CONFORMATIONAL CHANGES AND SPECTROSCOPIC STUDY OF POLYETHYLENE GLYCOL AND CALF THYMUS DNA COMPLEX

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF APPLIED SCIENCES

OF

NEAR EAST UNIVERSITY

by

ALI M. BENTALEB

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FORTHE DEGREE OF MASTER

in

BIOMEDICAL ENGINEERING

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DECLERATION

I hereby declare that all information in this thesis document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have cited referenced all material and results that are not original to this work. Portions of the work described herein have been published elsewhere and are listed below.I also declare that the work presented in this thesis is the result of my own investigations and where the work of other investigators has been used, this has been fully acknowledged within the text.

Name, Last name :Ali Bentaleb

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ABSTRACT

Control of conformational and morphological features of biocomplex formation play an important role in gene therapy applications. In this study, the influence of different PEG-400 concentrations, different pH values, incubation time and thermal stability of ctDNA on the PEG-ctDNA biocomplex have been studied by using FTIR, UV-VIS NIR spectrophotometer, and TEM. UV-VIS NIR absorption analysis indicated that PEG forms complex with ctDNA not by via intercalative interaction. The results of thermal denaturation studies showed that an increase in the PEG-ctDNA melting temperature able to stabilized PEG-ctDNA biocomplex helix. The FTIR analysis results indicated that PEG binds with ctDNA by weak to moderate complex formation with both hydrophilic and hydrophobic contacts through ctDNA base pair, with little binding preference towards phosphate backbone of ctDNA helix. The results showed that the binding reaction of PEG and ctDNA proceeds rapidly at room temperature and complexation formation vary by time after PEG and ctDNA are mixed together and kept almost constant for at least 10 minutes. TEM micrographs showed that the addition of PEG to ctDNA causes condensation of ctDNA with PEG molecules in irregular aggregate structure. These results have potential applicability for a variety of gene delivery systems based on PEG-ctDNA biocomplex, due to their well known conformational, spectroscopic and morphologic properties.

Key words : Polyethylene Glycol 400, Calf thymus DNA, Uv-visible, FTIR, TEM.

ÖZ

Gen tedavi uygulamalarında, biyokompleks oluşumunun yapısal ve morfolojik özelliklerini kontrol edebilmek önemli rol oynamaktadır. Bu çalışmada, PEG-ctDNA biyokompleksi farklı PEG-ctDNA oranları, pH değerleri, reaksiyon süreleri ve ctDNA'nın ısı kararlılığı, FTIR, UV-VIS NIR spektrofotometre ve TEM metodları kullanılarak çalışılmıştır. PEG'un ctDNA ile biyokompleks oluşturma yönteminin interkalativ etkileşim yolu ile olmadığı, UV-VIS NIR absorpsiyon analiz sonuçları ile işaret edilmektedir. Isı denaturasyon çalışmaları, PEG-ctDNA biyokompleksinde erime sıcaklığı artışının sarmaldaki PEG-ctDNA biyokompleksinin oluşturduğu kararlılığın artmasından kaynaklandığını göstermektedir. FTIR analiz sonuçları ise, PEG'un ctDNA ile hidrofilik ve hidrofobik zayıf ve orta dokunuşlarla kompleks oluşturup, ctDNA sarmalının fosfat yapısına bağlanmayı çok az tercih ettiğini göstermiştir. Sonuçlar, oda sıcaklığında Peg ve ctDNA'nın birbirleri ile etkileşiminin hızla ilerlediğini ve biyokompleks oluşumunun zamanla en az 10 dakika sbit kaldığını göstermektedir. TEM mikrograflar, PEG ilavesinin ctDNA'nın yapısında yoğunlaşmaya neden olup, düzensiz yapılar oluşturduğunu işaret etmektedir.

Bu çalışmadaki sonuçlar, PEG-ctDNA biyokompleksinin pek çok gen tedavi sistemlerinde uygulanma potansiyelinin yüksek olduğunu göstermektedir.

ANAHTAR KELİMELER: Gen tedavisi, PEG, ctDNA, Biyokompleks, nütral polimerler.

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ABBREVIATIONS

PEG	Poly Ethylene Glycol
ctDNA	Calf Thymus Deoxyribo Nucleic Acid
FTIR	Fourier Transform Infra Red
TEM	Transmission Electron Microscope
PEI	Poly Ethylene Imine
EPR	Electron Paramagnetic Resonance
PBS	Phosphate Buffered Saline
UV VIS	Ultra Violet and Visible Light
AT	Adenine-Thymine
GC	Guanine-Cytosine
mPEG	Monomethoxy Poly Ethylene Glycol
SPION	Super Paramagnetic Iron Oxide Nanoparticles
Tm	Melting Temperature
DTGS	Deuterated Triglycine Sulphate

CHAPTER 1 INTRODUCTION

1.0 Overview

Interactions of DNA with various molecules are interesting because of its importance as biomolecular and biochemical tool for many biomedical applications, such as visualization of DNA [1], DNA hybridization [2], DNA biosensors [3,4], action mechanisms and determination of some DNA targeted drugs, origins of some diseases, and developing gene and drug delivery systems [5]. Therefore, deeper understanding DNA interaction patterns, and forces involved based on the study of molecules that bind to DNA, is of prime importance [5], due to several reasons: (i). The molecule interact with DNA requires a knowledge of how the structure of the molecule related to the specificity, and affinity of binding. (ii). Identifying the forces and energetics involved in the interaction to unraveling the mystery of molecular recognition in general and DNA binding in particular. Several synthetic polymers play a major role as a biomaterials and vehicles for many drug delivery systems, and selecting biopolymer molecules that bind genomic DNA to form a complex is a central requirement for gene delivery system development, necessitating new *in vitro* methods for rapid and low-cost assessment of the binding affinity and location of molecule along DNA molecules. Many applications of DNA-polymer complex have already been demonstrated and characterized. Among synthetic polymers, polyethylene glycol (PEG) show potential applications in different biotechnical, industrial, and clinical applications including biosensors[6], gene and drug delivery system development, because of its solubility, non toxicity and biocompatibility [7,8]. Therefore, PEG is extensively investigated polymer for modification of biological macromolecules and surfaces for many pharmaceutical formulation and biotechnical applications [9,10].

1.1 Research Needs for Gene Therapy

The optimization of DNA and cationic polymer complexation is crucial for non viral gene delivery. Although physiochemical characterization of interaction between DNA and cationic polymers as has attracted more attention. The literature on the effect of non charged (neutral) on DNA complexation is still scarce, in addition the detailed structural analysis of PEG complexes with Calf thymus DNA (ctDNA) is still an area of further characterization and investigation for optimum biomolecular product output for various applications such as gene therapy [11]. ctDNA (DNA isolated from thymus organ) was used for many scientific experiments, because Thymus has a very yield of DNA approximately 2.542 w/w, furthermore ctDNA was found effective as a cancer therapy when complexed with cationic liposomes. Several of characterization methods are used to investigate micro, and nano-scale structures of biological materials at the morphological and /or molecular levels. These include Uv-Visible Spectrophotometer (UV-Vis), Fourier Transform Infra Red **(**FTIR), and Transmission Electron Microscopy (TEM). The spectra analysis and light absorbance measurement of organic compound are routinely carried out by a spectrophotometer, which is set to measure how much light is absorbed or transmitted at the optimal wavelength. It stands to reason that there is proportionality between how much of the compound is present and how much light is absorbed. If there is twice as much of the compound, twice as much light will be absorbed. This Absorption of electromagnetic radiation by organic molecules is restricted to certain functional groups (chromophores) that contain valence electrons of low excitation energy. The Uv-Visible spectrum of this organic molecule containing these chromophores is complex, because of the superposition of rotational and vibrational transitions on the electronic transitions gives a combination of overlapping lines, and this appears as a continuous absorption band. DNA absorbs light in the ultra violet range of the electromagnetic spectrum at 260 nm, the wavelength at which the light is absorbed is a function of molecular structure of DNA (nitrogenous bases A, G, C and T) [12] UV-spectrum of DNA is also sensitive to pH and **π**-bonding in the amine bases of DNA due to ability of nitrogenous bases of DNA to be protonated, therefore neutral pH normally was used in biological media. As well as the qualitative studies of DNA with other molecules may also be carried out using uv visible spectroscopy technique for monitoring DNA reactions with other biologically interesting molecules, to obtain the information about the possible interaction, and behavior of classical electrostatic interactions is the hyperchromism and blue shift of the absorption bands of the complexes and DNA. In addition, hydrophobic

associations study of aromatic rings of the complex (if any) with the hydrophobic interior of DNA may also be possible when observation of hyperchromism and blue shift [12]. FTIR spectroscopy is another absorption/transmission method used in this study to probe chemical bonds and their crowding environment in molecular system *versus* time in DNA-PEG interaction. It is a chemical analysis method of choice used to rapidly identify substances [13], it produces their molecular fingerprint, and absorption peaks correspond to normal mode frequencies of the molecular bonds making up the material, an interferometer is used to encode the detected signal which is digitally Fourier transformed to produce an FTIR spectrum (absorbed intensity *versus* wave number) [13,14]. In current study, transmission electron microscopy (TEM) is also proposed as another technique to obtain images for complex samples using certain stain in order to enhance the contrast, and to observe any changes at nano size scales.

1.2 Problem Statement

The macromolecular analysis of DNA interaction with other molecules such as drugs, organic dyes, polymers and metals, has been an intensive topic for decades, because it provides insight into the screening and design of novel and/or more efficient molecular targeting of DNA [15]. Moreover, study on the properties of polymers and their interaction with DNA is highly significant and important in developing new gene therapy treatments or other biomedical applications. Recognition of DNA binders involves a complex interplay of different interactive forces. It includes intercalation, and hydrophobic interaction along the minor and major groove of DNA, strong electrostatic interaction arising from the exterior sugar-phosphate backbone and intercalative interaction between the stacked bases pairs of native DNA from the major grooves [16-18]. Poly ethylene glycol (PEG) or poly ethylene oxide (PEO) is a hydrophilic, neutral, intrinsically flexible polymer available over a wide range of molecular weights. PEG is often called amphiphilic, since it is soluble in both water and many organic solvents. Especially its water solubility, combined with non-toxic properties, has allowed PEG to become one of the most prominent polymers in biotechnical and biomedical researches. Binding affinity, interaction mode of PEG to DNA are not well known. Therefore the spectroscopic study of this subject is of a great importance because it exists at the interface of chemistry, physics, and biology, and many biomedical, and pharmaceuticals application such as

anticancer, antibiotics, antivirals, MRI contrast medium, and biosensors, exert their primary effects based on reversible and irreversible interactions with DNA. The variety of analytical techniques have been developed for characterization and identification of the interaction between DNA and molecules with relative advantages and disadvantages [18-23]. However, most of these methods suffer from high cost, low sensitivity and procedural complication. Up to now, electro-chemical methodologies have attracted appreciable attention for direct monitoring and characterize DNA targeting compound interaction to obtain quantitative analysis information in pharmaceutical formulations and biological fluids, due to the specificity and high sensitivity. [24,25] In addition, the electro chemical methods can serve as a versatile and illuminating model of biological system in a similar way to the real interaction occurring in the living cells. [26] In this study the interaction mechanism between DNA and the PEG 400 can at least be elucidated by three different techniques, using UV-visible, FTIR spectroscopic, and TEM. The results will be obtained by all these techniques are significance due to major reasons: (1). Enhance our understanding of PEG as biopolymer for some biomedical applications such as drug delivery, and gene therapy. (2). Elucidation the chemical structure of PEG-ctDNA complexes under certain conditions. (3). The design of a specific drug molecule having affinity for DNA needs a knowledge how the structure of the molecule or the drug is related to the specificity, affinity of binding, and what structural modifications could result in a molecule with desired qualities. (4). Identifying the forces, energies involved in chemical interactions are essential to understand molecular recognition in DNA binding. (5). Using efficient different characterization techniques in the current study, will offer platform closely related to the structure and morphology formed by DNA interaction with polymers.

1.2 Research Aims

The main aims of the current study described in this thesis are :

1. To assess the influence of pH, incubation time, DNA denaturation, and the ability of PEG 400 ratios to form complexes with ctDNA, by varying the pH of the medium, incubation time, melting temperature, and PEG ratios, and analyzing the resulting effects on the binding affinity, and complex morphology.

2. To optimization of ctDNA-PEG complexation.

3. To evaluate synthesized biocomplex by non destructive diagnostic equipments including UV visible NIR spectroscope, FTIR spectroscope, and transmission electron microscope (TEM).

4. To compare synthesized complex data with literature corresponded material.

1.3 Research Objectives

The main objective of this thesis is to investigate the complex relation between the macromolecular architecture of ctDNA-PEG, and its conformation in different pH medium, incubation time, melting temperature, polymer:DNA ratios behavior, and microscopical conformation using TEM. The current work has been divided into several chapters including, (I) Experimental investigation using different characterization methods, and (II) Theoretical modeling and comparison to existing experimental data. In Chapter 2, elucidate the topic related literature review of this work, and an overview to characterize techniques that have been used in this work. Chapter 3, describes the materials and methods which include an introduction to (polymers, substrates) and techniques used in this work are presented, as well as detailed description of sample preparation for each technique. Chapters 4 will be presenting the experimental results obtained for each technique have been used (UV-vis, thermal study, FTIR, and TEM) under different environmental conditions. The last part is Chapter 5, which summarizes, conclusion of main findings, and recommendations for future potential studies of this work.

CHAPTER 2 LITERATURE REVIEW

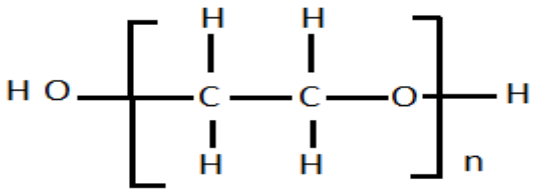
2.0 Introduction

The incorporation of poly ethylene glycol (PEG) into molecule is an important approach being developed for several applications, which involves attachment of PEG to drug molecules, and has great potential for improving pharmokinetic and pharmodynamic properties of delivered drugs [27]. Thus PEG has varied uses in the biopharmaceutical field, including drug delivery (e.g. treatment of hepatitis C), laxatives, cell immobilization (as adhesion promoters), and encapsulation of islets of langerhans for treatment of diabetes. It is also used as a carrier material for encapsulated cells for tissue engineering purposes [28,29,30]. Therefore PEG, with its biocompatibility, flexibility and stealth properties is an ideal material for use in pharmaceutical applications. Polyethylene glycol which has a monomeric repeat unit has been also incorporated into DNA complexes of several cationic polymers, including poly methacrylate [30], poly ethylene imines (PEI) [31,32], poly L-lysine (PLL) [33], chitosan [34], and poly amido amines (PAA) [35]. PEG reduces the surface charge of the complexes, which in turn reduces cytotoxicity [36]. The shielding effect of PEG also reduces the interaction between the complex and blood components (plasma proteins and erythrocytes), and can prolong circulation of the complexes in the blood stream [37]. PEG is non toxic, thus ideal for biological applications, and can be injected into the body without adverse effects. The incorporation of PEG into drug molecules can prevent salt induced aggregation through steric stabilization [37]. Additionally, PEG is often used as a spacer for targeting ligands since the shielding effect of PEG is able to decrease nonspecific interactions with negatively charged cellular membranes, which results in reduction of nonspecific cellular uptake [38]. Another important application of PEG has been described by Zalipsky et al.[39,40]. to engineer multifunctional pharmaceutical nanoparticulates by using PEG conjugates with special properties such as pH sensitivity. The concept of synthesizing cleavable PEG-lipid polymers, the linkage employs a *p*- or *o*-disulphide of a benzyl urethane which when subjected to mild

reducing conditions present in endosomal compartments of cell releases PEG from the conjugate [40].

2.1 Chemical Properties of PEG

PEG is linear, uncharged, hydrophilic polymer, refer to repeating of ethylene glycol units with hydroxy groups on both sides.fig 2.1. The molecular weight of PEG vary and ranging from 500 Da up to 30 kDa in both linear or branched chains [40, 41].



Figuer 2.1 Chemical formula of poly(ethylene glycol) (PEG) [42]

The numbers (n) which is often exists in chemical formula of PEG indicate its average molecular weights (MW) of this PEG, and for instance, PEG with (n = 9) have an average molecular weight of approximately 400 Daltons, and named PEG 400.[40,41,42]. Therefore there are several forms of PEG available in the market which also depend on the initiator have been used in polymerization process of those polymers. Monofunctional methyl ether PEG (mPEG) is an example of these polymer fig 2.2. Poly ethylene glycol linked together by chemical linkers, and before coupling, PEG must be activated using chemical leaving group [43].

