Synthesis and Surface Modification of Graphite Oxide-Cellulose Composites for Solid Phase DNA Extraction Applications

A Doctoral Dissertation by

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Abstract

The extraction of DNA is the most vital technique which is widely used in genetic engineering. It is the key process of the recovery, purification and industrial applications of DNA. Generally, silica matrices are the most utilized solid support material due to their unique property of selective DNA binding. The major advantage of silica matrices belongs to its capability to fix DNA on a solid support in a quick and convenient process. However, the adsorption force of DNA based Si-OH functional groups is much greater than that of desorption force, as a result, the DNA extraction efficiency of silica surfaces is limited.

Chapter 1 began with a general introduction of various DNA extraction methods, protocol types, fundamentals and applications. The purpose of this study is to invent a new material with suitable functional groups for high DNA extraction efficiency. Therefore, in this study, I have proposed Graphite oxide (GO)/Cellulose composite as an innovative solid based support for DNA extraction. Graphite oxide production and DNA characterization methods and devices are explained in Chapter 2. In chapter 3, my study focused on preparing GO/Cellulose composite and applying single strand DNA (ssDNA) on the surface. The concentration of GO within the composites were ranged from 0 – 6 wt. %. The highest binding capacity was achieved with 4.15 wt. % GO, where the extraction efficiency was reported as 660.4 ng/µl. The results were compared to commercial silica spin column and showed that the extraction efficiency was 50% higher than that of conventional silica column with similar DNA purity.

In order to examine the extraction efficiency of DNA in practical usage, genomic DNA samples were applied. In Chapter 4, the extraction of genomic DNA by GO/Cellulose composite was reported. Genomic DNA samples were obtained from forensic DNA sources such as cigarette bud paper, nail, chewing gum, animal tissue and human hair. The bulk % of

Graphite oxide was fixed at 4.15% in the composite where the extraction efficiencies of all samples were 4 to 12 times higher than that of commercial silica spin column. The purity of extracted DNA was also evaluated. Purity of DNA samples extracted from commercial silica spin column were between 1.5 - 8.37 value where samples extracted from GO/Cellulose composite were 1.6 - 2.0 value ranges which indicating that extracted DNA is more suitable for further applications.

On the other hands, magnetic particles have been developed for purification of genomic or plasmid DNA from different biological sources since it can provide decent amount of DNA extraction in direct application with rapid recovery. Hence, in chapter 5, GO/Cellulose/Magnetic composites were prepared. The mass % of Graphite oxide was keeped at 4.15% and concentration of magnetite inside the composite ranged between 0.2 – 3.98 wt. %. The extraction efficiency was 150 times higher than that of commercial magnetic beads, while the purity of DNA was ranged between 1.7-1.8 value ranges.

Finally, chapter 6 summarized the performance and extraction efficiency of GO/Cellulose composite on each type of DNA samples. The results demonstrated that it might be a promising and efficient solid phase material for DNA extraction applications. In this study, GO/Cellulose composite is one of the first researches in the field of applying carbon based material for DNA extraction. Compare to the most common DNA extraction material, silica films, GO/cellulose composite showed higher DNA binding capacity and extraction yield with a simple and environment friendly production. The results observed that (1) It was found that the binding capacity of DNA increased with increasing weight percentage of GO inside the composite, (2) GO/Cellulose composite showed high extraction efficiency not only for ssDNA, but also for Genomic DNA samples, and (3) GO/Cellulose/Magnetite composite exhibits superior extraction efficiency compared to that of commercial magnetic bead product.

List of Abbreviations and Symbols

CNF	Carbon Nano Fiber
CNT	Carbon Nano Tube
CTAB	Cetyltrimethylammonium bromide
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic Acid
dsDNA	Double strand DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
EtBr	Ethidium Bromide
FESEM	Field Emission Scanning Electron Microscopy
GC	Glassy Carbon
GuHCl	Guanidine Hydrochloride
GO	Graphite Oxide
MCC	Microcrystalline cellulose
MNP	Magnetic nanoparticles
MWCO	Molecular weight cut-off
PB	Phosphate Buffer
PCR	Polymerase Chain Reaction
PU	Polyurethane
RGO	Reduced Graphene Oxide
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy

ssDNA	Single strand DNA
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
UV-Vis	Ultra Violet-Visible spectroscopy
XPS	X-ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Background and Motivation of DNA Extraction

Purification of biomolecules is the most important method used in genetic engineering as it is the key of downstream processes, biotechnological applications, diagnostic developments, paternity determinations, and forensic science applications. Among all biomolecules, deoxyribonucleic acid (DNA) has a special property by existing in any living or preserved organism and carrying inheritance. DNA purification can be divided into two categories regarding to their lengths; (1) the purification of short DNA structure and (2) the purification of long DNA structure from various organisms. In general, four stages are required for the purification process: (1) distraction of tissue or the cell, (2) denaturation of nucleoprotein structures, (3) inactivation of enzymes like DNase and (4) washing the contamination free environment. At the end, DNA should obtain without other impurities. High quality and good integrity of DNA is very important for further experiments.[1, 2, 3, 4]

