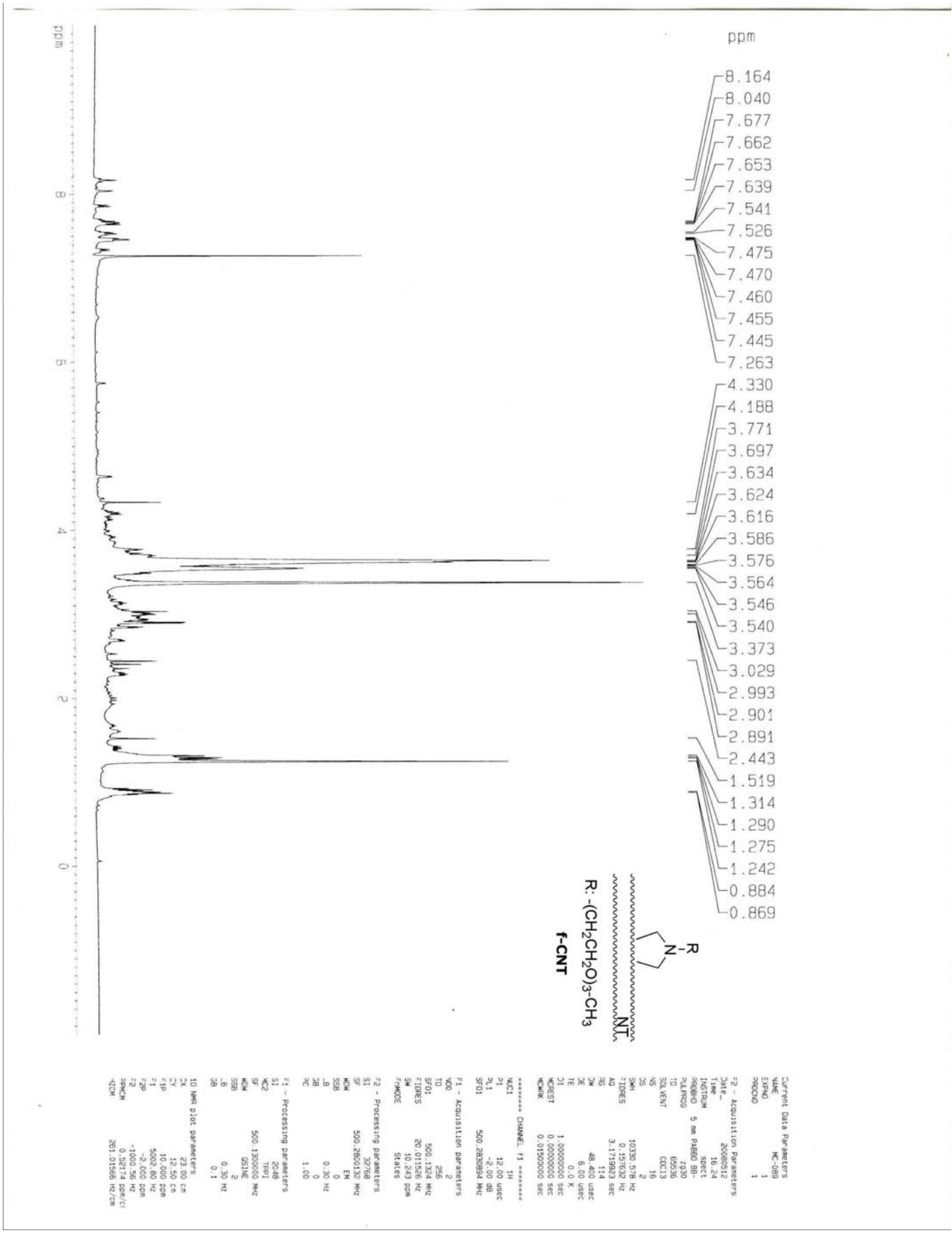
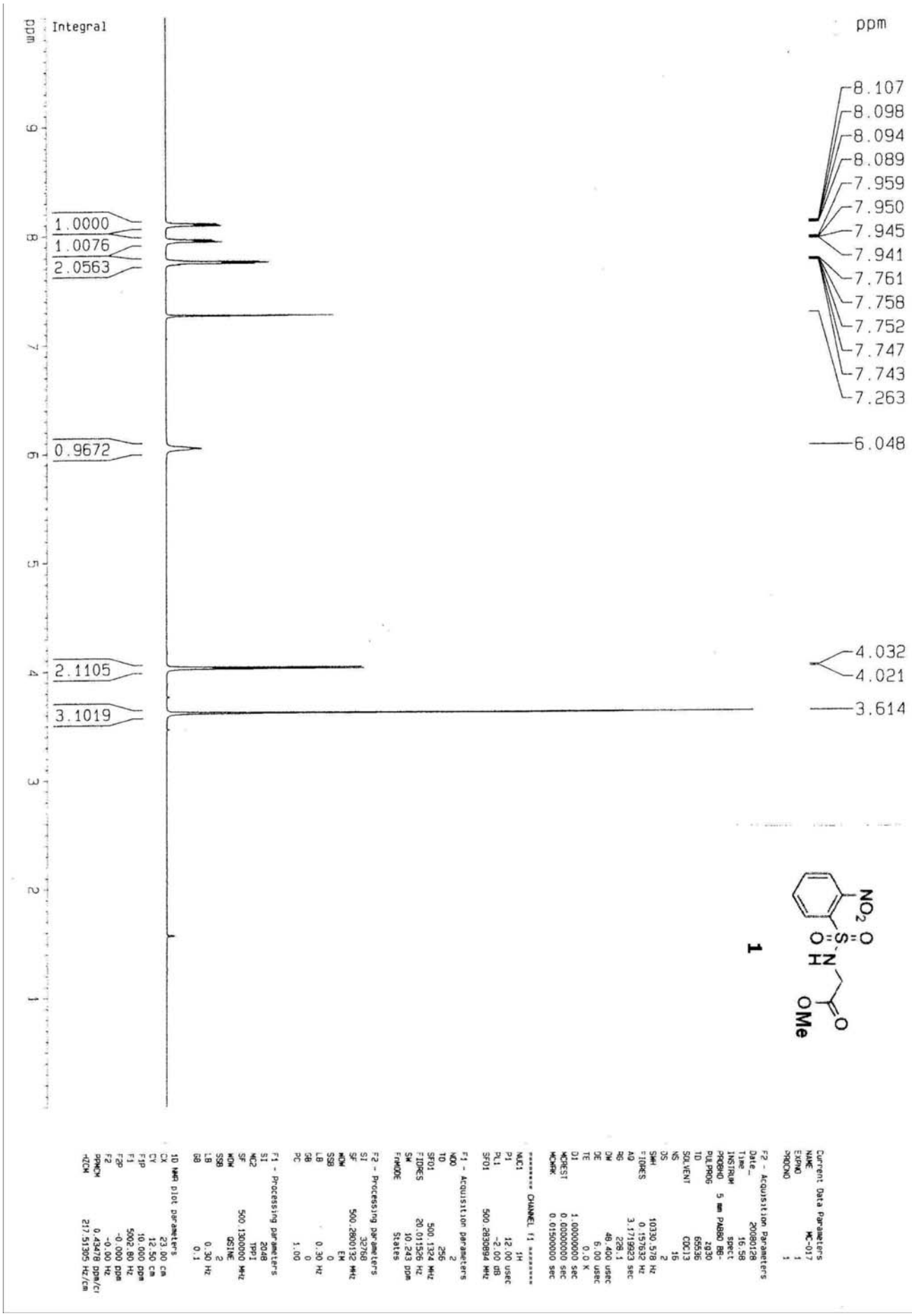
Appendix: NMR Spectra

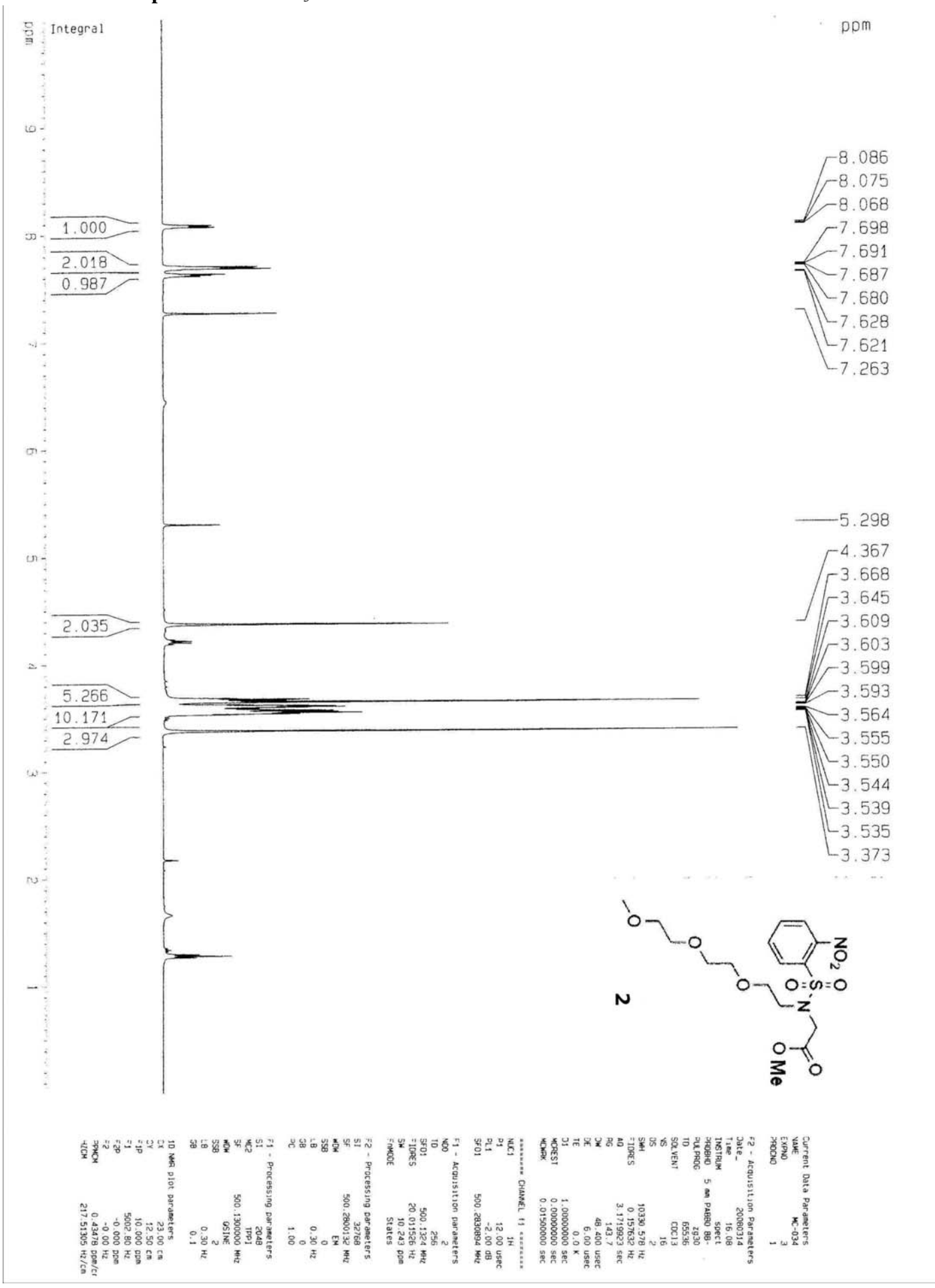
^{1}H NMR of **f-CNT** in $CDCl_3$



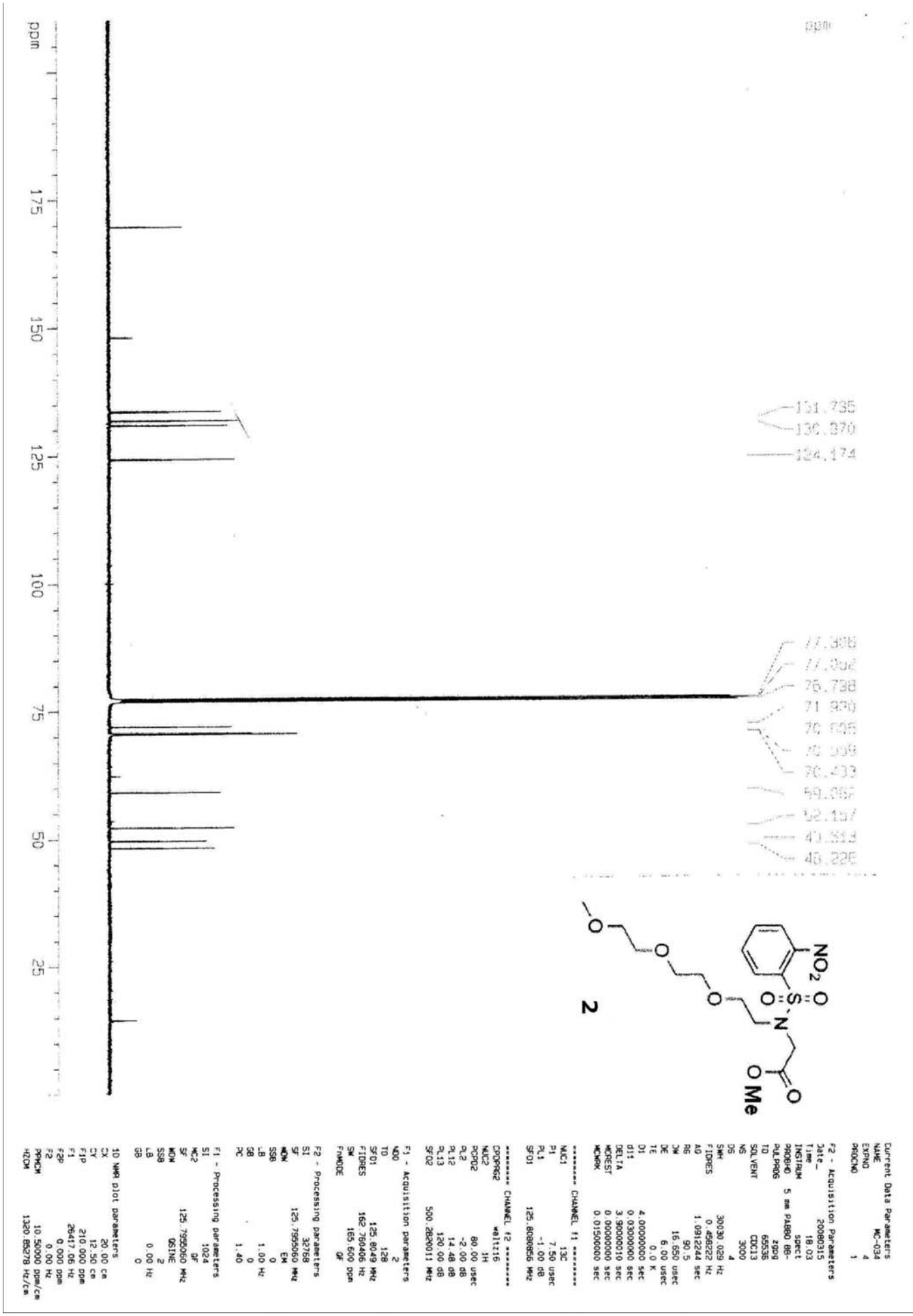
^{1}H NMR of **Compound 1** in $CDCl_3$