CH3O-(CH2CH2O)*n* –CH2CH2-OH

Figure 2.2 Chemical structure of monomethoxy polyethylene glycol (mPEG) [42]

PEG is amphiphilic polymer, This means its soluble in many solvents such as water, benzene, dichloromethane, and insoluble in diethyl ether and hexane. It may chemically coupled to hydrophobic molecules to produce non ionic surfactant. The molecular mass of PEG play major role in PEG toxicity, therefore the molecular mass of PEG which is recommended for *in vivo*

applications are ranged between 0.4-10 kDa [44], due to low molecular mass PEG less than 0.4 kDa are degraded by alcohol dehydrogenase enzyme to toxic metabolite, and higher molecular mass PEG more than 10 kDa has slow kidney clearance. [45]

2.2 PEGylation

PEGylation is a technique defined as conjugation of PEG molecule to any particle surface [46]. As a technology was first developed by Davis et al. in the 1970 [47]. In order to conjugate the PEG chains onto proteins, peptides or particle surfaces, it is necessary to have PEG activated with a functional group at one or both of the ends. The choice of the functional group is influenced by the functional groups available on the molecule of interest. In proteins or peptides the side chain amino groups (lysine, arginine), sulfhydryl (cysteine), hydroxyl (serine, threonine), carboxy (aspartic acid, glutamic acid) or N-terminal amino and C- terminal carboxy can be considered. Where as in the case of glycoproteins, the hydroxyl groups can be utilized. The majority of the cases for PEGylation of proteins or peptides make use of available primary amine groups from lysine, arginine or the N-terminal amino group [48]. Zalipsky et al. have also described the synthesis of detachable PEG-lipid polymers cleaved by cysteine [49]. The linkage employs a p- or o-disulphide of a benzyl urethane which when subjected to mild reducing conditions present in endosomal compartments of cell releases PEG from the conjugate. Thus there is an array of available PEGylation chemistry to tailor the requirements for drug molecules, proteins, peptides or any particulate drug delivery systems where long-circulation is desired. FDA-approved PEGylated products clearly indicate the improved therapeutic efficacy of the drugs using this technology Table 2.1. Even though many studies have been conducted demonstrating the theoretical and commercial usefulness of PEGylation technology there are many more untapped applications that are still to be explored [49].

2.2.1 Limitation of PEGylation Process

Despite some PEGylation strategies have had no effect on transfection efficiency *in vitro* or *in vivo* [36], others have reported that PEGylation resulted in poor transfection [30], presumably due to interference with complexation [32]. These effects due to PEGylation have been

associated with the extent of PEGylation, which may shield the surface charge [35], thus reducing cell binding and transfection, or alternatively, induce membrane leakage, resulting in enhanced cytoplasmic release.

PEG Conjugate	Drug Name / FDA Approved Date	Bioactivity of Native Agent	Main Effect of PEGylation	Medical Indication
ADA (adenosine deaminase)	PEGADEMASE 1990	Enzyme replacement	Longer half life, reduce immune response	SCID as result of ADA deficiency
Asparginase	PEGASPARGASE 1994	Hydrolyze asparagines ,on which leukemic cells are dependent	Longer half life, reduce immune response	chemotherapy combination, acute lymphoblastic leukemia
Granulocyte colonoy-stimulating factor	PEGFILGRASTIM 2002	stimulation of neutrophil production	Longer half life, reduce immune response	Prophylaxis against neutropenia
Interferon alpha 2b	PEGINTERFERON alpha2b 2001	Antiviral cytokine	Slower clearance, increase bioavailability	Hepatitis C with normal liver function
Interferon alpha 2ba	PEGINTERFERON alpha2ba 2002	Antiviral cytokine	Slower clearance, increase bioavailability	Hepatitis C with compensated liver disease
Stealth PEG liposomes for delivery of doxorubicin	CAELYX, DOXIL 1990	Antitumor anthracycline	Slower clearance, max. distribution into tumor	Kaposi sarcoma, refractory ovarian cancer

Table 2.1 PEGylated pharmaceutical products [49]

2.3 PEG a Polymer as a Carrier in Gene Therapy

The basic concept of gene therapy involves the treatment of human diseases by inserting genetic material to specific cell types in order to correct or supplement defective genes responsible for disease development [50]. Progress in the clinical development of this approach has been hindered by the inefficient transport of plasmid DNA/oligonucleotides through the cell membrane. Therefore, the success of gene therapy is largely dependent on the development of efficient gene delivery vehicles. There are two types of carriers used in experimental gene

therapy protocols, viral and non-viral vectors, both of which present specific advantages and disadvantages [51]. The search for non-viral vectors began when viral vectors met with serious draw backs such as high risk of mutagenicity, immunogenicity, low production yield, and limited ability to carry long gene sequences [52]. Several approaches have been tested in order to circumvent problems associated with each type of non-viral gene delivery vehicles [53,54]. The use of polymeric materials as delivery vehicles has been well established and widely used to improve therapeutic potential of peptides, proteins, small molecules and oligonucleotides [55–58].The spontaneous formation of polyplexes by the interaction of negatively charged phosphate groups of DNA/oligonucleotides and positively charged polymers under physiological salt conditions and the successful transport of these polyplexes to cells has been demonstrated [59–61]. Since DNA molecules condensed with low molecular weight cations are susceptible to aggregation under physiological conditions [62], advanced polymeric gene delivery systems employ macromolecules, with high cationic charge density, that can protect the DNA from degradation [63]. So, this has necessitated attempts towards modification of spermine with a view to developing high molecular weight copolymers [64]. Jere *et al.* have reported synthesis of a poly (β- amino ester) of spermine and poly (ethylene glycol) (PEG), which showed higher degree of safety and transfection efficiency in comparison to polyethyleneimine, when studied in 293T human kidney carcinoma cells [63]. Vinogradov et al. [65] reported that poly (ethylene glycol)-spermine complexes are less stable in the presence of low molecular weight electrolytes compared to the PEG-PEI complexes. Coupling the copolymer with hydrophilic compounds, such as PEG, might reduce non-specific interaction of the copolymer with blood components as well as make it water soluble. PEGylation of synthetic polymers such as dendrimers is shown to reduce toxicity and increase biocompatibility and DNA transfection [66-69]. Similarly, the effect of PEGylation on the toxicity and permeability of biopolymers such as chitosan has been reported [70]. It has been reported that PEG induces significant changes in DNA solubility and structure under given conditions. DNA concentration, pH, ionic strength of the solution and the presence of divalent metal cations have been shown to impact PEG DNA precipitation [71,72].

2.4 Lipoplexes and Polyplexes

In order to facilitate the effective transfer of non-viral DNA into the cells, synthetic vectors improving the admission of DNA into the cell and protecting it from undesirable degradation were created. The most used are derived from lipids or synthetic polymers. Plasmid DNA can be covered by lipids into organized structures such as liposomes or micelles. This complex (DNA with lipids) is called a lipoplex [73]. Lipoplexes can be divided into two types anionic and neutral liposomes. Vectors based on a complex of polymers with DNA are called polyplexes. Most of them consist of cationic polymers and their production is regulated by ionic interactions. In contrast to lipoplexes, some polyplexes (polylysin) are not able to release intravesicular DNA into the cytoplasm [74].

2.5 PEG in Development of MRI Contrast Media

An interesting application of PEG is developing magnetically sensitive micelles, super paramagnetic iron oxide nanoparticles (SPION) were incorporated into PEG-PE based micelles to form stable SPION-micelles. SPION have excellent MRI contrast properties, however, they are not stable in physiological systems and show aggregation [75,76]. PEG-lipid based micellar formulation not only prevented the SPION from aggregation but also improved its MRI signal. Because of the small size and long-circulating property, SPION-micelles can be targeted passively by EPR effect. SPION- micelles can also be targeted to the disease site under influence of external magnets. Moreover to prepare actively targeted MRI contrast agents, SPION-micelles can be easily surface-modified by active targeting ligands [76].

2.6 Role of PEG in Biosensor Development

Biosensors are diagnostic tools used for the rapid detection of metabolites, drugs, hormones, antibodies and antigens [77,78]. Traditional biosensors are composed of disposable sensor elements containing molecular receptors immobilized by adsorption, covalent cross linking or entrapment. [79]. Biosensor have been developed by grafting biotin labeled, 3400 molecular weight with poly ethylene glycol to silicon surfaces to produce a dense PEG monolayer with functionally active biotin. These surfaces have been activated with antibodies through the strong streptavidin-biotin interaction by simply incubating the surfaces with antibody-streptavidin conjugates. The stability of the biotinylated PEG monolayers produces a sensing element that can be regenerated by removal of the streptavidin conjugate and stored in a dry state for

extended periods of time [80]. Another study demonstrate that PEG-based biosensor chips to measure and study interactions between proteins and heparin offer an alternative to dextran-based chips when an analyte interacts non specifically with a dextran matrix. PEG-based chips are easy to prepare and afford high baseline stability. And offer excellent binding sensorgrams for the Interaction between factor P and heparin using these chips. Other heparin-binding proteins examined in this study have also exhibited significant non specific interactions with dextran matrix [81]. PEG residues have been also reported extensively in the literature as having inherent capabilities to reduce non-specific protein binding, and improve immunoassay sensitivity in sensing applications, and hence have become more attractive for biomedical research, biosensors, and pharmaceutical applications [82,83]. PEG is a neutral, non-toxic polymer with the capability of improving a material's affinity for water, helping to create a microenvironment conducive for protein stabilization and improved biomolecular interactions. The feasibility of immunosensors based on capacitance measurements on semiconductor-immobilized antibody-electrolyte heterostructures using PEG has been investigated [84-86]. Capacitance measurements on biosensors succeed only if the successive biomolecular layers grafted onto the heterostructures are sufficiently electrically insulating and retain their recognizing ability, the results show the possibility of developing a differential capacitive biosensor [87].

2.7 DNA Overview

A single cell is all it takes to create a human being. With the exception of red blood cells, each cell in our body contains a nucleus which holds our genetic blueprints known as DNA (deoxyribonucleic acid). The primary purpose of DNA is to make copies of itself. DNA is comprised of 3 key elements; nucleobases (bases), sugar and phosphate. There are 4 bases or nucleotides Fig.2.3 Adenine (A), Thymine (T), Cytosine (C) and Guanine (G) [88]. Each base will attach to a sugar molecule that is attached to a phosphate molecule. The sugar and phosphate form the backbone of DNA while the various combinations of bases attached to sugar are what provide the biological diversity between all living beings, and each base has a complimentary base which it can pair up with. The base pair rules are such that adenine pairs with thymine and cytosine with guanine. Each base pair with another base through the use of hydrogen bonds. Two hydrogen bonds comprise the A-T bond while three hydrogen bonds are required for the C-G bond. Due to the increased number of hydrogen bonds between cytosine and guanine, it is more difficult to break apart than the adenine-thymine base pair. This base pairing allows DNA

to be composed of two strands linked together, also known as hybridization. A DNA sequence has two ends, known as 5` and 3`. The 5` and 3` refers to the position of the carbon atoms in the sugar ring of DNA. The two strands of DNA line up in an anti-parallel fashion, such that one strand is in the 5` to 3` direction while the other is in the 3` to 5` direction.

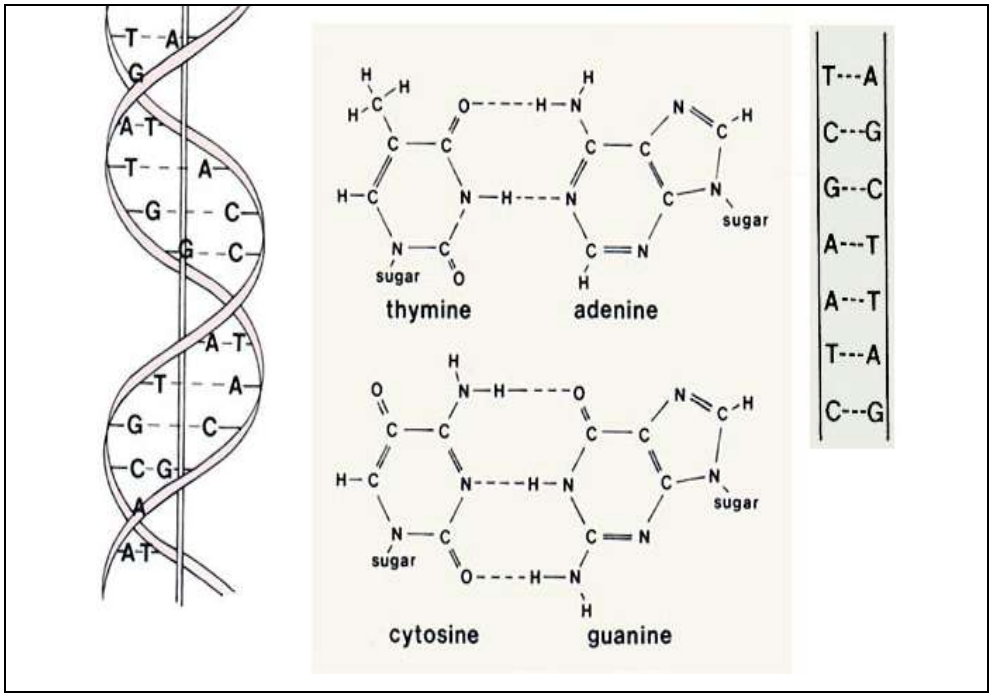


Figure 2.3 Molecular structure DNA showing base pairs and nucleotide of DNA

The hybridization of these two strands forms a double-helix structure, which was discovered in 1953, by James Watson and Francis Crick. The pairing of DNA is such that if the DNA sequence of one strand is known, it is easy to determine the sequence of the complimentary strand, due to the base pairing rules of AT, CG [88].

2.8 The DNA-Molecule Forces Binding

DNA as carrier of genetic information is a major target for drug molecules interaction, because of the ability of these drugs molecules to interfere with transcription (gene expression and protein synthesis) and DNA replication. Understanding the forces involved in the binding of certain molecules to DNA is of prime importance, molecule bind to DNA both covalently as well as non-covalently [89]. Covalent binding in DNA might be irreversible and invariably leads to complete inhibition of DNA processes and subsequent cell death. Cis-platin is a famous

covalent binder used as an anticancer drug [90]. While the non covalently bound drugs mostly fall under tow classes fig.2.4, Intercalation and groove binding [91].

2.8.1 Intercalation

The binding of molecules to double stranded DNA including intercalation between base pairs has been a topic of research for over 40 years. For the most part, however, intercalation has been of marginal interest given the prevailing notion that binding of small molecules to protein receptors [92]. It is largely responsible for governing biological function. Intercalation involves the insertion of a planar molecule between DNA base pairs Figure 2.4, which results in a decrease in the DNA helical twist and lengthening of the DNA [93].

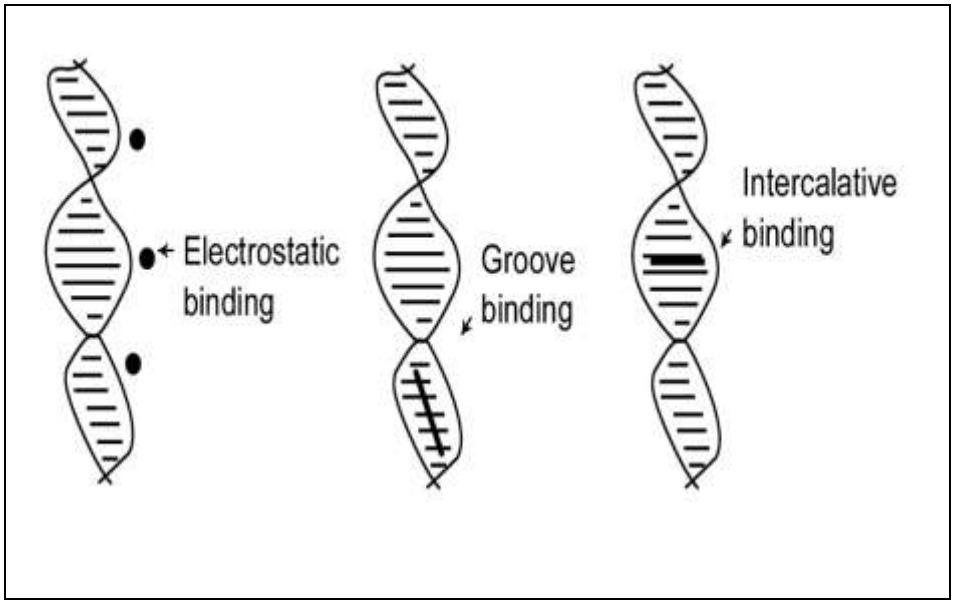


Figure 2.4 Three major binding modes for the binding of bases to DNA: Intercalation, outside groove binding and outside binding [92]

Although intercalation has been traditionally associated with molecules containing fused bi/tri cyclic ring structures, atypical intercalators with non fused rings systems may be more prevalent than previously recognized [93]. Moreover, DNA intercalators have been used extensively as antitumor, antineoplastic, antimalarial, antibiotic, and antifungal agents, not all intercalators are genotoxic (defined by the ability to alter a cell's genetic material

as a means of inducing a toxic effect). The presence of basic, cationic, or electrophilic functional groups is often necessary for genotoxicity [94]. Intercalation as a mechanism of interaction between cationic, planar, polycyclic aromatic systems of the correct size (on the order of a base pair) was first proposed by Leonard Lerman in 1961.

2.8.2 Groove Binding

In groove binding molecules are usually crescent shaped, which complements the shape of the groove and facilitates binding by promoting van der Waals interactions. Additionally, these molecules can form hydrogen bonds to bases, typically to N3 of adenine and O2 of thymine. Most minor groove binding drugs bind to A/T rich sequences. This preference in addition to the designed propensity for the electronegative pockets of AT sequences is probably due to better van der Waals contacts between the ligand and groove walls in this region, since A/T regions are narrower than G/C groove regions and also because of the steric hindrance in the latter, presented by the C2 amino group of the guanine base. However, a few synthetic polyamides like lexitropsins and imidazole-pyrrole polyamides have been designed which have specificity for G-C and C-G regions in the grooves. Groove binding, unlike intercalation, does not induce large conformational changes in DNA and may be considered similar to standard lock-and-key models for ligand macromolecular binding [95]. Groove binders are usually crescent-shaped molecules that bind to the minor groove of DNA. They are stabilized by intermolecular interactions and typically have larger association constants than intercalators (approximately 1011 M-1), since a cost in free energy is not required. for creation of the binding site [95]. Like intercalators, groove binders also have proven clinical utility as anticancer and antibacterial agents, as exemplified by mitomycin (which is also a DNA cross linker) [96]. Notably, the anthracyclines, a class of clinically important compounds with antineoplastic and antibacterial properties, take advantage of both modes of binding as they possess an intercalative unit as well as groove-binding side chain [97].