In 1869 Friedrich Miescher has successfully purified DNA from leucocytes cells. The intention of his research was to determine the chemical composition of leucocytes cell and to understand the fundamental principles of life, he hoped to understand the mechanism by purifying DNA molecule from the cell. He used lymph nodes as cell models to extract DNA. However, lymph nodes are difficult samples to purify. Furthermore, the amount of DNA was insufficient for further experiments. Thus, he changed his cell model and switch to leucocytes. At the beginning, he focused on the protein structures and could show the protein components inside the leucocytes and explained the structure of cell cytoplasm. Throughout his researches, he found a molecule that its solubility was changing according to pH value of the solution. Crude precipitate of the DNA molecule was observed at the very first time in this experimental section. After, Miescher has created a new protocol to separate and purify this

new molecule from protein molecules, cell's nuclei and cytoplasm. Although his first attempts was failed because of insufficient amount and low quality level of samples, his later attempts succeeded to develop a new protocol to collect bigger amounts of extracted nuclein. This nuclein molecule is called "nucleic acid" by Richard Altman in 1889.[5]

After Miescher's achievements, other researchers have started to study on better and advance DNA extraction protocols. The first DNA purification experiments were developed according to density gradient centrifugation strategy. Following these methods, solubility strategies has also been developed respectively for longer and shorter DNA structures in alkaline buffers. Currently, there are many purification methods to obtain high purity with high quality DNA from various sources. Briefly they can be divided them into liquid based and solid support based extraction protocols.[3]

1.2 Liquid based Extraction Materials

1.2.1 Alkaline Extraction Method

Alkaline extraction method is based on denaturation of bacterial cultures (1 to 500 mL) and purification of DNA by using alkaline solutions and sodium dodecyl sulfate (SDS). While alkaline solution denature the bacterial cell walls, protein molecules, heavy chromosomal DNA and circular DNA with covalently closed structure can stay intact inside the bacteria. The denatured molecules make large complexes with SDS. By using centrifugation, denatured material can be removed and DNA is recovered from the supernatant.[6, 7]

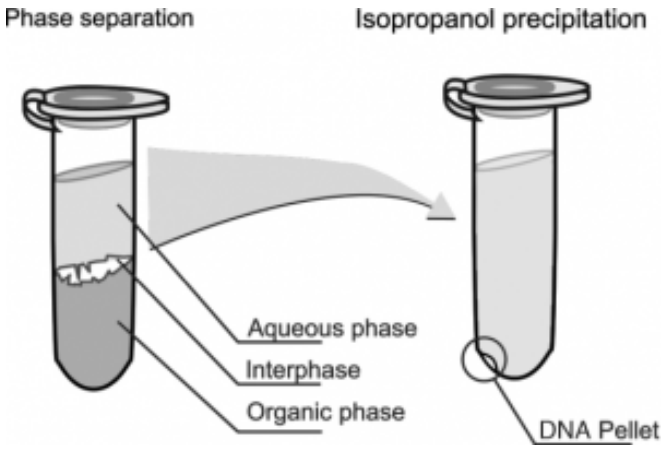

Figure 1.1- Phenol-Chloroform method is one of the first extraction method used at molecular biology[8]

1.2.2 Phenol-Chloroform Extraction

Phenol-chloroform extraction method, sometimes referred as organic extraction (Fig. 1) is one of the widely and longley used DNA purification method in molecular biology. Even though the required chemicals are flammable, corrosive and toxic, it is still the most common liquid based extraction due to its high DNA extraction capacity. Biomolecules like protein, lipid or carbohydrates, are washed away by the removal of the aqueous phase with the mixture of chloroform and phenol. Biphasic emulsion is created when chloroform and phenol were added to the solution. By centrifugation these two emulsion layer can be separated. Top layer of this emulsion is going to contain the DNA molecule inside and by adding high concentrations of salt and ethanol or isopropanol. DNA can be settled down from the top layer easily. After centrifugation DNA can be obtained and should be washed by 70% ethanol in order to remove the remaining salt from the DNA molecule. Then DNA molecules are dissolved and sterilized with TE buffer, AE buffer or water for further experiments.[9, 10, 11, 12]