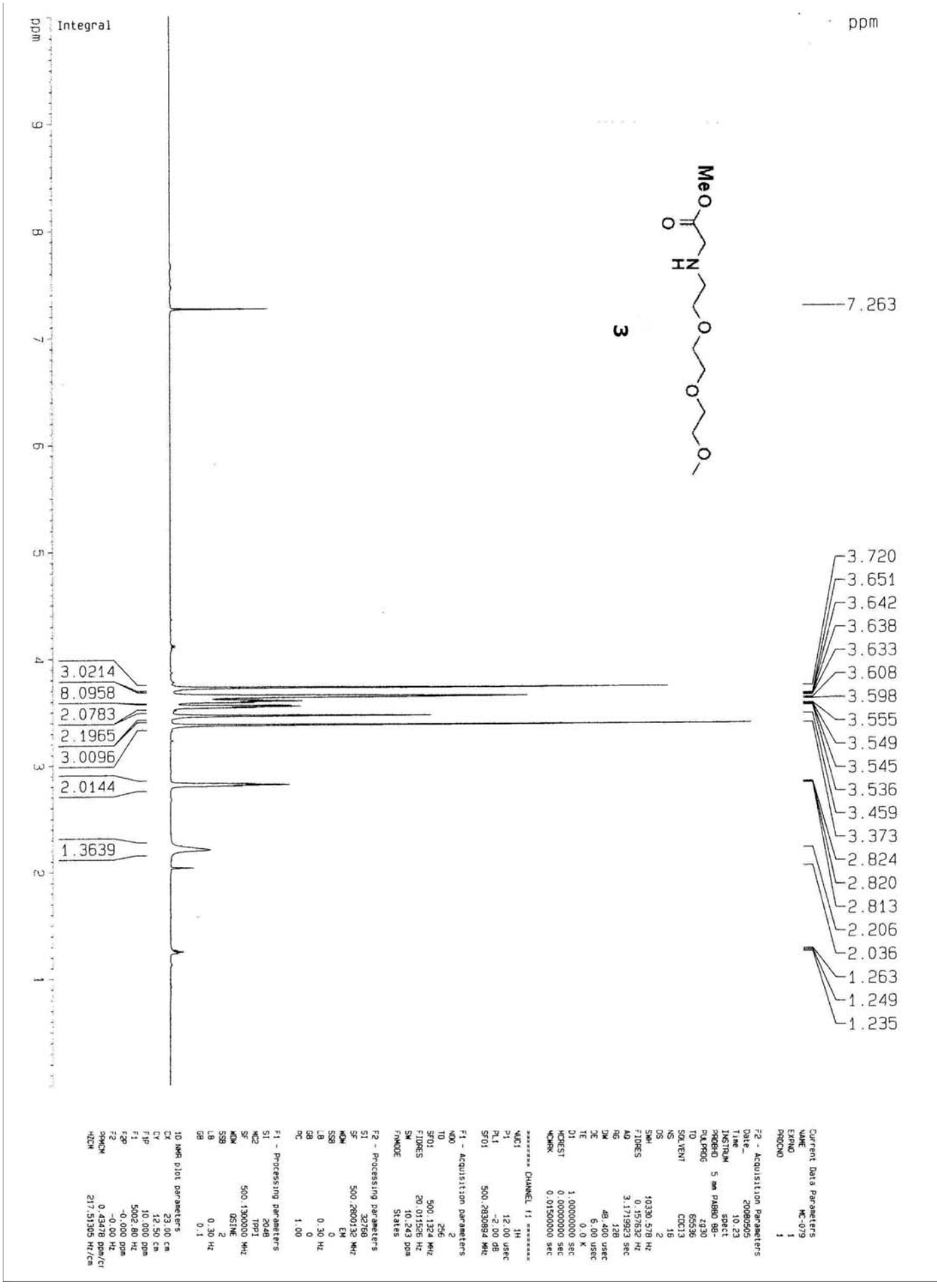
^{1}H NMR of **Compound 2** in $CDCl_3$



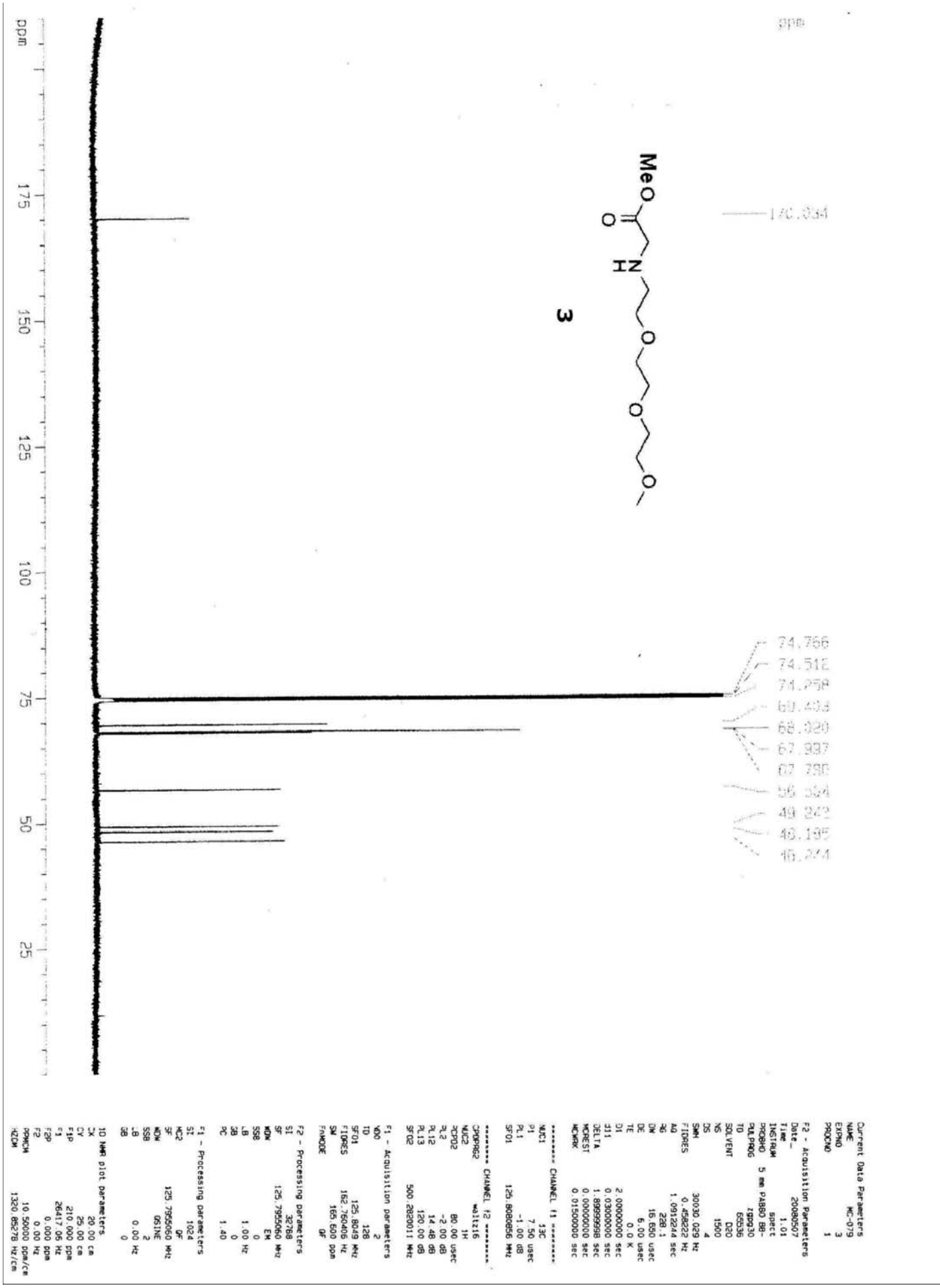
^{13}C NMR of **Compound 2** in $CDCl_3$



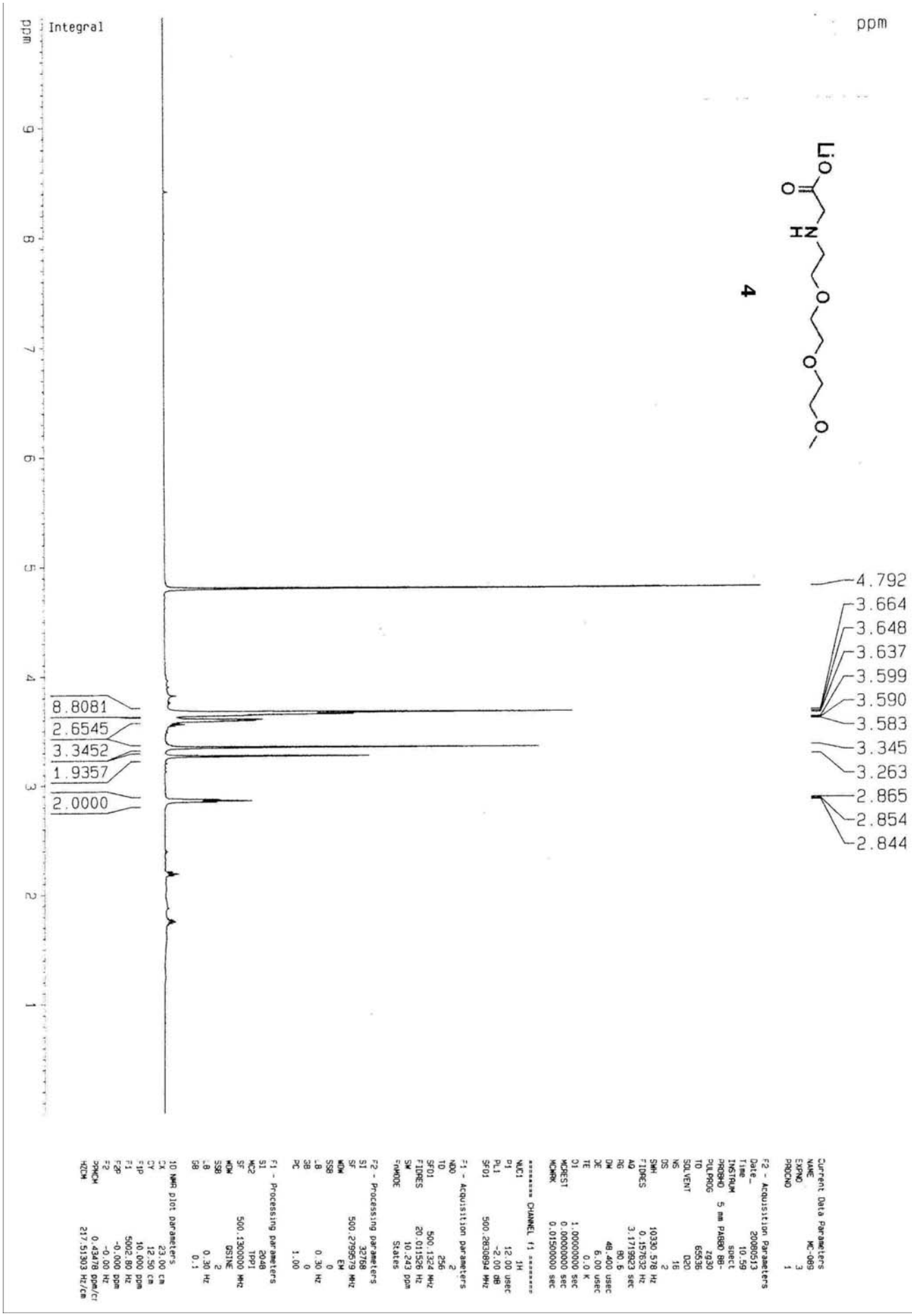