2.8.3 Hydrogen Bonding

The presence of hydrogen bonding is of great importance in a range of molecules. For instance the biological activity of DNA relies on this type of bonding [98]. Hydrogen bonding is defined

as the attraction that occurs between a highly electronegative atom carrying a non-bonded electron pair (such as fluorine, oxygen or nitrogen) and a hydrogen atom, itself bonded to a small highly electronegative at type bonding interactions between water molecules, this is an example of intermolecular hydrogen bonding. It is also possible for hydrogen bond to form between appropriate groups within the same molecule. This known as intra-molecular hydrogen bonding, like in protein structure [99]. A variety of analytical techniques have been developed for characterization and identification of the interaction between DNA and small molecules with relative advantages and disadvantages [100-106]. However, most of these methods suffer from high cost, low sensitivity and procedural complication. Up to now, electrochemical methodologies have attracted appreciable attention due to the inherent specificity and high sensitivity. Direct monitoring, simplicity and low cost facilitate to investigate the drug-targeting compound interactions and obtain the quantitative analysis information in pharmaceutical formulations and biological fluids [107,108]. On the other hand, the electrochemical system can serve as a versatile and illuminating model of biological system in a way to the real action occurring in the living cells in vivo [109,110]. The interaction mechanism can at least be elucidated in three different ways, involving the use of drug- and/or DNA-modified electrodes and interaction in solution [110].

2.9 Non Viral Gene Therapy System

Several non viral gene delivery systems have been an increasingly proposed strategy as safer alternatives to viral vectors [111]. The advantages and limitations of each method for gene delivery have been well known. It is important to point out that therapeutic applications of these non viral gene delivery systems are rather limited despite the progress in vector design and the understanding of transfection biology. Continuous effort to improve currently available systems and to develop new methods of gene delivery is needed and could lead to safer and more efficient non viral gene delivery. Non viral vectors should circumvent some of the problems occurring with viral vectors such as endogeneous virus recombination, oncogenic effects and unexpected immune response. Further, non viral vectors have advantages in terms of simplicity of use, ease of large-scale production and lack of specific immune response. These techniques are categorized into two categories, Naked DNA delivery by a physical method, and Delivery mediated by a chemical carrier [111,112]

2.10 Gene Packaging Strategies

The basic design criteria for any synthetic gene delivery system includes the ability of this system to protect DNA from extracellular/intracellular nuclease degradation, condensing the bulky structure of DNA to appropriate length scale for cellular internalization, and lastly ability to neutralize the negative charge phosphate backbone of DNA. Therefore several of gene packaging methods are relied on three strategies: electrostatic interaction, encapsulation, adsorption [113].

2.10.1 Electrostatic Interaction

Polymeric molecule have been developed to neutralize the anionic nature of DNA to drive complexation via electrostatic interaction at a sufficient charge ratio which can condense DNA [113], to appropriate size for cellular internalization by endocytosis, macro pinocytosis, and phagocytosis. Despite of the benefits of this method, other limitations are raised due to the presence of positive charges of cationic polymer which lead to cyto-toxicity and the strong electrostatic interaction may lead to difficulties of DNA release.

2.10.2 Encapsulation

In this approach DNA were encapsulate within a micro spherical biodegradable structure, most of these biodegradable polymers can be hydrolytically degraded and readily cleared from the body. This degradation can be modulated by various factors such as polymer properties, composition, and particle size formulation [113]. The main limitations of this approach is shear stresses, organic solvents, temperature, low encapsulation efficiency, DNA degradation due to low pH microenvironment, and DNA bioavailability due to incomplete release from polymer [114,115].

2.10.3 Adsorption

This approach involve marriage of the two previous techniques, which including adsorption of cationic moieties to the surface of biodegradable particles to which DNA can electrostatically bind [114-116]. This approach can offer increase of DNA amount available to release.

2.11 DNA Characterization Techniques

The characterization by absorption spectroscopy and electronic microscopy in DNA binding studies is a very useful technique. Because the interactions of molecules with DNA are subjects that exist at the interface of chemistry, physics, and biology. Many anticancer, antibiotic and antiviral pharmaceuticals exert their primary biological effects by reversibly interacting with DNA [117,118]. Therefore, the study of the action mechanism, trend in DNA-binding affinities and optical properties of molecules with DNA is of significance in the better understanding of their clinical activities and rational design of more powerful and selective anticancer pharmaceuticals. Absorption scattering and transmission of various electromagnetic radiations are used as spectroscopic characterization methods. The most known of these methods are UV Visible NIR, FTIR, and TEM [119].

2.11.1 UV-Visible Spectroscopy

UV– Vis absorption spectroscopy is a powerful tool for studying biological systems. It often provides a convenient method for analysis of individual components in a biological system such as proteins, DNA, and metabolites [120]. It is sensitive to formation of complexes and can be used to evaluate their association constants, to define the size of the binding site and the sequence specificity on the basis of the shape and positions of maximums of corresponding spectra. [121-123]. It can also provide detailed information about the structure changes and mechanism of action of molecule-DNA [124, 125, 126]. As well as this technique is sensitive to the π-bonding in the amine bases of DNA. The π-bonding absorption line occurs at a wavelength around 260 nm for the various nucleotides figure 2.5. UV-Vis NIR absorption technique is also sensitive to the presence of two amino acids forming the proteins: Tryptophan and Tyrosine. Note that the absorption signal from proteins (observed around the 280 nm absorption line) is 40 times smaller than that from DNA (observed around the 260 nm absorption line) for comparable concentrations. The absorbance is typically kept between 1 and 10 in order to avoid signal saturation effects This is done by adjusting the sample thickness and concentration. The UV-Vis NIR absorption spectroscopy method can also be used to distinguish among the possible macromolecular conformations: alpha helix, beta sheet or random coils [127,128,129].

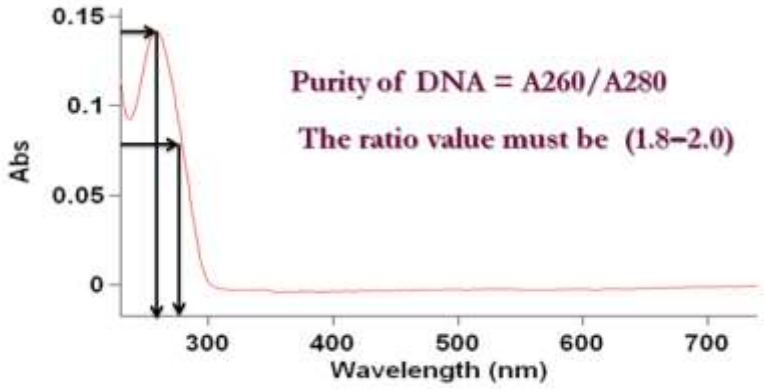


Figure 2.5 DNA purity determination using spectrophtometer

The basic component of spectrophotometer Figure 2.6. Includes a radiation source, a monochromator, a sample cell, and a detector. To minimize errors in spectrophotometer, samples should be free of particles, cuvets must be clean, and they must be positioned reproducibly in the sample holder. Measurements should be made at a wavelength of maximum absorbance [130]. Simply stated, spectroscopy is the study of the interaction of radiation with matter. Radiation is characterized by its energy, E, which is linked to the frequency, **ν**, or wavelength**, λ**, of the radiation by the familiar Planck relationship:

$$\lambda \nu = h \nu = hc/\lambda \qquad \text{Eq 2.1}$$

Where *c* is the speed of light, and h is Planck's constant. Absorption of light is commonly measured by absorbance (*A*) or transmittance (*T*) defined as:

$$A = \log (P_0 / P) \qquad \text{Eq 2.2}$$

$$T = P_0 / P \qquad \text{Eq 2.3}$$

Where P_0 is the incident irradiance and *P* is the exiting irradiance. A absorption spectroscopy is useful in quantitative analysis because absorbance is proportional to the concentration of absorbing species in dilute solution (Beer`s law):

$$A = \varepsilon bc \qquad \text{Eq 2.4}$$

Where b is pathlenght, c is concentration, and **ε** is the molar absorptivity (a constant of proportionality) [130].

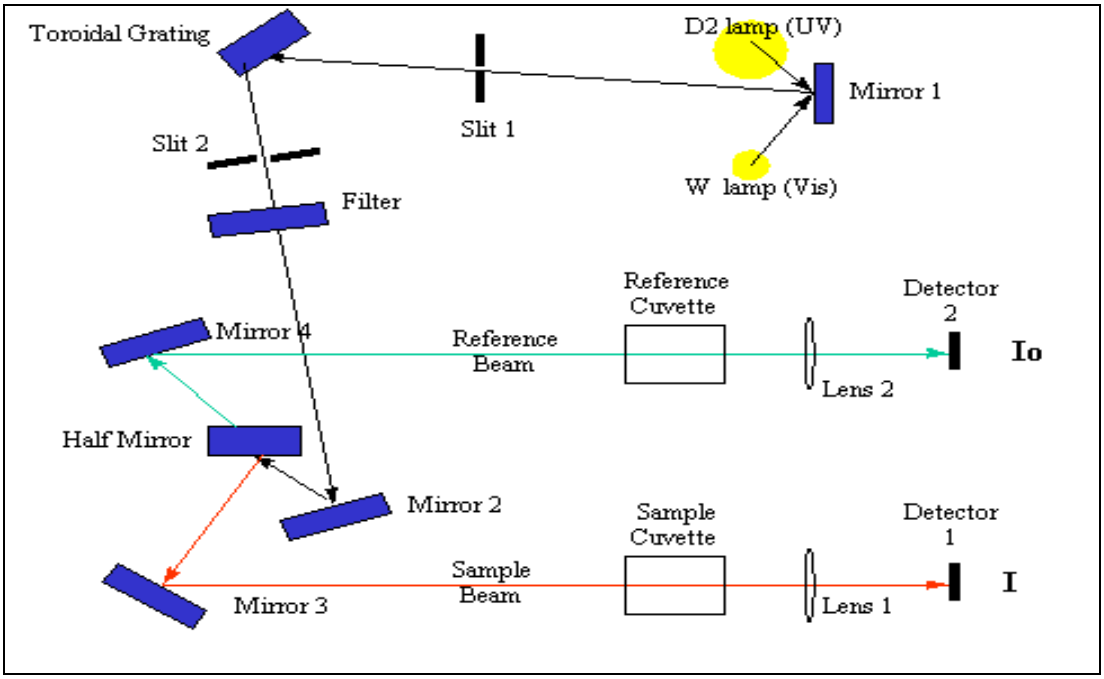


Figure 2.6 Illustration of principle of uv visible spectrophotometer. [130].

2.11.2 Thermal Stability and Denaturation of DNA

The nucleic acids are characterized by the ability of an individual molecular strand to specifically pair with a second strand using the intrinsic pairing capabilities of the nucleotide bases to form a double stranded, helical structure. The stability of the double stranded structure is critically important for many aspects of nucleic acid metabolism. Strand separation must occur for DNA replication, for DNA repair and for the transcription of DNA into RNA [131]. When duplex DNA molecules are subjected to conditions of pH, temperature, or ionic strength that disrupt hydrogen bonds, the strands are no longer held together. That is, the double helix is denatured and the strands separate as individual random coils. If temperature is the denaturing agent, the double helix is said to melt. The course of this dissociation can be followed spectrophotometrically because the relative absorbance of the DNA solution at 260 nm increases as much as 40% as the bases un stack [132]. This absorbance increase, or hyperchromic shift, is due to the fact that the aromatic bases in DNA interact via their *p* electron clouds when stacked together in the double helix. Because the UV absorbance of the bases is a consequence

of π electron transitions, and because the potential for these transitions is diminished when the bases stack, the bases in duplex DNA absorb less 260-nm radiation than expected for their numbers. Un stacking alleviates this suppression of UV absorbance. The rise in absorbance coincides with strand separation, and the midpoint of the absorbance increase is termed the melting temperature (T_m). The relative GC base pair content of DNA, and ionic strength of DNA sample solution are both important factors that play major effect in melting temperature values and DNA denaturation, because of the number of hydrogen bounds that hold base pair together. In GC base pair there are three hydrogen bounds, while in AT base pair are only two hydrogen bounds. [133,134], as well as lowering the ionic strength of DNA solution was found lowering the melting temperature of DNA, because the ions lead to suppress the electrostatic repulsion between negative charges of phosphate back bone groups in the complementary DNA strand. Therefore, when the concentrations of the ions concentration is raised up, so the T_m is increase as well [135]. The renaturation of DNA is the opposite process of denaturation where two single strands of DNA reconnect again into double helix DNA by lowering temperature, so the complementary base pairs are holed again. Renaturation is depend on DNA concentration and time due to imperfection of DNA, thus the process occur more quickly if the temperature is warm enough for diffusion of large DNA molecule.[135]

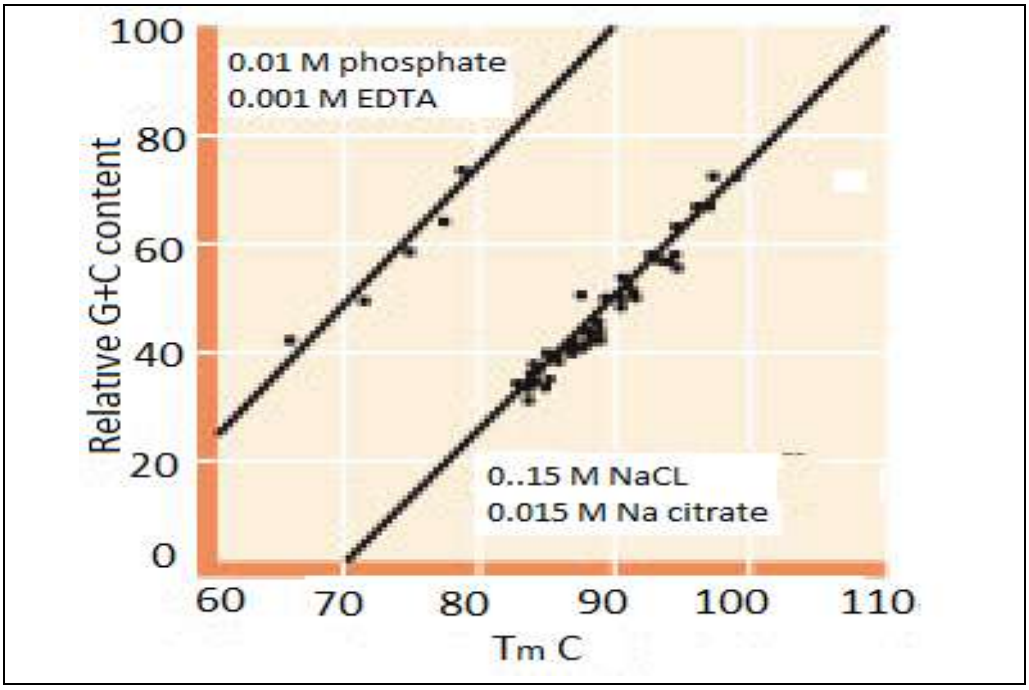


Figure 2.7 Dependence of melting temperature on relative (G + C) content in DNA.(J. Marmur and P. Doty, Journal of Molecular Biology 5(1962)120).

2.11.2.1 The Melting Temperature (T_m)

The melting temperature (T_m) is the temperature at which the molecule is half denatured. This represents the point at which enough heat energy is present to break half the hydrogen bonds holding the two strands together[133]. The melting temperature depends on both the length of the molecule, and the specific nucleotide sequence composition of that molecule. Because cytosine / guanine base-pairing is generally stronger than adenosine / thymine base-pairing, the amount of cytosine and guanine in a genome (called the GC content) can be estimated by measuring the temperature at which the genomic DNA melts. Higher temperatures are associated with high GC.