1.2.3 Etidium Bromide – Caesium Chloride Gradient

Etidium Bromidee – Caesium Chloride (EtBr-CsCl) gradient is suitable for large scale bacterial culture DNA extraction. At first, the samples are precipitate by alcohol. In the next step DNA can be purified with EtBr-CsCl gradient by centrifuging and resuspension. In this method, (Fig. 2) CsCl disintegrate and Cs atoms creates different gradient inside the solution,

due to the strong centrifugation force and the DNA will migrate to the level as it has similar density same with its density. Based on the strong centrifugation force, protein, DNA and RNA can be successfully purified. After the extraction, butanol is used to eliminate the remaining Etidium Bromide, while dialysis is applied to extract the CsCl from the solution. It is regarded to be a difficult, expensive and labor intensive protocol compared to other purification methods.[1, 4, 13]

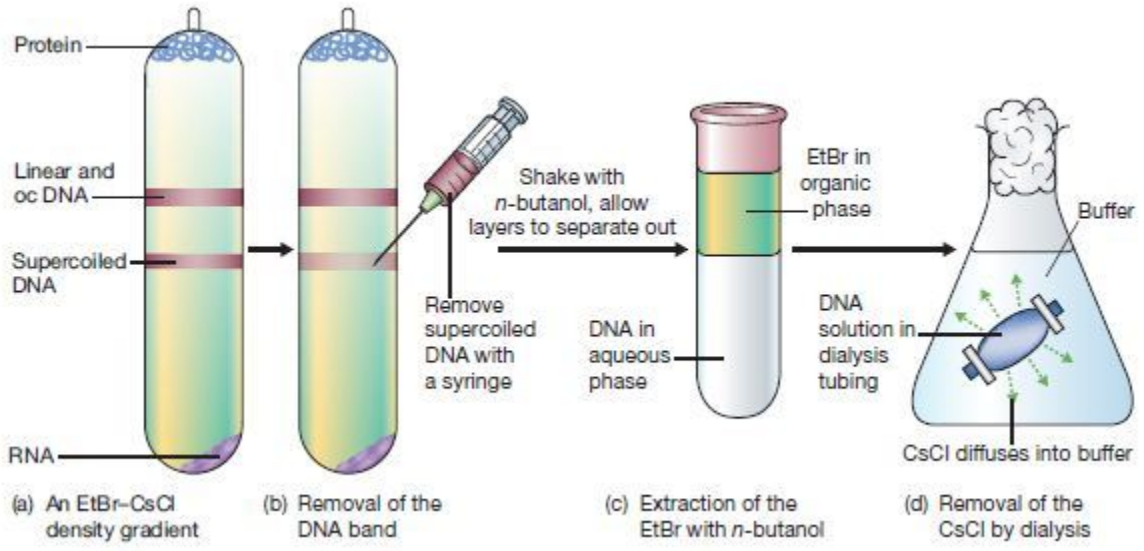

Figure 1.2- EtBr-CsCl extraction method is time consuming and labor intensive method.[14]

1.2.4 Cetyltrimethylammonium Bromide Extraction Method

Cetyltrimethylammonium bromide (CTAB) extraction method (Fig. 3) is widely used for samples which consist of a large amount of polysaccharides such as plant cells or Gram-negative bacteria. The principal of this method relies on CTAB's special ability to precipitate with DNA and acidic polysaccharides at various ionic solution concentrations. At low ionic solutions, CTAB precipitate with DNA and acidic polysaccharides where proteins and polysaccharides are free of CTAB free at the same environment. At high ionic solutions CTAB will not bind with DNA, instead, it creates protein-CTAB structures. In the later steps, organic solvents and alcohol based solutions are used for precipitation in order to remove

insoluble material through centrifugation. Other soluble materials and proteins can be separated by adding chloroform and applying centrifugation. After this step DNA can be purified from the supernatant and washed with water to eliminate contaminant salts and stored in Tris-EDTA solution or sterile distilled H_2O for further experiments.[15, 16]

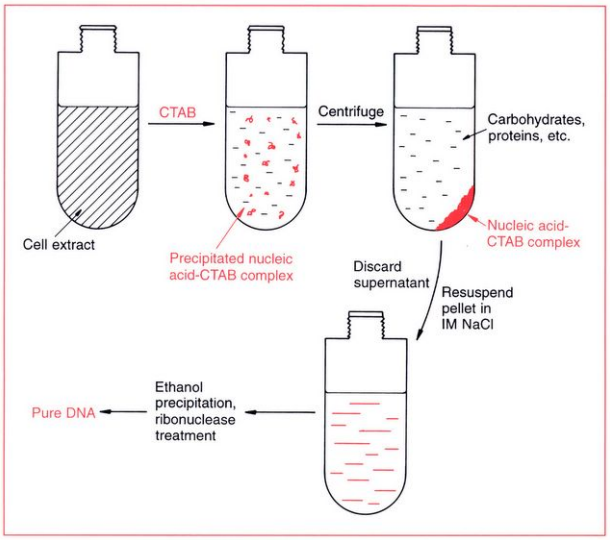

Figure 1.3- CTAB extraction methods give a great advantage if the DNA source sample consist plant cells inside.[17]