^{1}H NMR of **Compound 3** in $CDCl_3$



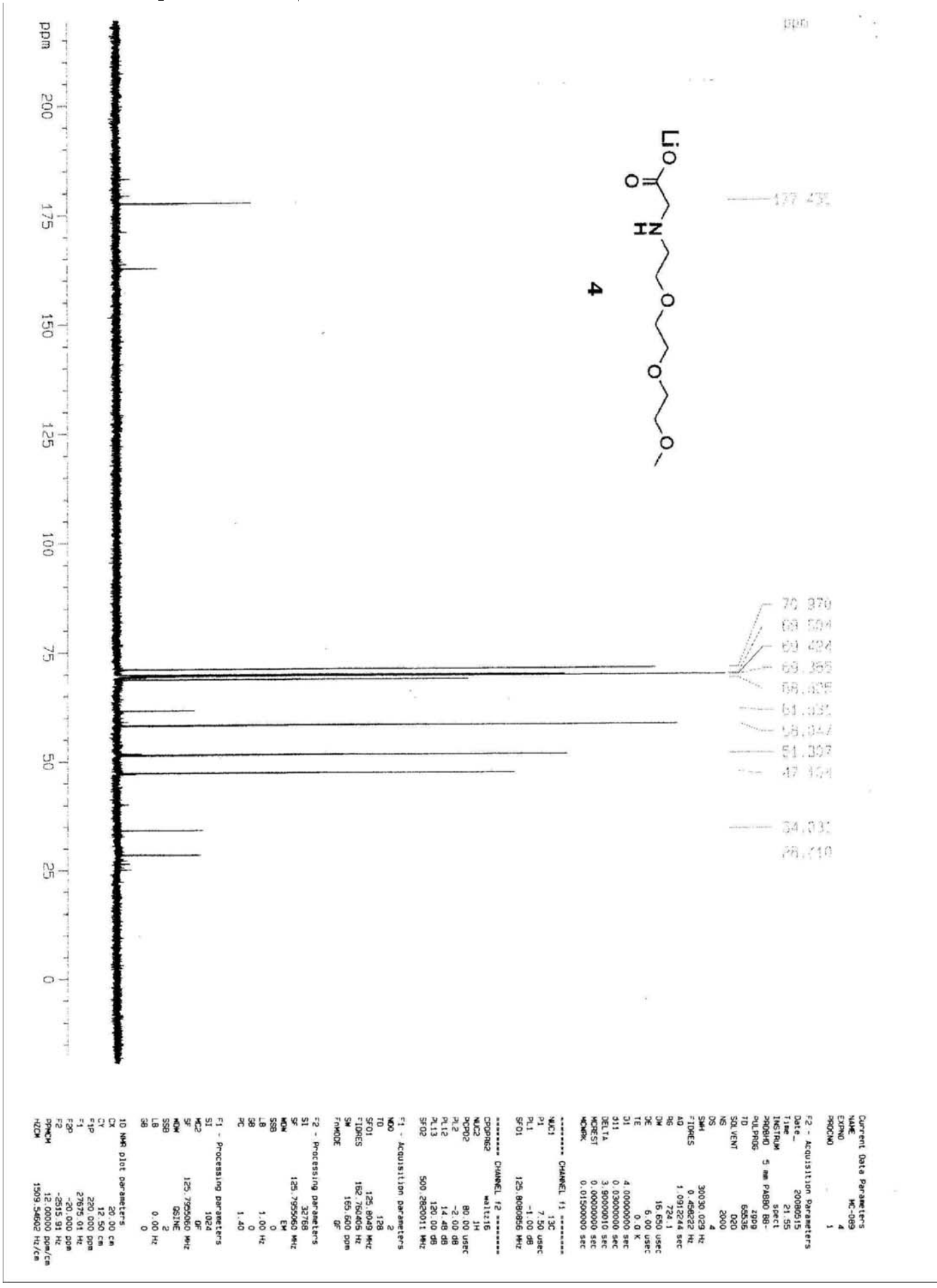
^{13}C NMR of **Compound 3** in $CDCl_3$



^{1}H NMR of **Compound 4** in D_2O



^{13}C NMR of **Compound 4** in D_2O



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PROCNO 1
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PULPROG zgpg
TD 65536
SOLVENT D2O
NS 2000
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.0912244 sec
RG 724.1
DW 16.650 usec
DE 6.00 usec
TE 0.0 K
D1 4.00000000 sec
D11 0.03000000 sec
DELTA 3.90000010 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
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NUC1 13C
P1 7.50 usec
PL1 -1.00 dB
SFO1 125.8080856 MHz
-------- CHANNEL f2 --------
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -2.00 dB
PL12 14.48 dB
PL13 120.00 dB
SFO2 500.2820011 MHz
F1 - Acquisition parameters
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FIDRES 182.760405 Hz
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FnMODE QF
F2 - Processing parameters
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SF 125.7955960 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
F1 - Processing parameters
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MC2 QF
SF 125.7955960 MHz
WDW GENNE
SSB 2
LB 0.00 Hz
GB 0
1D NMR plot parameters
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CY 12.50 cm
F1P 220.000 ppm
F1 27675.01 Hz
F2P -20.000 ppm
F2 -2515.91 Hz
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HZCM 1509.54602 Hz/cm