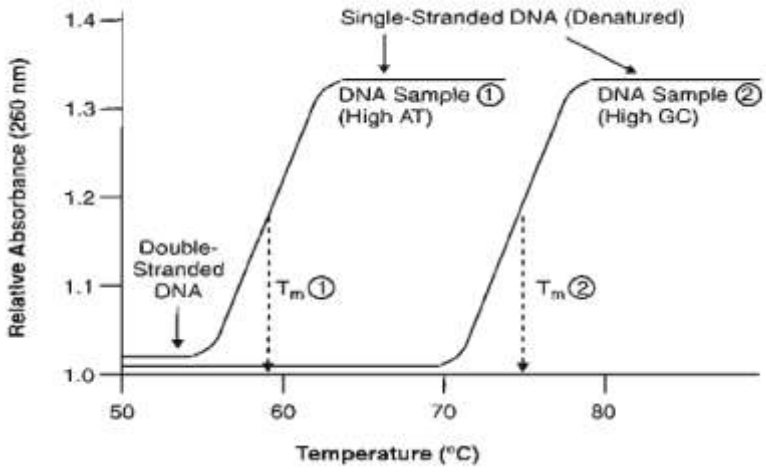


Figure 2.8 Importance of GC content in DNA denaturation (B. Hansen, L Jorde. USMLE step1. Biochemistry notes. (2002)

2.11.3 Fourier Transform Infra Red (FTIR)

FTIR spectroscopy is technique based on absorption-transmission used to probe chemical bonds and characterization of materials [136]. It is not providing only information about the composition and the structure of molecules, but also morphological information and their crowding environment in molecular systems. FTIR is a chemical analysis method of choice used to rapidly identify substances [136], it produces their molecular fingerprint, and absorption peaks

correspond to normal mode frequencies of the molecular bonds making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. In recent years, the Fourier transformed infrared spectroscopy is often applied in studies of biological materials such as drug or polymers on cellular level [137]. Several studies were conducted using FTIR Their aim were vary from examine the molecular interactions of molecule with DNA in aqueous solution at physiological pH, and to note if infrared microscopy can be used in radiation DNA damage detection. Statistical analysis shows sensitivity of this technique the divergences between spectra of irradiated by deferent dosages of protons and control cells. Fitting analysis allows to follow small changes in spectra. Presented results prove that FTIR spectroscopy could be useful tool in DNA damage study in single cells [138]. Now with modern software algorithms, FTIR becomes an excellent tool for quantitative analysis. The original infrared instruments were of the dispersive type. These instruments separated the individual frequencies of energy emitted from the infrared source. This was accomplished by the use of a prism or grating. An infrared prism works exactly the same as a visible prism which separates visible light into its colors (frequencies). A grating is a more modern dispersive element which better separates the frequencies of infrared energy. The detector measures the amount of energy at each frequency which has passed through the sample. This results in a spectrum which is a plot of intensity vs. frequency. Fourier transform infrared spectroscopy is preferred over filter methods of infrared spectral analysis for several reasons such as their wide applicability, it is a non-destructive technique, it provides a precise measurement method which requires no external calibration, it can increase speed, collecting a scan every second, it can increase sensitivity, it has greater optical throughput, and it is mechanically simple with only one moving par. Besides these intrinsic advantages (of the known as dispersive infrared spectroscopy), the more recent infrared spectroscopy by Fourier transform (FTIR) has additional merits such as: higher sensitivity, higher precision (improved frequency resolution and reproducibility), quickness of measurement and extensive data processing capability (as FTIR is a PC based technique, it allows storage of spectra and facilities for processing spectra). The term Fourier transforms, Infrared spectroscopy originates from the

fact that a Fourier transform (a mathematical algorithm) is required to convert the raw data into the actual spectrum [139]. This technique can be used for solid, liquid or gas. IR spectra originate in transitions between two vibrational levels of a molecule in the electronic ground state and are usually observed as absorption spectra in the infrared region [140].

3.11.3.1 The Principle of FTIR

FTIR stands for Fourier Transform Infra Red, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample, some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum [136]. This makes infrared spectroscopy useful for several types of analysis such as identify unknown materials, determine the quality or consistency of a sample, and can determine the amount of components in a mixture.

3.11.3.2 Basic Theory of FTIR

Infrared spectroscopy has been a workhorse technique for materials analysis in the laboratory for over seventy years. An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis. The original infrared instruments were of the dispersive type. These instruments separated the individual frequencies of energy emitted from the infrared source. This was accomplished by the use of a prism or grating. An infrared prism works exactly the same as a visible prism which separates visible light into its colors (frequencies). A grating is a more modern dispersive element which better separates the frequencies of infrared energy. The detector measures the

amount of energy at each frequency which has passed through the sample. This results in a spectrum which is a plot of intensity versus frequency [141].

3.11.3.3 Importance of FTIR in DNA Study

DNA may studied by using IR spectroscopy [142,143], the spectra of nucleic acids may be divided into the modes due to the constituent base, sugar and phosphate groups. The bases (Thymine, adenine, cytosine, guanine and uracil) give rise to purinic and pyrimidinic vibrations in 1800-1500 cm^{-1} range and these bands sensitive markers for base pairing and base stacking effects. Bands in the 1500-1250 cm^{-1} region of nucleic acids are due to the vibrational coupling between a base and sugar, while in 1250-1000 cm^{-1} range sugar-phosphate chain vibrations are observed. These bands provide information about backbone conformations. In the 1000-800 cm^{-1} region, sugar/sugar-phosphate vibrations are observed [143]. Major IR of nucleic acids are listed below in the table 2.2. Advantages of FTIR are very interesting when you come to the laboratory scale applications; it may produce results in short time with high sensitivity, and it is considered simple to use instrument and has self calibration facility during the running mode. The spectral resolution is the same throughout the entire spectral range. In the other hand, many limitations of FTIR can be faced. It cannot detect atoms or mono atomic ions because single atoms do not contain chemical bonds, therefore, do not possess any vibrational motion. Consequently, they absorb no infrared radiation, for example, homo-nuclear diatomic molecules- molecules comprised of two identical atoms, such as N2 and O2, do not absorb infrared radiation. The spectra obtained from samples are complex, and difficult to interpret because it is hard to know which bands are from which molecules of the sample. Its usage in aqueous solutions is also difficult to analyze by means of infrared spectroscopy. Water is a strong infrared absorber in specific wave number ranges, thus it masks regions of the sample spectrum. FTIR spectrometers are a single beam technique and the samples and the background are measured at different times. In order to eliminate the instrumental and environmental contributions to the spectrum, the sample spectrum is divided by the background spectrum. However, spectral artifacts can appear in the sample spectrum as a result of instrumental or the environmental changes of water vapor and carbon dioxide concentration during the time between the sample and background. The water vapor in the air (humidity) absorbs mainly in the regions 1270-2000 cm-1 and 3200-4000 cm-1).

Wave number (cm^{-1})	Assignment
2960-2850	CH2 stretching
1705-1690	RNA C=O stretching
1660-1655	DNA C=O stretching; N-H bending; RNA C=O stretching
1610	C=C imidazol ring stretching
1578	C=N imidazol ring stretching
1244	RNA PO2 asymmetric stretching
1230	DNA PO2 asymmetric stretching
1218	RNA C-H ring bending
1160,1120	RNA ribose C-O stretching
1089	DNA PO2 symmetric stretching
1084	RNA PO2 symmetric stretching
1060,1050	Ribose C-O stretching
1038	RNA ribose C-O stretching
1015	DNA ribose C-O stretching RNA ribose C-O stretching
996	RNA uracil ring stretching; uracil ring bending
970,916	DNA ribose-phosphate skeletal motions

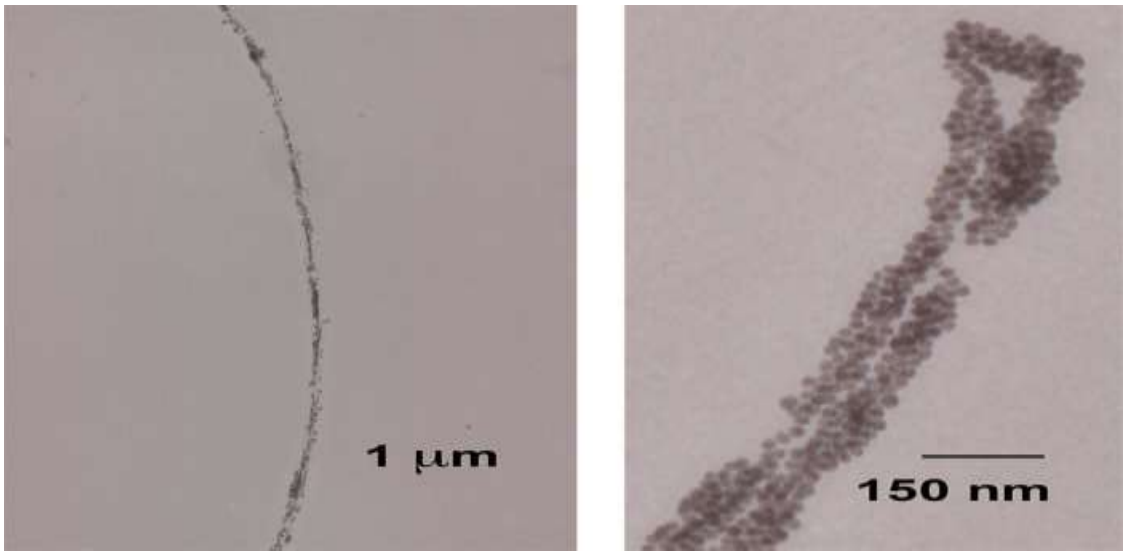
Table 2.2 Major infrared bands of nucleic acids [142]

2.11.4 Transmission Electron Microscope (TEM)

Electron microscopy can observe smaller size scales down to one nanometer. Electron microscopy is used in the transmission mode (TEM) for thin samples or in the scanning mode (SEM) to image surfaces. Samples are stained in order to enhance the contrast [144].

2.11.4.1 Basics of TEM

The basic principle of TEM is similar to that of optical microscopy; but uses electrons instead of light, a set of lenses and an adjustment system to enlarge the image taken from a specimen. Electron microscopy uses electrostatic lenses to focus the electron beam and can achieve magnifications a thousand times greater than optical microscopy fig. 2.9. A beam of electrons is transmitted through an ultra thin specimen then focused and magnified to form an image which is displayed on an imaging screen. The magnification of electron microscopes can be as high as 2 million times. Most ranges in the nanometer scale can be observed [144]. In TEM Electrons are emitted by a cathode (usually a tungsten filament) by applying high voltage between two electrodes. The electron beam is focused and made to go through electrostatic lenses to produce an image which is recorded by hitting a fluorescent screen fig. 2.10, a photographic plate, or a light sensitive sensor [144,145].



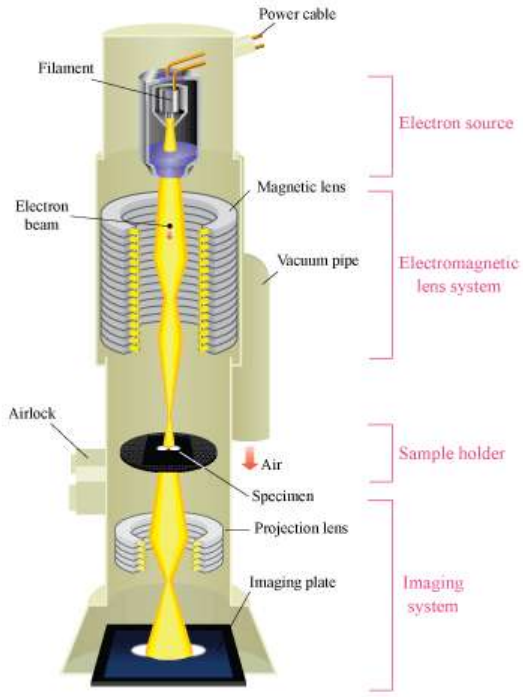


Figure 2.9 Transmission Electron Microscopy (TEM) images of a long DNA molecule that has been decorated electrostatically with positively charged gold nanoparticles.

Figure 2.10 the basic component of TEM

2.11.4.2 Electron Source in TEM

The electron source consists of a cathode and an anode. The cathode is a tungsten filament which emits electrons when being heated. A negative cap confines the electrons into a loosely focused beam figure. The beam is then accelerated towards the specimen by the positive anode. Electrons at the rim of the beam will fall onto the anode while the others at the center will pass through the small hole of the anode. The electron source works like a cathode ray tube [145].

2.11.4.3 TEM Principle Work

TEM works like a slide projector. A projector shines a beam of light which transmits through the slide. The patterns painted on the slide only allow certain parts of the light beam to pass through. Thus the transmitted beam replicates the patterns on the slide, forming an enlarged image of the

slide when falling on the screen. TEMs work the same way except that they shine a beam of electrons (like the light in a slide projector) through the specimen (like the slide). However, in TEM, the transmission of electron beam is highly dependent on the properties of material being examined. Such properties include density, composition, etc. For example, porous material will allow more electrons to pass through while dense material will allow less. As a result, a specimen with a non-uniform density can be examined by this technique [145,146,147].

CHAPTER 3 MATERIALS AND METHODS

3.0 Introduction

This chapter describes the general material and experimental methods employed in the preparation of complexes of polyethylene glycol and DNA, and their spectroscopic and microscopic characterization. Basic principles of the techniques that have been used in this study such as FTIR, UV visible and TEM were described the previous chapter. Specific materials and methods are outlined separately for each experimental study in the following chapters.

3.1 Experimental Material

3.1.1 Buffers and Salts

Unless otherwise stated all buffer salts and reagents used in the spectroscopy techniques were obtained from National Medical Research Center NMRC- Al Zawya-Libya. All water used in the experiments was obtained from an GFL purification system. Water and buffers were filtered through a filter membrane with 0.2 μm pore size prior to use. Phosphate buffered saline (PBS) was prepared by dissolving one tablet (Sigma-Aldrich) in 200 ml water. For spectroscopic studies PBS was diluted to 10% of solution strength, thus, reducing the ionic strength. The TEM experimental work conducted by staining the samples, and positive staining is the advised in studying the biological samples. The most commonly used stain in TEM is uranyl acetate, the saturated uranyl acetate in 50% ethanol staining solution has been prepared by adding 5g of uranyl acetate to 50% ethanol / purified water in a fume cupboard and rolling the bottle for 30 minutes whilst keeping away from strong lighting especially direct sunlight.

3.1.2 Calf Thymus DNA

Highly polymerized linear calf thymus (ctDNA) was purchased from Sigma-Aldrich with molecular weight 10-15 million Daltons prior to activation (nicking) as reported by sigma. It was received as freeze-dried material, which was reconstituted with water, before being further purified by precipitation with dry ice-cooled ethanol/3 M sodium acetate (90/10) to 1 ml of DNA solution 1 mg ml^{-1}. Relative purity and exact DNA concentration were determined using a (UV absorbance) wavelength scan from 200 to 400nm and fixed wavelengths 260 and 280 nm. Relative purity can be determined by calculating the ratios of absorbance at 260 and 280

nm. Ideally, this should be 1.8. Therefore, the purity can be determined from (A_{260}/A_{280}) / 1.8. The actual concentration of the DNA solution can be determined using the following equation:

$$\text{Actual DNA Conc. } (\mu g/ml) = \frac{50 \times (A_{260}/A_{280}) \times dilution\ \ of\ \ DNA\ \ sample}{1.8} \qquad \text{Eq. 3.1}$$

These samples were further diluted to 20 $\mu g ml^{-1}$ with 10 % w/v phosphate buffered saline (PBS, 0.014 M NaCl, 0.001 M phosphate, pH 7.4) before use. Serial of dilutions were also prepared based on the concentration suggested in different experimental protocols.

3.1.3 Polyethylene Glycol 400

Polyethylene glycol 400 (PEG) was obtained from NMRC and has been purchased from PS Park scientific limited-UK, phosphate buffer solution pH =7.4 (Sigma), Hcl 1M (sigma), NaOH 1M (Sigma).

3.2 Experimental and Methods

3.2.1 Preparation PEG-DNA ratio samples

It should be understood that polymer (polyethylene glycol) to DNA ratios are presented in this study as polymer monomer to DNA nucleotide molar ratio (mol/mol) in terms of monomer: nucleotide for a series of PEG (5 µl, 15µl, 20µl, 130µl, 230µl) for 20µl ctDNA. The amount of polymer needed to achieve a certain ratio was calculated according to the equation below:

$$\text{Amount of polymer} = \frac{polymer\ \text{monomer Molecular weight}}{\text{DNA nucleotide molecular weight}} - \times \text{Ratio} \times \text{amount} \qquad \text{Eq 3.2}$$

The average molecular weight of nucleotide is 308.

3.2.2 Preparation of PEG-DNA Complexes

PEG was prepared to a concentration that allowed PEG and ctDNA solutions to be combined in equal volume. The quantity of PEG required producing a complexes of a given PEG to DNA ratio was calculated using the equation 2.2. All PEG-ctDNA complexes were routinely prepared by the addition of a single aliquot of PEG solution to buffered DNA solution.

3.2.3 Experimental Details of PEG-ctDNA Study

Polymer solutions were prepared directly as described using Equation 2.2 to appropriate concentrations before adding to the DNA solution an equal volume was required to prepare samples of a specified polymer: DNA ratio. Further dilution by using 10% PBS for those samples was prepared under different pH environments.

3.2.4 UV-visible experimental Instrument

The absorption and transmission of various electromagnetic radiations are used as spectroscopic characterization methods. The most known of these methods is UV-visible wavelength ranges. The UV visible absorbance spectra for PEG with ctDNA with complex interactions were obtained and scanned between 200nm–800nm using UV-visible–NIR spectrophotometer from Varian model-Cary 5000 with slit of 2 nm, Quartz cuvette of 1cm were used.

3.2.5 Preparation of PEG-ctDNA for UV-visible

Sample of ctDNA was prepared according to Eq.3.1 in phosphate buffer solution (pH 7.4) at 22 C º with stirring for 10 minutes to ensure the formation of a homogeneous solution. The sample was then determined spectrophotometrically at 260 nm. PEG 400 were also prepared directly as described using Eq.3.2 to appropriate concentrations before adding to the DNA solution an equal volume was required to prepare samples of a different PEG:DNA ratio, and pH medium.

3.2.6 Thermal Analysis of DNA Using UV-Visible

In the current work experiments carried out for determining the melting temperature for ctDNA in the absence and presence of PEG, measurements were carried using UV-visible **s**pectrophotometer from Varian model Cary 5000, the system equipped with a thermo electrically controlled cell holder and quartz cell with a pathlenght of 1.0 cm. Absorbance versus temperature, data profiles were recorded after every 0.15 ◦C and it were obtained by plotting the absorbance measured at 260 nm with a heating rate of 0.25 ◦C/ min. The fraction of melted base pairs, θ, was calculated from the standard formula [24,39,40].

$$\theta = \frac{A - AL}{\mathrm{AU} - \mathrm{AL}} \qquad \text{Eq. 3.3}$$

Where A, AL and AU are sample absorbance, absorbance of the lower baseline, and the absorbance of the upper baseline respectively. (Tm**)** is defined as the temperature for which θ was 0.5. The hyperchromicity [18,30,41,42] of the samples was calculated using:

$$\% H_{260} = \frac{AU - AL}{AL} X 100 \qquad \text{Eq. 3.4}$$

3.2.7 Determination of Melting DNA Temperature (T_m)

20 µl DNA solution mixed with 1 ml of phosphate buffer (0.01M phosphate, 0.1M NaCl, at pH 7.4). Melting curves were recorded by starting at a temperature well below the T_m and linearly increased the temperature well above the T_m (dissociation segment). The temperature was then decreased at the same rate until the starting temperature was reached again (association segment). T_m experiments were directed and controlled by a computer with DNA melting software, which is supplied by Varian. Melting curve experiments were performed spectrophotometrically that was based on A_{260} profile, performed using the Cary 5000 UV-visible spectrophotometer by measuring the change in absorbance at 260 nm with temperature increase from 5 ^{O}C to 100 ºC at a rate of 0.25 ^{O}C / minute. The sample was cooled at a rate of 0.25 ^{O}C / minute. Melting temperatures (T_m) were determined by taking the point at half the curve height. The melting curve obtained by plotting relative absorbance at 260 nm against temperature. T_m was calculated by taking the point at half the curve height.

3.2.8 Fourier Transform Infra-Red instrument

FTIR spectra were obtained with Varian 660-IR spectrophotometer equipped with DTGS (deuterated triglycine sulphate) detector and KBr beam splitter assembly. Spectra were collected after three certain incubation times of PEG with DNA solution. Background spectra were collected before each measurement. Interferogram were accumulated over the spectral range 4000-400 cm^{-1} with a nominal resolution of 2 cm^{-1} and a minimum of 50 scans. The water subtraction was carried out with 0.1 mM NaCl solution as a reference at pH 7.4. [138], all measurements were carried out at room temperature and controlled ambient humidity of 43% RH. The difference spectra of [(ctDNA solution + PEG)-(ctDNA solution)] were then obtained, the spectra are smoothed using Savitzky - Golay procedure [148].

3.2.9 Preparation of PEG-ctDNA for FTIR

Complex samples PEG/ctDNA for FTIR were prepared by rapid addition of Equal volumes of ctDNA to PEG solution and kept for three incubation times (Zero, 1hr, 48 hrs) to allowing reaction. The composition of complexes is defined by the complex charge ratio, which is the ratio of PEG (positive charge) to DNA negative charge. To improve the stability of the complexes, samples were prepared in un buffered purified water, except complexes formed for FTIR analysis. These complexes were prepared in buffer pH 7.4. The plots of wave number and absorption of ctDNA in plane vibration related to AT, GC base pairs and PO2 stretching vibration versus PEG were obtained after peak normalization.

3.2.10 Transmission Electron Microscope

TEM techniques were used for the study of the PEG-ctDNA biocomplex. Complex samples were analyzed in microscopy department, National Institute of Research Egypt, using a JEOL JEM-1010 TEM (Jeol), operating at a voltage of 80 kV. Micrographs were taken at magnifications ranging between 60, 000 and 100, 000 x**.**

3.2.11 Preparation of PEG: ctDNA Complexes for TEM

The TEM of PEG-ctDNA biocomplex samples were prepared as described earlier in the current chapter, and detailed TEM requirement protocols is produced as the sample solution containing sufficient PEG to give the required polymer repeating-unit DNA nucleotide ratio was added to ctDNA, (2.5 μg), in 10 % PBS, such that the final DNA concentration was 10 μg ml^{-1}. The sample was leaved for vortex mix briefly and incubated for 30 minutes at room temperature to allow complex formation. For TEM imaging as 10 μl drop of sample was placed onto a copper grid and left for 1 minute, followed by removal of excess liquid by blotting with filter paper. This process was repeated, and after air-drying, the grid was inverted onto a drop of a saturated solution of Uranyl acetate in 50 % alcohol. This was covered and left for 20 minutes to stain, before washing with 50 % alcohol and two stages of demonized water, blotting between each wash. The grid was then allowed to dry in air before imaging was performed. Control grids with polymer solution only and buffer solution only were also prepared and examined.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 UV-Visible Characterization

UV-visible spectroscopic investigation of the polymer and DNA binding is one of most useful and powerful techniques in biotechnology [149,150], it's extensively used to detect and determine the mechanism of action, binding mode, and stability of DNA molecule. This technique is sensitive to the π-bonding in the amine bases of DNA, and also sensitive to the presence of two amino acids forming the proteins such as Tryptophan and Tyrosine. It

can also provide detailed information about the structure, interaction mode, and mechanism of action of DNA-molecule, which involving a strong stacking interaction between the molecules and the base pairs of DNA. It is known that the aggregation state of molecular chains in polymer solutions changes with their concentrations [151], and according to the literature studies intercalation into DNA base pairs is characterized by red shift and hypochromic effects in the absorption spectra. The groove binding is characterized by no or minor changes of UV-visible spectra, occasionally with some hyperchromicity [152], outside binding with self stacking shows quit similar characteristics as the intercalation binding mode but lesser extent [153]. In this study the influences pH medium and PEG ratio on the ctDNA-PEG complex formation was studied using uv-vis spectroscope. In uv-vis spectroscopy characterization technique the binding of these molecules to the DNA usually results in hypochromic or hyperchromic effect, a broadening of the envelope and red shift or blue shift of complex absorption band. Poly ethylene glycol is basically hydrophilic polymer; it has hydroxy groups on both end sides, and generally has higher poly dispersity and high molecular weight since polymerization can occur at both ends of the polymer. Polyethylene glycol which has the monomeric repeat unit has also been incorporated into DNA complexes of several cationic polymers, including poly methacrylate, Polyethyleneimine , Poly L Lysine, chitosan, and poly (amido amine)s [30-35]. Beside that PEG has the ability to reduce the surface charge of the complexes. Therefore the investigating the effect of PEG: DNA ratio and different pH medium to produce stable complex is of great important to study, using UV-visible spectroscope. The aim of using Uv-visible spectroscopy in current experiment was to elucidate the influence of PEG to DNA ratios, and pH medium on optimization of PEG-ctDNA complex. Method and the principle of this technique has been described previously in chapter 2.11.1, and chapter 3.2.4.

4.1.1 Effect of Different Ratios of PEG-ctDNA

The absorption results for each PEG and ctDNA samples at buffer solution pH 7.4 are shown in table 4.1. From the UV-visible spectral analysis it was seen that PEG-ctDNA with a ratio 1:1 (20µl) at 285 nm and the absorption was 0.472. The continuous increasing of PEG ratio (30 µl, 130 µl, and up to 230 µl) or decrease (5 µl, 10 µl) hardly showing any significant changes on the absorption and without any noticeable spectral shift.

Sample No.	PEG	ctDNA	Ratio	pH	λ (nm)	Abs
S1	5 µl	20µl	1:4	7.4	258 nm	0.459
S2	15 µl	20µl	3:9	7.4	258 nm	0.480
S3	20 µl	20 µl	1:1	7.4	258 nm	0.472
S4	30 µl	20µl	3:2	7.4	258 nm	0.461
S5	130 µl	20µl	13:2	7.4	258 nm	0.458
S6	230 µl	20µl	23:2	7.4	258 nm	0.444

Table 4.1: Serial samples of PEG –DNA in buffer medium pH 7.4 at spectral between 200-800 nm

4.1.2 Effect of Different pH Medium on ctDNA-PEG

The results and discussion on the effect of pH on the PEG-ctDNA interaction at different pH medium are presented in this part. PEG is a non charged polymer with two hydroxyl group in both ends. Therefore, the pH of the solution has a notable influence on the DNA-molecule binding characteristic and molecular mechanism, also the varying in pH medium study is an efficient method to distinguish binding modes between molecule and DNA [154], which will result in the changes of the shape and wavelength position of the molecular absorption features of DNA at different pH. At the same time, the different pH of the solution must have influence on the conformation of DNA and the interaction between the PEG and DNA. As well as and according to the literatures, intercalation into DNA base pairs is characterized by a red shift and hypochromic effects in the absorption spectra, while outside binding with self

stacking shows quite similar characteristics as intercalative binding mode but to lesser extent. In our experiment based on the influences of pH medium on PEG-ctDNA interaction and complex formation was tested and characterized by uv-vis spectroscopy and spectral results, and absorption for both conditions was shown in tables 4.2, 4.3. As seen in the Sample of PEG – DNA tested in acidic medium pH 1.2, was shown a little peak shift 264 nm (bathochromic) tables 4.2, and the absorption was 0.828. While in alkaline medium the sample of PEG-ctDNA was shown blue shift 256 nm (hypochromic) tables 4.3 and absorption was 1.041.

Sample No.	**PEG**	**ctDNA**	**pH**	**WL (nm)**	**Abs**
S7	230 µl	20µl	1.2	**264 nm**	0.828

Table 4.2: PEG –DNA complex in acidic pH medium at spectral between 200-800 nm

Sample No.	**PEG**	**ctDNA**	**PH**	**WL (nm)**	**Abs**
S8	230 µl	20µl	14.00	**256 nm**	1.041

Table 4.3: PEG –DNA complex in alkaline pH medium at spectral between 200-800 nm

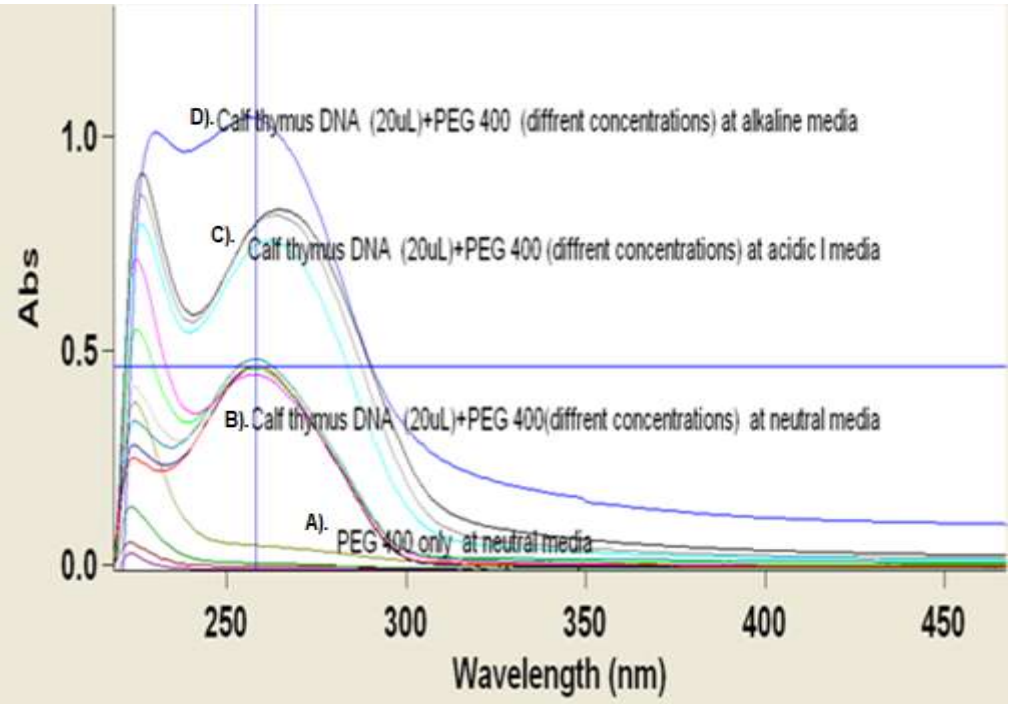


Figure 4.1 Uv-visible spectral analysis for A). PEG, B). PEG-ctDNA in neutral medic, C). PEG-ctDNA in acidic medic. D). PEG-ctDNA in alkaline medic.

4.2 Thermal Denaturation (Tm) Studies

Thermodynamic study of DNA will be really important for understanding the biological effects of polymer on DNA. It is known that the interactions of DNA with molecule have three general models: (I) Electrostatic interaction, which is from the negative-charged nucleic sugar–phosphate structure; (II) binding hydrophobically against the minor or major grooves; and (III) intercalation between the stacked base pairs of native DNA. Therefore the thermal behavior of DNA in the presence of PEG can give insight into their conformational changes when temperature is raised, and offers information about the interaction strength of drugs with DNA. It is well known that when the temperature in the solution increases, the double stranded DNA gradually dissociates to single strands. According to the literature the interaction of intercalators generally results in a considerable increase in melting temperature (Tm). In addition, upon interaction with a cationic species the double helix stability increases [155] and so does the DNA melting temperature. Although the increase in denaturation temperature is not specific to any particular type of non covalent interaction, the Tm values give some indications regarding the binding mode. Large increases in melting temperature are observed only

for the strongest type of interaction. The aim of this experiment was to elucidate the effect of PEG on thermal stability, and determine melting temperature of ctDNA in absence and in presence of PEG using UV-visible spectrophotometer equipped with a thermocouple attached to the sample holder, This technique will provide an overview of the mode of action and force binding between PEG and DNA. The method, and principle of this technique has been described previously in chapter 2.11.2, and chapter 3.2.6, 3.2.7.

4.2.3 Effect of PEG on Thermal Denaturation of ctDNA

The experiments were carried out for 20 µl ctDNA in the absence and presence of 20 µl of PEG and ctDNA at buffer solution (pH 7.4). The melting plot of DNA was monitored by plotting the UV maximum absorption of DNA at 260 nm *versus* temperature (ramped from 40 to 100 ^{O}C). The Tm was taken as the midpoint of the hyperchromic transition. In the present case, Tm of ctDNA in the absence of any added PEG has been found to be 45.26 ± 0.5 ^{O}C fig.4.2. An increase in the DNA-PEG melting temperature 48.50 ± 0.5 ^{O}C for the above-mentioned concentrations was observed fig. 4.3. The comparison between tow Tm values clearly show that the PEG is able to stabilize DNA helix [156].

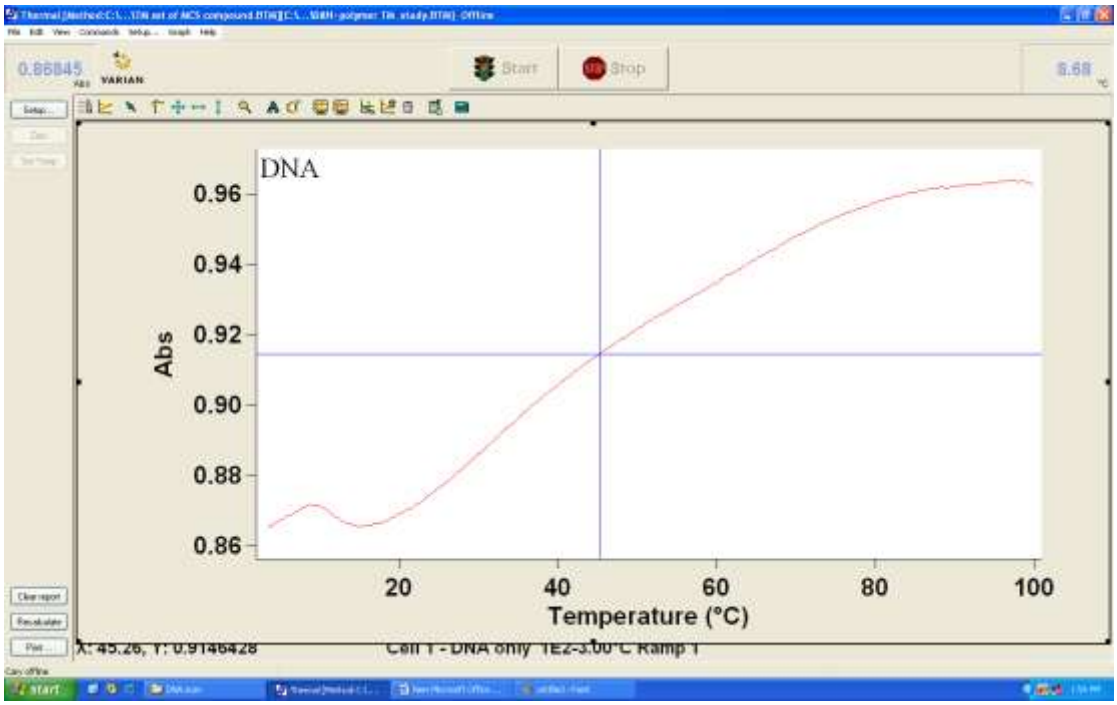


Figure 4.2 Thermal denaturation of free ctDNA showing melting curve dissolved in PBS pH 7.4
Tm was found =45.26 ^{O}C

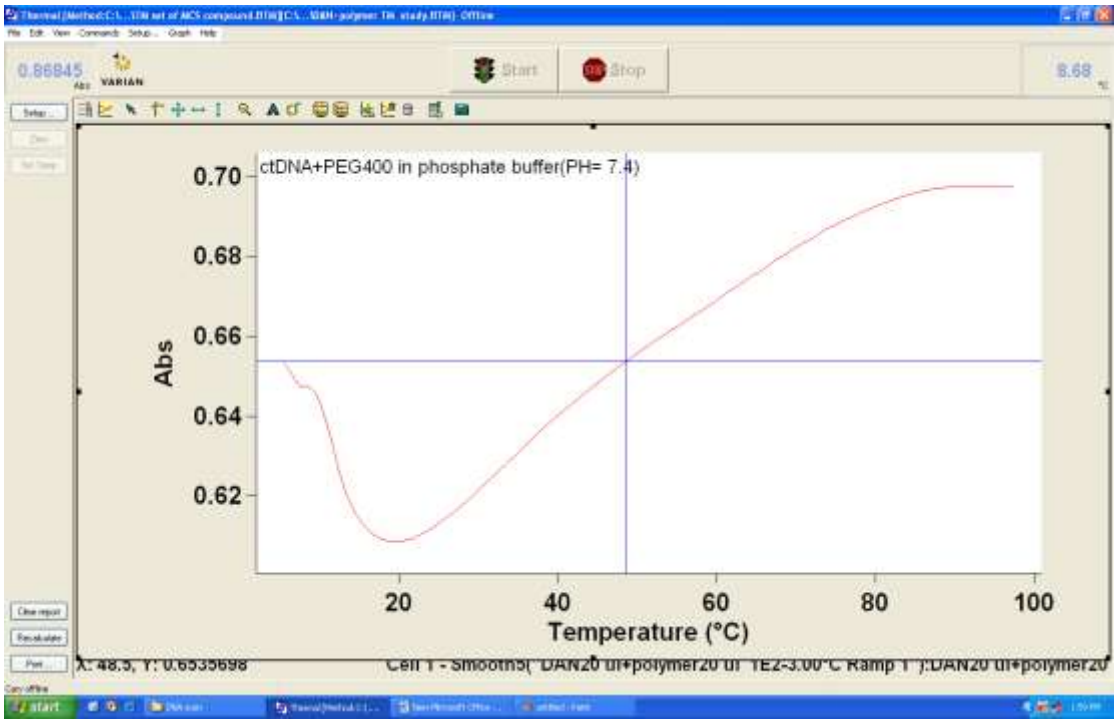


Figure 4.3 Thermal denaturation of ctDNA with PEG 400 showing melting curve dissolved in PBS pH 7.4Tm was found =48.50 ^{O}C

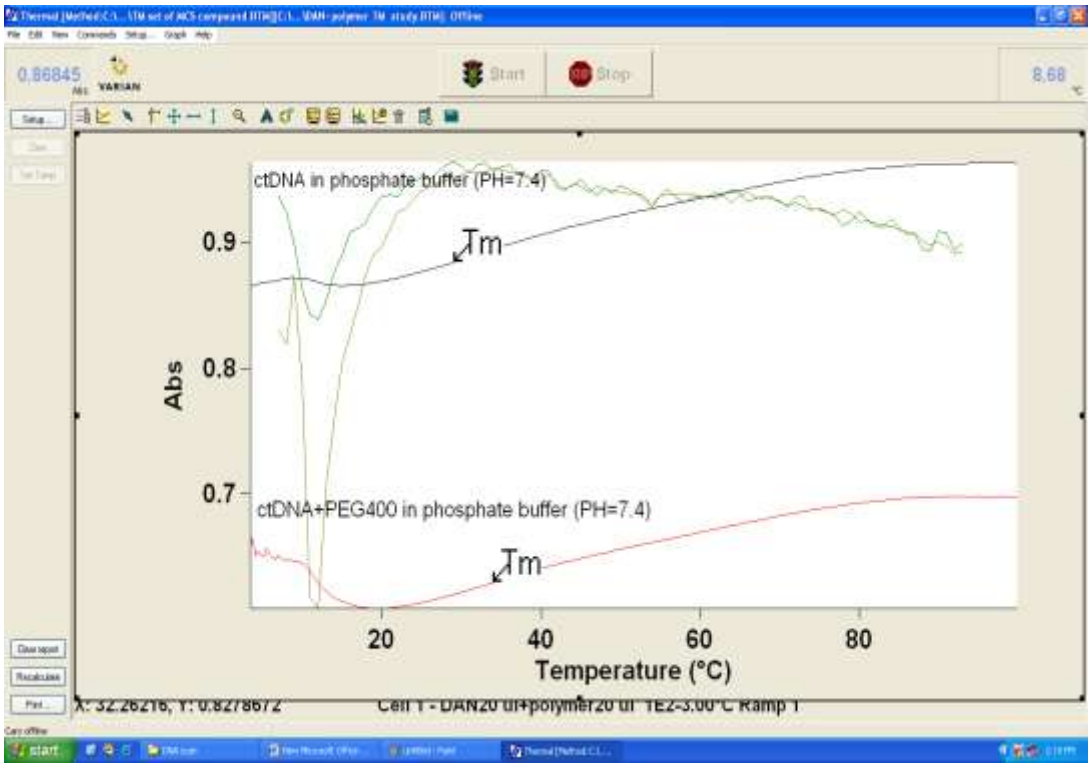


Figure 4.4 Thermal denaturation and melting curve showing the comparison between ctDNA In absence and presence of PEG dissolved in PBS pH 7.4

4.3 FTIR Characterization

FTIR spectroscopy is well established experimental techniques, and recognized as a valuable tool for the examination of DNA [157], protein conformation in H2O-based solution, and analysis of secondary structure of polymers, polypeptides and proteins, and as well as in deuterated forms and dried states, resulting in a greatly expanded use in studies of DNA secondary structure and protein dynamics in the past decade. In this work, the main aim of this experiment was to present the results, and discuss the influence of incubation time on the chemical binding of polyethylene glycol 400 to the ctDNA using FTIR spectroscopy technique. The FTIR spectroscopic method and the principle of this technique have been already described previously in chapter 2.11.3, and chapter 3.2.8.

4.3.1 FTIR Characterization of PEG and ctDNA

The IR spectral features, finger print, and major peaks for pure PEG 400 fig**.** 4.5, fig 4.6, pure ctDNA fig**.** 4.7, and fig 4.8 were all obtained and presented below. As demonstrated in spectrum analysis of pure PEG 400, it was showed many characteristic peaks at (885, 944, 1096, 1146, 1287, 1296, 1349, 1556, 1772, 2710, 2886, and 3711 cm^{-1}). The strong absorptions of PEG are assigned to the -CH2CH2- stretching around 2886 and 3749 cm^{-1} which demonstrates the presence of saturated carbons (CH2CH2)n-. fig**.** 4.8 shows the FTIR finger print spectrum of ctDNA without the addition of PEG. As can be seen, ctDNA possess characteristic peaks at (1086, 1219, 1491, 1611, 1637, and 1650, 1716 cm^{-1}). Ring vibrations of nitrogenous bases (C=O, C=N stretching), PO2 stretching vibrations (symmetric and asymmetric) and deoxyribose stretching of DNA backbone are limited in the spectral region 2000- 750 cm^{-1}. Therefore, this particular region is of interest here in this study. The vibrational bands of DNA at 1716, 1651, 1611, and 1491 cm^{-1} are assigned to guanine (G), thymine (T), adenine (A) and cytosine (C) nitrogenous bases, respectively figure 6.3, 6.4. Bands at 1219 and 1086 cm^{-1} are related to phosphate asymmetric and symmetric vibrations, respectively. These are the major bands of pure DNA which are monitored during this study. Changes in these bands (shifting and intensity) upon incubation time were further studied.

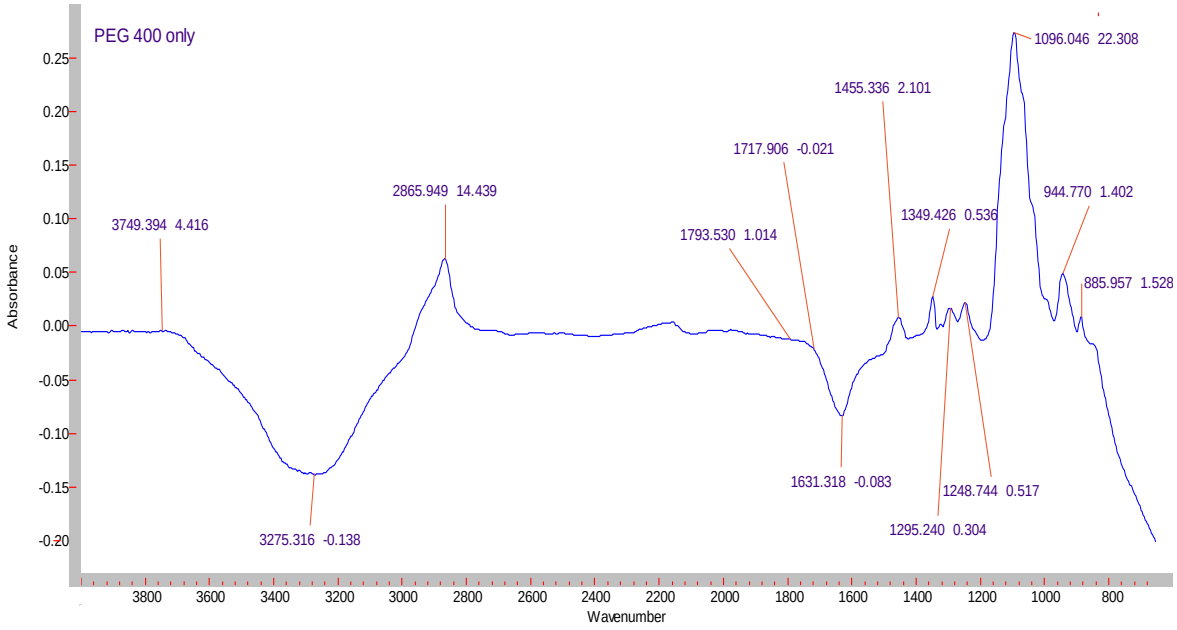


Figure 4.5 FTIR total spectra of PEG 400 (4000-800 cm^{-1})

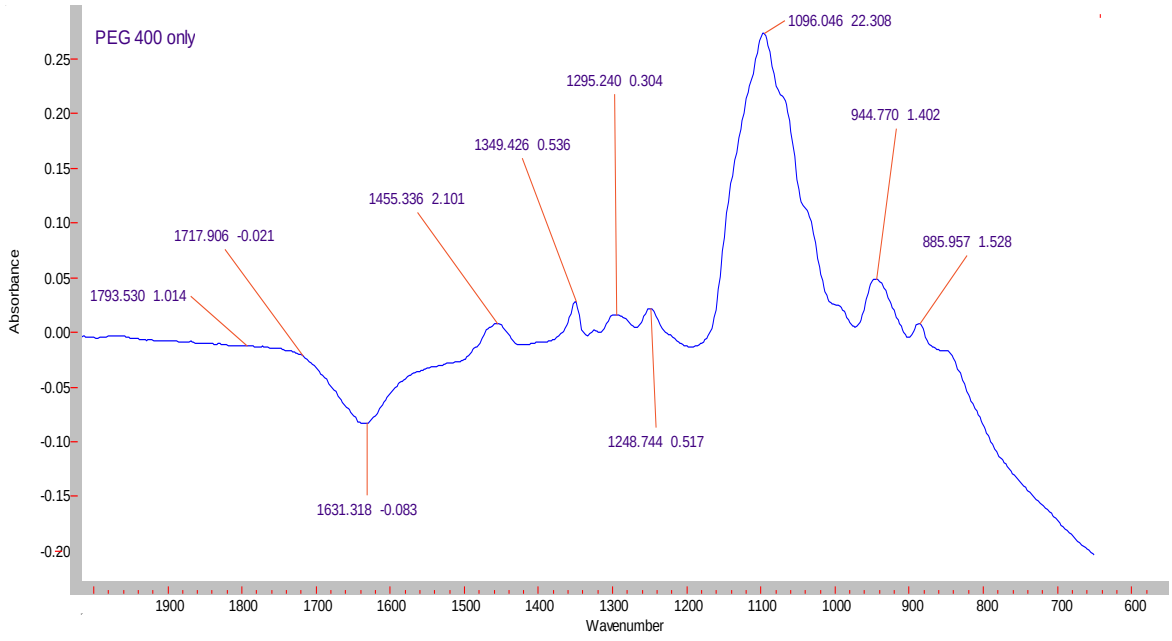


Figure 4.6 FTIR finger print of pure PEG 400 (2000-800 cm^{-1})

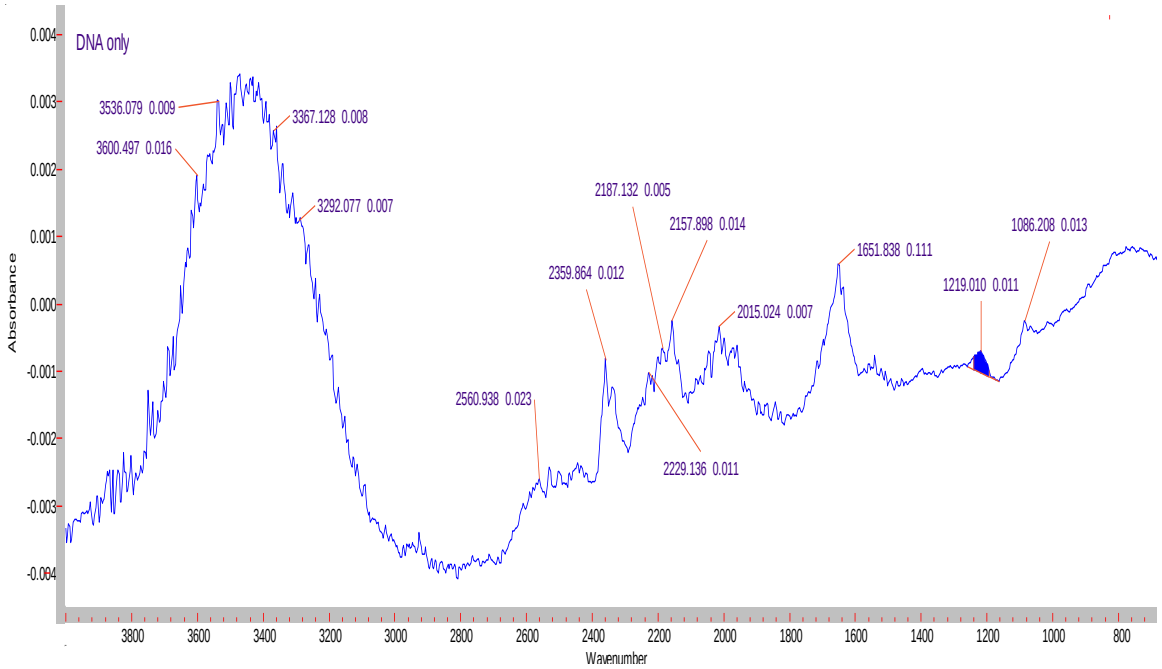


Figure 4.7 FTIR total spectra of ctDNA, (4000-800 cm^{-1})

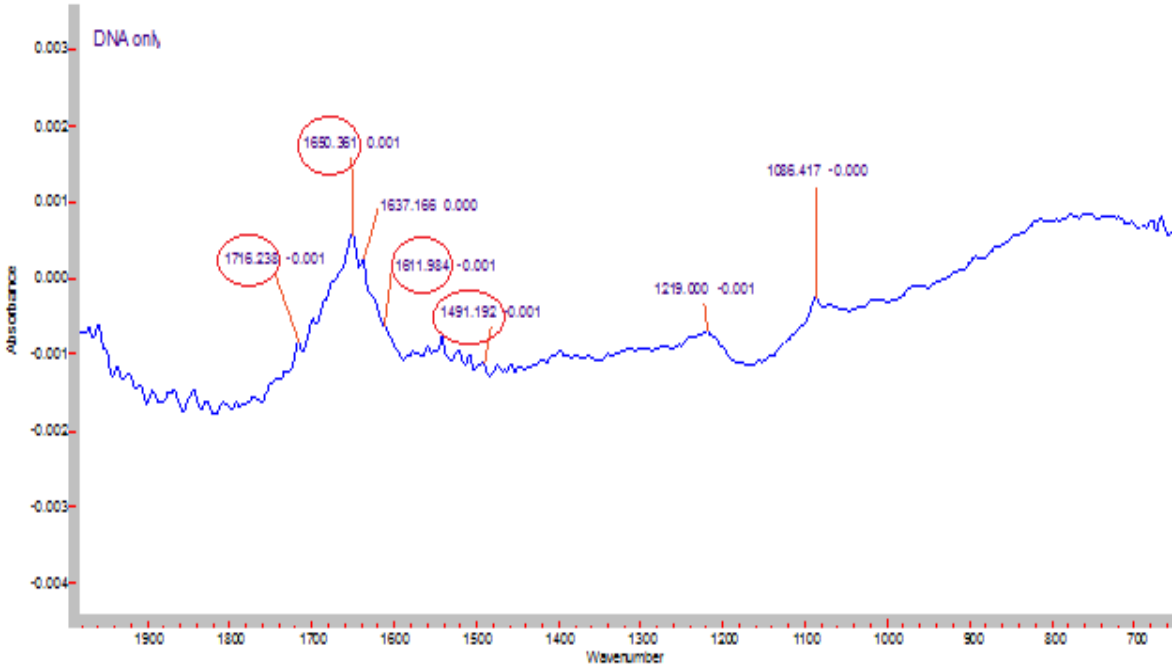


Figure 4.8 FTIR finger print of pure ctDNA (800-2100 cm^{-1}).

4.3.2 Effect of Incubation Time on FTIR spectra of PEG-ctDNA

To determine the effect of incubation time on the chemical binding between PEG and ctDNA, FTIR Background analysis for pure PEG 400, and pure ctDNA were previously collected. Spectral analysis of PEG-ctDNA complex samples were also obtained at zero time, 1hour, and after 48 hours (fig. 4.9, 4.10, 4.11, 4.12, 4.13, 4.14) at 1:1 ratio in buffer pH 7.4. As can been seen several new absorption peaks have been seen at (2878, 2914 cm^{-1}) after zero time, and (2879, 2915 cm^{-1}) after 1-hour, and (2879, 2929 cm^{-1}) after 48 hours. These peaks corresponding to some of the characteristic peaks of PEG 400 because these peaks were not present in the pure PEG sample, and the only difference between is the addition of PEG to ctDNA, which attributed to the effect of incubation time on the chemical binding of PEG to the ctDNA.

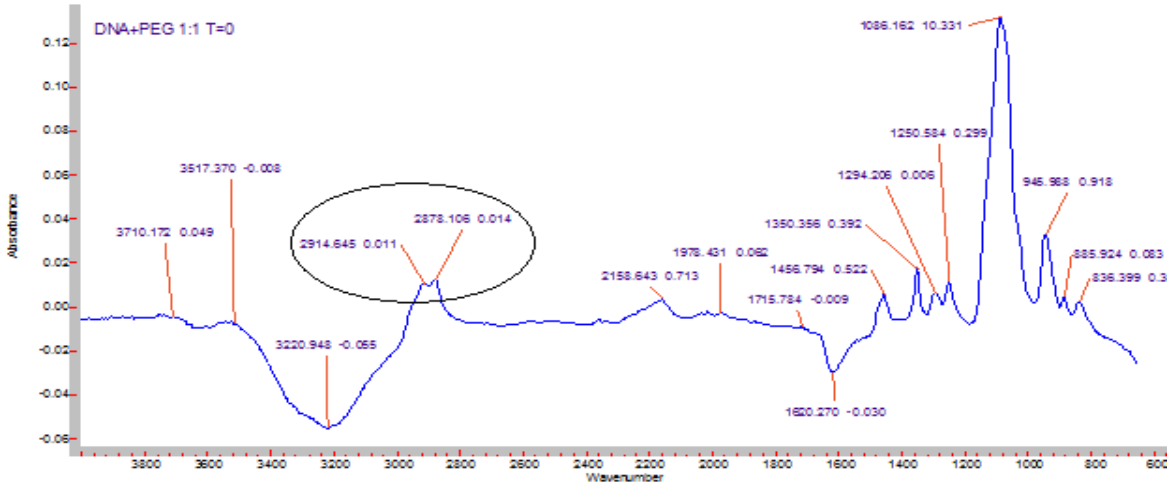


Figure 4.9 FTIR total spectra of PEG 400 AND ctDNA at zero time, (800-4000 cm^{-1})

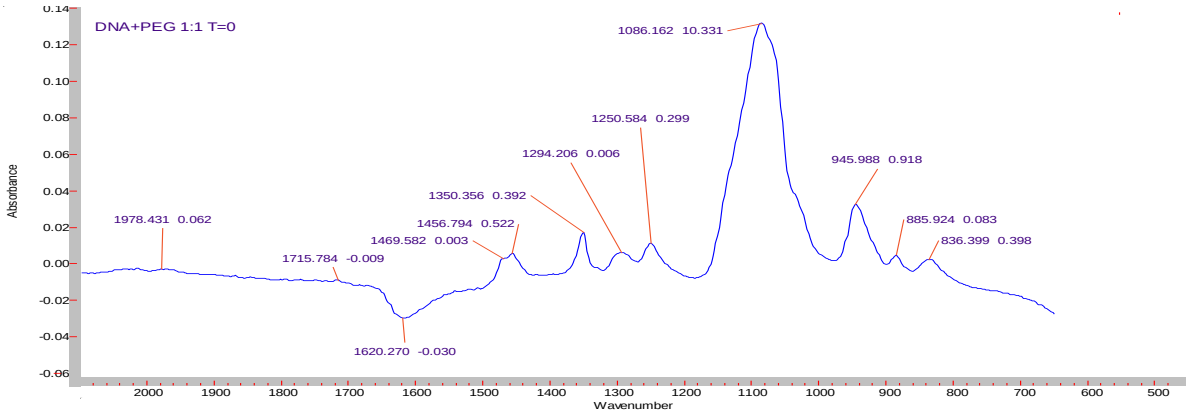


Figure 4.10 FTIR finger print of PEG 400 and ctDNA at zero time. (800-2100 cm^{-1})

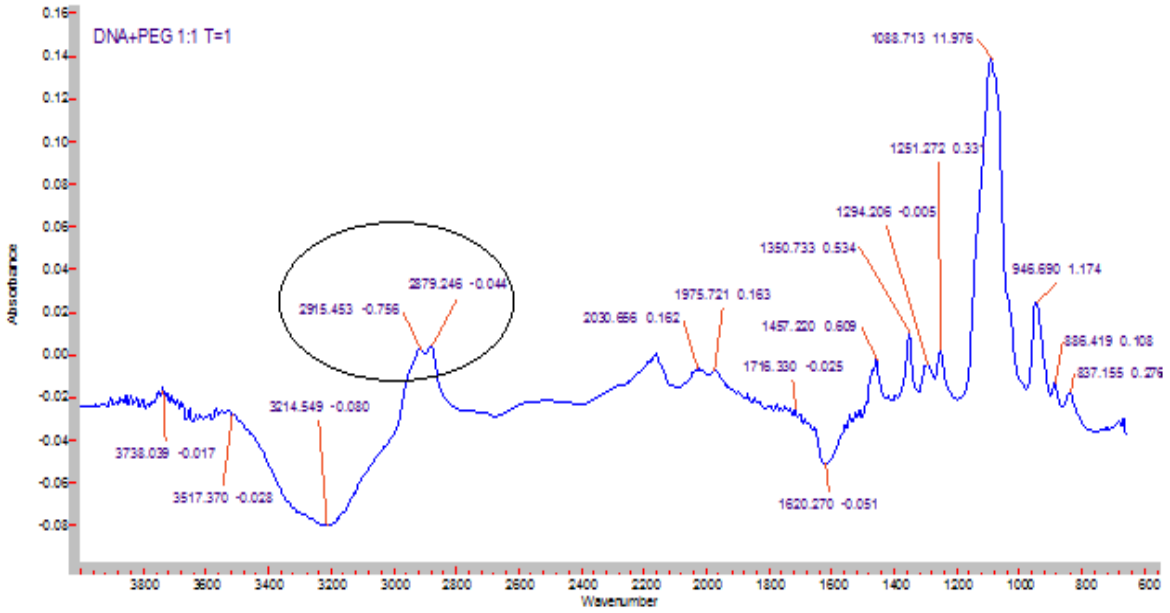


Figure 4.11 FTIR total spectra of PEG 400 AND ctDNA after 1 hour, (800-4000 cm^{-1})

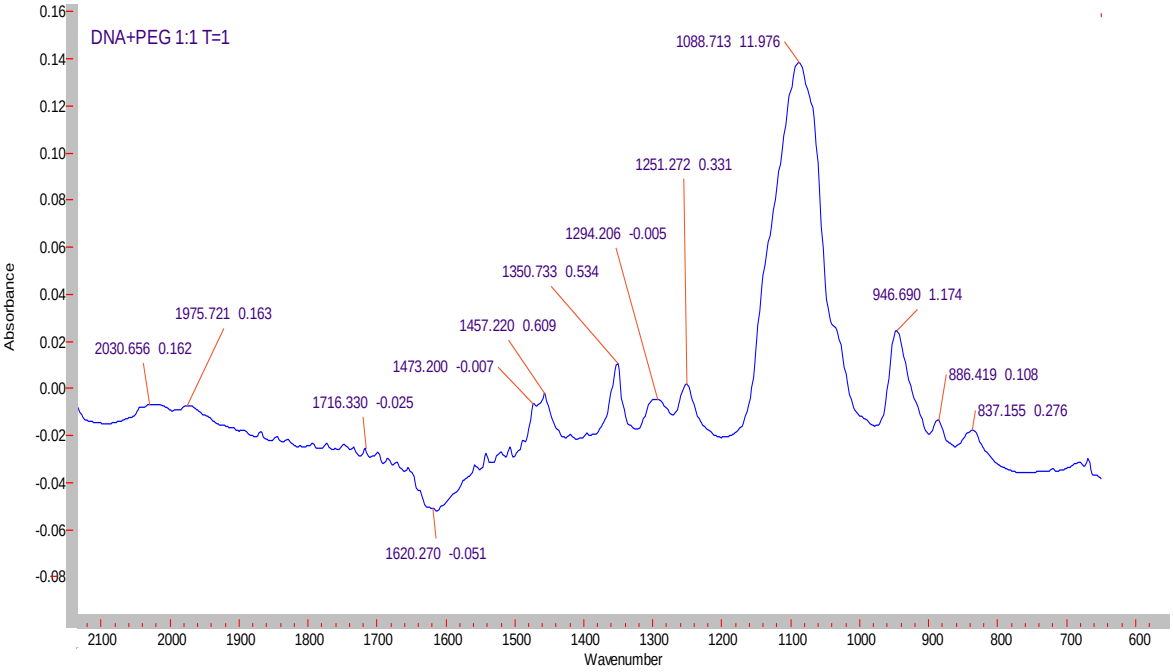


Figure 4.12 FTIR finger print (800-2100 cm^{-1}) of PEG 400 and ctDNA after 1 hour.

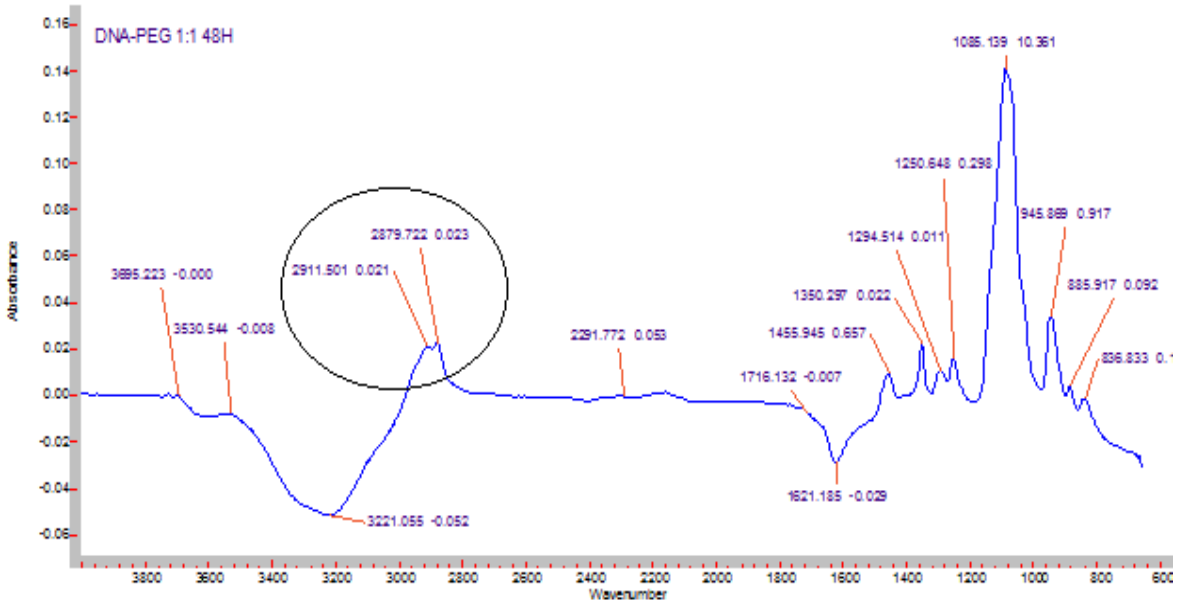


Figure 4.13 FTIR total spectra of PEG 400 AND ctDNA after 48 hours, (800-4000 cm^{-1})

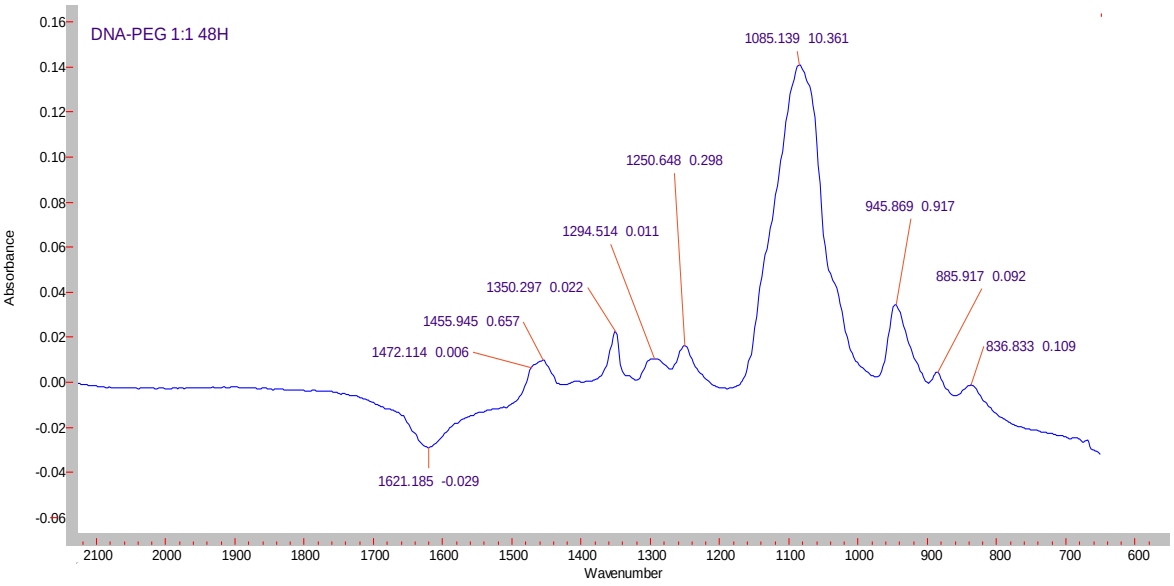


Figure 4.14 FTIR finger print (800-2100 cm^{-1}) of PEG 400 and ctDNA after 48 hours.

4.3.3 Determination the Binding Sites of PEG with ctDNA

Interaction of PEG with phosphate backbone of ctDNA was evident from an increase in the absorption and shifting of the PO_2 antisymmetric band at 1219 cm^{-1} in the spectra of the PEG-ctDNA complexes. The PO2 band at 1219 cm^{-1} after PEG was added shifted toward higher

frequencies after zero time (1250 cm^{-1}), and to (1251 cm^{-1}) after 1hour, and return to (1950 cm^{-1}) after 48 hours. While the band at 1086 cm^{-1} related to the phosphate symmetric stretching vibration was exhibited a minor absorption changes and shifting to a lower frequency at 1085 after 48 hours, higher frequency at 1088 after 1 hour, and with no shifting changes after zero time upon PEG-DNA complexation. Some evidences for PEG with base binding was observed from the spectral changes for free ctDNA upon PEG complexation. After zero time a minor shifting of the bands at 1716 cm^{1}(guanine) toward higher wave number 1717 cm^{-1} , and to 1699 cm^{-1} after 1 hour, and to 1786 cm^{-1} after 48 hours. For cytosine at 1491 cm^{-1}, it has been seen that there is high band shift toward lower wave number after zero time (1456), after 1 hour (1457), and after 48 hours 1455. Over all these changes could be attributed to direct PEG binding to the base pairs of the ctDNA. To determine the presence of hydrophobic contact in the PEG-ctDNA complexes, the spectral changes of the polymer antisymmetric and symmetric CH2 stretching vibrations, in the region of 3000 cm^{-1}, 2800 cm^{-1} were examined by infrared spectroscopy. The CH2 bands of the free PEG located at 2865 cm^{-1} shifted to higher wave number at 2879 cm^{-1} after ctDNA was added at zero time, 1 hour, and 48 hours. Also there is a new peak has been seen at 2914 cm^{-1} formed after zero time, and 2915 cm^{-1} after 1 hour, and 2929 cm^{-1} after 48 hour. Shifting of the PEG antisymmetric and symmetric CH2 stretching vibrations in the infrared spectra the PEG-ctDNA complexes suggests the presence of hydrophobic interactions via polymer aliphatic chain and hydrophobic region in DNA.

4.4 TEM Characterization

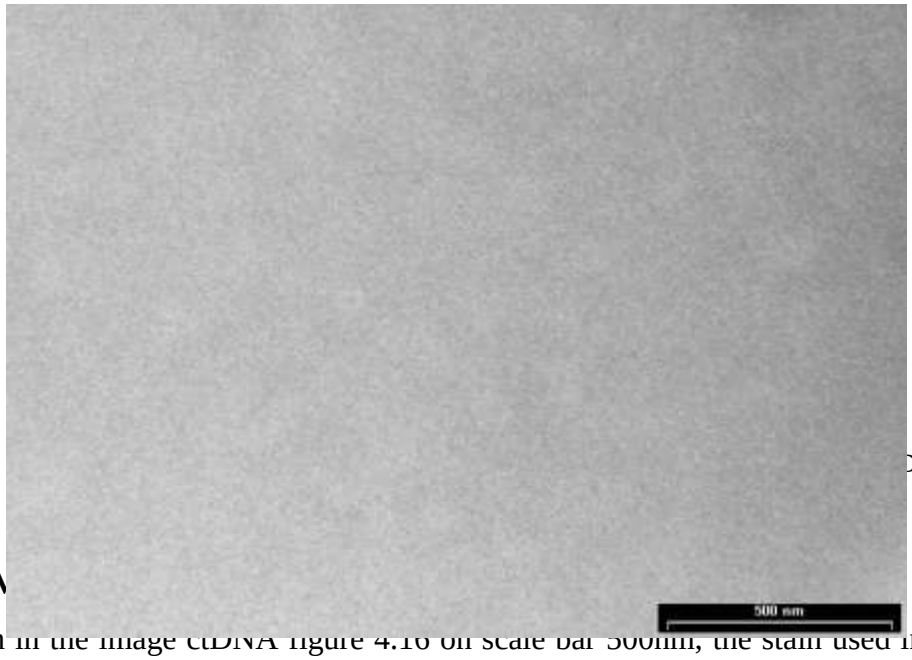
TEM is able to routinely resolve details finer than 0.5 nm and capable of operating at magnifications considerably higher than the 60,000 times. These magnification and resolving power allow the visualization of smallest biological structures, such as viruses. TEM utilizes energetic electrons to provide morphologic, compositional and crystal graphic information on samples. At a maximum potential magnification of one nano- meter, TEMs are the most powerful microscopes and produce high resolution and two dimensional images which allow for a wide range of educational, science and industry applications. TEM gives the local changes and anisotropy of a sample visually. The TEM image contains much information on the morphology and structure of a sample. To some extent the analysis of the image could be separated into distinguished contrast and low distinguished contrast. Although, microscopy as

a visual method is generally qualitative rather than quantitative analysis, a large number of dimensional details can be manipulated and analyzed. TEM can be categorized into different classes according to the contrast scheme employed; the contrast best suited to TEM is Zernike phase contrast and differential interference contrast, since electron scattering by objects exerts only change in phase and not intensity of electron waves in general. This may be the fundamental cause of image contrast; always a major issue in TEM [158]. Grids are used to hold and support samples of DNA-polymer, copper grids are preferred for a such use because they dissipate heat and electrostatic charges resulting from exposure to the electron beam. However, copper grids react with some reagents and certain buffer components. Therefore, in order to minimize unwanted reactions, provide sufficient support to the studied samples, obtain artifact free and gain images of sufficient contrast and resolution, support films with high transparency to electrons should be used [159-161]. Fragile and very thin samples are unstable under the electron beam without such support films. For example, the examination of specimens such as cell fragments, bacteria and glycol methacrylate usually requires the use of support film. A film material should have a relatively low atomic number and density and should be very thin (10-20nm) to gain contrast and resolution, and to avoid attenuation of the intensity of important structural details in the image [161,162]. In addition, the application of selective staining is necessary to increase the local electron scattering power of the specimen sufficiently so that an appreciable increase in image contrast will result. Its density should be higher than that of the embedding medium. Although, there is no electron staining reaction that is absolutely specific for biological polymer, Uranyl salts stain nucleic acids by ionic association with phosphate group [163]. The aims of using TEM were to elucidate the morphological structure and characteristics PEG and ctDNA. Moreover, it is expected to study the biocomplex surface structure of PEG: ctDNA and verify its structure from individual polymer and DNA itself. The protocols of preparation TEM samples has been explained in the chapter three, which allowing to produce the current chapter result and reproduce them also.

4.4.1 TEM Characterization of Grid Control Morphology

TEM images of PEG, ctDNA, and PEG-ctDNA samples were obtained after air drying on cooper grid. As shown in figure 4.15 an image of a TEM copper grid, the images were taken at a high magnification of about 60 000x times. This images was taken to make the needed

comparison between the control (cooper grid), ctDNA individual molecules,, PEG molecule figure 3.3, and complex morphology of ctDNA-PEG.



DNA.

4.4.2 TEM

As shown in the image ctDNA figure 4.16 on scale bar 500nm, the stain used in this image was uranyl acetate, which resulted in positively stained regions in the grid. This data reveals a diverse population of morphologies of DNA morphology, including open loops, toroid, and other relaxed shapes. The DNA molecules ware appears as dark objects on a lighter background. The visualization of DNA in the current study in accordance with many other studies in published literature [143,143], that appropriate magnifications were essential and electron scattering with endogenous atoms of biomolecules in the entire specimen, moreover, electron scattering could be due to exogenously induced by heavy metals atoms. Finally the image provided obvious relaxed super coiled structure of DNA morphology.

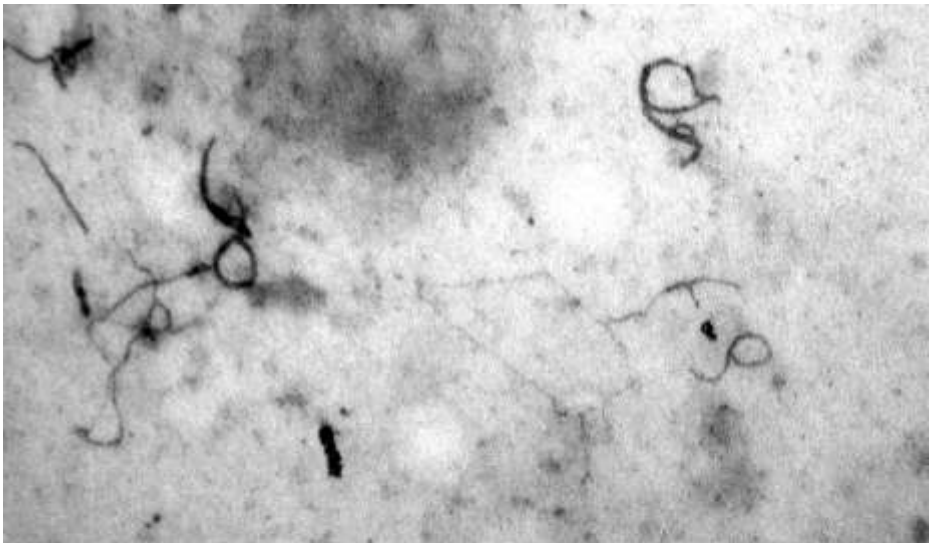
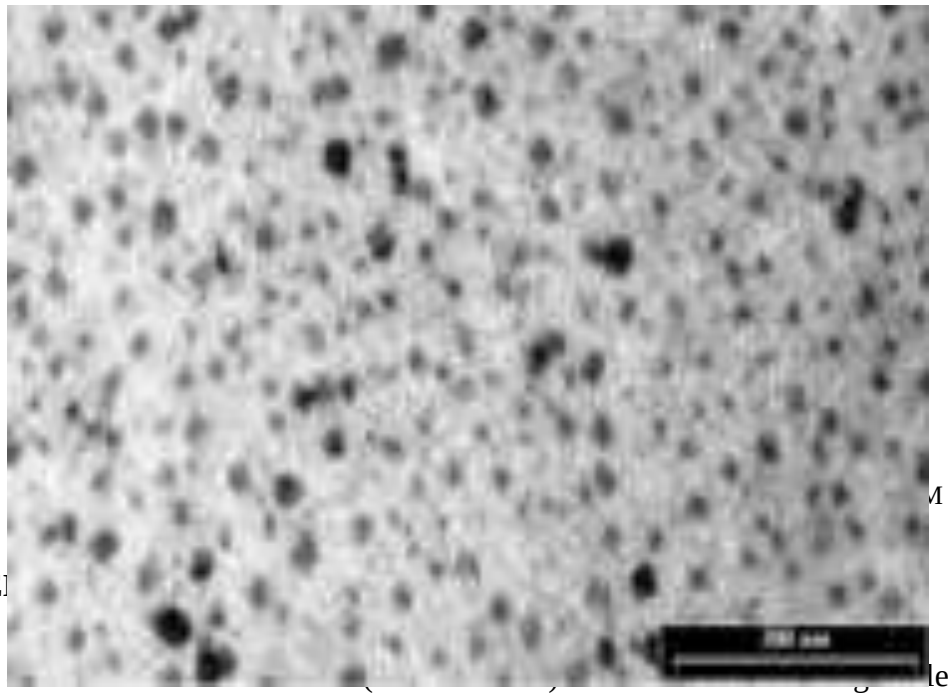


Figure 4.16 TEM image for ctDNA stained with urinyl acetate.
Scale bar 500 nm Mag. 80 000x

4.4.3 TEM Characterization of PEG 400

Image of PEG polymer stained with a uranyl acetate stain seen in figure 4.17 a large number of black globular apparent individual PEG molecules are observed; also small islands of aggregated materials are also seen. The particles were distributed all over the field of the grid. A globular structure was the predominant morphology. We note also that PEG 400 was observed to form globule structures under the pH 7.4 environmental medium and TEM grid preparation procedure. The morphology of polymer were occurred in high reproducibility, this could be the physical structure of polymer is absolutely thin, therefore, the penetration depth of electrons are greater than the layer thickness.



4.4.4 TE *M*

In figure ( ) le structures under the buffer solution, while in figure 4.16 the image showed diverse structural population of

morphologies of DNA. The addition of PEG 400 to a sample of ctDNA at physiological environment pH 7.4 with 1:1 ratio Figure 4.18 has been analyzed microscopically using TEM; it was shown that the addition of PEG causes conformational change of ctDNA and express different morphological structure such as toroidal and rod-like particles) with PEG particles in irregular aggregate structure, whereas individually appeared relaxed structures. The relative % populations of ctDNA morphology complexed with PEG could not be measured under the PEG-induced complexation conditions. Therefore the analysis of the formed complexes by TEM resulted in morphology with no significant complex structures observed. Generally, the TEM results suggest that presence of the salt content of PBS (10 mM) (pH 7.4) containing 147 mM NaCl, results in shielding of the charge of anionic DNA and may alter to some extent the charge of PEG, therefore reducing or preventing complex formation. Similar observations have been reported showing that when NaCl concentration increases in PEG / DNA complex the binding is not completed in case of PEG: DNA complexes as may been seen in other cationic polymers with DNA even the PEG is a neutral charge polymer physically, this may explain why we observed such featureless images as seen even the formation of complex is observed clearly.

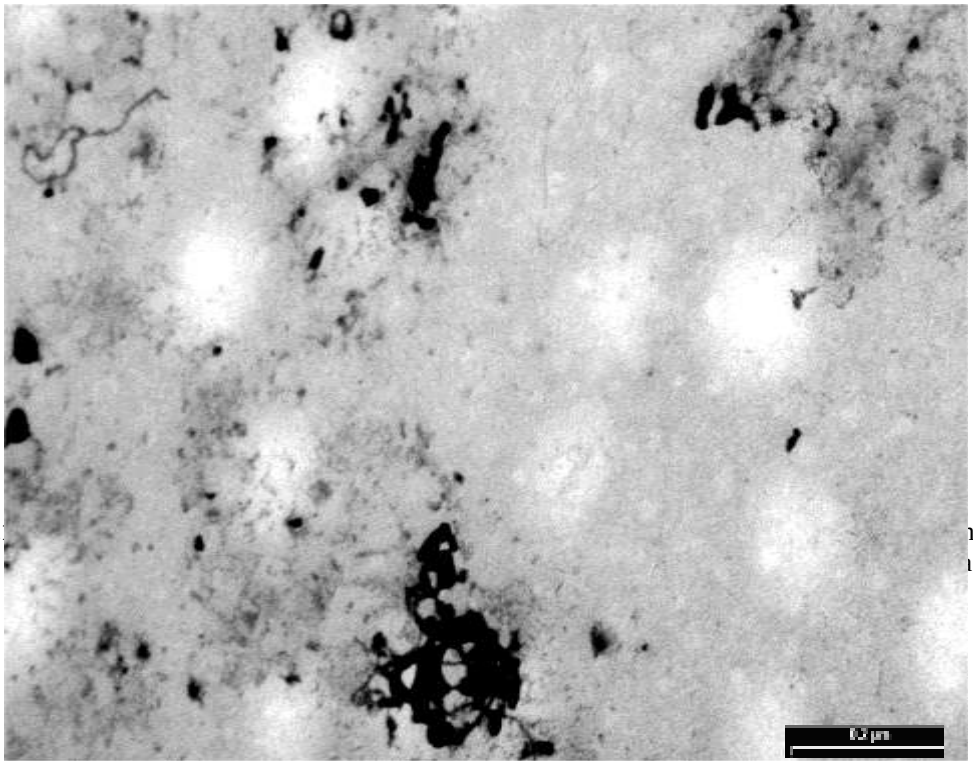


Figure 4. n 100 PBS. The ıge

CHAPTER 5 CONCLUSIONS AND FUTURE PRESPECTIVES

5.0 Conclusion

In the current study the behavior of PEG interaction with ctDNA has been investigated and characterized with different methods (Uv-visible, FTIR, and TEM) as a function of several parameters such as ratios, pH, incubation time, and stability of ctDNA in presence and absence of PEG (T_m). The UV visible spectral analysis was shown that PEG-ctDNA at ratio1:1 (20µl) has absorption 0.472 at wavelength 285 nm. While the continuous increasing or decrease on PEG ratio, hardly showing any significant changes. On the second experiment, the effect of pH medium on PEG-ctDNA interaction was also tested, the results shown red shift (bathochromic) in acidic medium. While in alkaline medium shown blue shift (Hypsochromic). The DNA denaturation and binding of PEG with DNA base pairs and stability of the complex formed has been also characterized through spectrophotometric to monitoring changes in the absorbance (red or blue shifts of spectrum) and the melting temperature (Tm) of DNA in the presence and absence of PEG. the results showed an increase in the DNA-PEG melting temperature. FTIR spectroscopy was used in current study as well, to probe the chemical bonds and their crowding environment in molecular systems. The binding characteristics and molecular mechanism of the interaction between PEG and ctDNA at various incubation times was studied. The fingerprint and total spectral analysis were obtained. The experiment shown the significant effect of incubation time in preparation biocomplex between PEG and ctDNA, and the structural analysis results was indicate that PEG binds with DNA, by weak to moderate complex formation with both hydrophilic and hydrophobic contacts through DNA base pair. In order to verify the morphology of complex formation and the interaction between ctDNA and PEG, samples of PEG and ctDNA, at physiological environment pH 7.4 with 1:1 ratio, have been analyzed microscopically using TEM. The images shown that the addition of PEG causes conformational change of ctDNA and express different morphological structure with PEG particles, in

irregular aggregate structure, whereas individually appeared relaxed structures. Based on these results, following conclusions have been drawn:

1. Increase or decrease of PEG ratio *versus* constant ctDNA has insignificant effect for optimizing the interaction, and complex formation.

2. pH-medium has a significant effect on the PEG-ctDNA interaction,

3. The UV VIS. absorption spectrum of the ctDNA showed that PEG is able to stabilize DNA helix structure.

4. The results showed that the binding of PEG with ctDNA proceeds rapidly at room temperature and complex formation vary by time after PEG and ctDNA are mixed.

5. The study shows the benefit of incubation time on the chemical binding of PEG to the ctDNA.

6. TEM shown that the addition of PEG to DNA causes condensation of ctDNA in irregular aggregate structure.

7. PEG 400 binds with ctDNA, with a weak to moderate binding force, and not via intercalative interactions. Perhaps, the binding is groove binding or electrostatic binding with both hydrophilic and hydrophobic contacts through the DNA base pairs, with a little binding preference towards phosphate backbone of DNA helix.

5.1 Future Prospective

Many new questions have arisen and paths to pursue discovered based on our findings of the research reported herein. The few of the key suggestions are outlined for the consideration of future researchers, they focus on gaining a better understanding of the DNA/PEG interaction and DNA complexation stability process by exploring the impact of the DNA length and sequence, PEG with different molecular weight, Increasing DNA ratio vs. PEG, and the influence of the preparation conditions. Ultimately, this work and expansion upon it may lead to a better understanding of DNA /PEG interaction which can be applied to

biotechnology applications such as gene delivery systems, antiviral agents and other applications. It may also help direct the development of better drugs based on the insight of polymer interactions with DNA.

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APPENDIX

Uv visible spectroscopy Scan Analysis Report

Report Time : Sat 18 Jun 01:31:24 PM 2011
Method: C:\ DNA calf thymus and PEG 400
Batch:
Software version: 3.10(339)
Operator: Ms.Amal+ Mr.Ali Bentaleb

Sample Name: Baseline 100%T

Collection Time	6/18/2011 11:31:30 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
216.000	1.624

Sample Name: Calf thymus DNA only (20uL)

Collection Time	6/18/2011 11:34:22 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.464
224.000	0.250

Sample Name: PEG 400 1UL

Collection Time	6/18/2011 11:39:03 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
223.000	0.028

Sample Name: PEG 400 6UL

Collection Time	6/18/2011 11:41:36 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
223.000	0.053

Sample Name: PEG 400 14UL

Collection Time	6/18/2011 11:44:39 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
223.000	0.137

Sample Name: PEG 400 100UL

Collection Time	6/18/2011 11:46:58 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
224.000	0.380

Sample Name: Calf thymus DNA (20uL)+PEG 400 (5UL)

Collection Time	6/18/2011 11:49:50 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.459
224.000	0.279

Sample Name: Calf thymus DNA (20uL)+PEG 400 (15UL)

Collection Time	6/18/2011 11:52:25 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.480
224.000	0.336

Sample Name: Calf thymus DNA (20uL)+PEG 400 (20UL)

Collection Time	6/18/2011 11:55:07 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs

258.000	0.472
224.000	0.365

Sample Name: Calf thymus DNA (20uL)+PEG 400 (30 UL)

Collection Time	6/18/2011 11:58:25 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.461
224.000	0.415

Sample Name: Calf thymus DNA (20uL)+PEG 400 (130 UL)

Collection Time	6/18/2011 12:01:03 AM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.458
225.000	0.550

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL)

Collection Time	6/18/2011 12:03:12 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
258.000	0.444
225.000	0.714

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL)at acidic media pH 3.03

Collection Time	6/18/2011 12:38:50 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
262.000	0.756
226.000	0.795

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL)at more acidic media pH

Collection Time	6/18/2011 12:41:47 PM
Peak Table	

Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
264.000	0.815
226.000	0.864

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL)at more acidic media pH1

Collection Time	6/18/2011 12:44:09 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
264.000	0.828
226.000	0.913

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL)at alkaline media pH

Collection Time	6/18/2011 1:01:26 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
256.000	1.041
230.000	1.008

Sample Name: Calf thymus DNA (20uL)+PEG 400 (230 UL) at alkaline media pH +**37.5 C**

Collection Time	6/18/2011 1:07:18 PM
Peak Table	
Peak Style	Peaks
Peak Threshold	0.01000
Range	800.000nm to 200.000nm

Wavelength (nm)	Abs
251.000	1.246