1.3 Solid Support Based Extraction Method

1.3.1 Glass Particles

Adsorption mechanism of glass particles is very similar to chromatography adsorption. DNA isolation can be conducted to silica glass, flint glass borosilicate glass or glass mixture. In these experiments, chaotropic salts are widely used to bind DNA molecules on common glasses and results showed that silica gel and glass particles are reliable materials for DNA purification under the presence of chaotropic salts.[13, 18, 19]

1.3.2 Diatomaceous Earth

Diatomaceous earth (Fig. 4) has been utilized for filtration and chromatography. Experiment results showed that DNA molecules can bind onto the particles through chaotropic salts and high silica content (94%). After binding DNA to the surface, other

impurities can be washing by ethanol. Finally DNA can be removed from the surface by applying of low ionic salt buffers or distilled water.[13, 20]

Figure 1.4- Diatomaceous earth consists a high amount of silica.[21]

1.3.3 Silica Matrices

The silica matrices are the most widely used solid support material in DNA extraction owing to their unique properties of selective DNA binding (Fig. 5). Silica matrices and various types of silica based materials can be prepared from different starting materials including glass fibers or filter papers. Extraction of DNA by silica matrices can be explained by the electronegative affinity between negatively charged phosphate groups on DNA and positive silica particles. Solutions or buffers with sodium ions attracting oxygen atoms inside the phosphate groups and create a cation bridge between DNA molecule and the silica. Under high salt conditions, sodium ions are removing the water molecules from the surface of silica by dissociating hydrogen bonds inside these molecules. As a result, DNA bonds tightly to the silica surface. The extensive washing by ethanol removes the impurities while DNA are safely fixed on the surface. The extracted DNA can be collected from the silica's surface by TE buffer, AE buffer or distilled water. [22, 23, 24, 25, 26, 27]

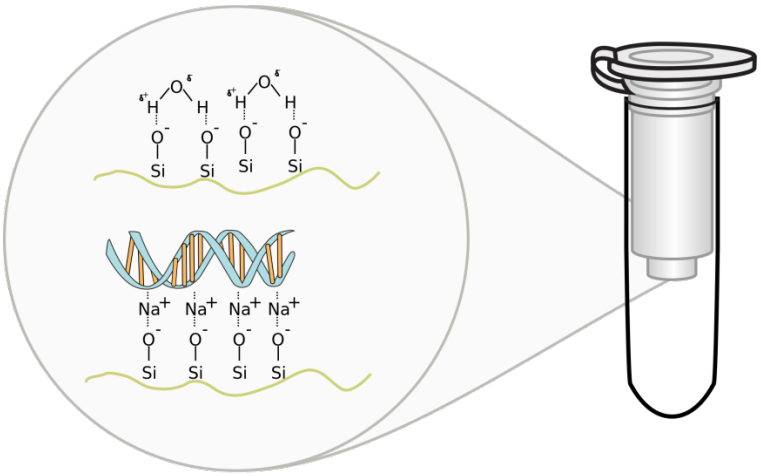

Figure 1.5- Silica spin columns are widely used in molecular biology applications. [28]

1.3.4 Anion Exchange Materials

The principal of this method is grounded on the interaction between phosphate backbone structure of DNA and the diethylaminoethyl cellulose (DEAE) groups. A large surface area, hydrophilic surface and high charge density is one of the important properties of these materials. Anion exchange materials can work with a various choice of pH conditions and salt concentrations that give them a great advantage for separating DNA from RNA, protein or other impurities. Anion exchange materials uses medium-salt buffers to remove the impurities from the surface. Then, DNA was extracted under high salt buffer solutions. Eventually, In order to bind and elute the DNA molecules ionic strength known solutions are required.[29, 30 13, 31]

1.3.5 Magnetic Bead

Magnetic beads are easy and efficient method to purify DNA molecules and there are many kits based commercial products available in the market. The kits consist suitable reagents to use with magnetic tools. The protocols have similarities with alkaline lysis procedures that followed with DNA attachment to a magnetic particle (Fig. 6). In order to catch DNA molecules and wash the impurities, a magnetic rod is using with a buffer provided. Generally, magnetic particles that are immobilized inside ligands or prepared with

biopolymers are showing higher affinity to target DNA. For a successful DNA extraction process, not only the magnetic properties, but the surface areas are equally important. Structures such as spherical beads are very favorable because of its large surface area and binding capacity. Furthermore, DNA binding capacity may be altered by “wrapping around” effect around the magnetic beads.

Another magnetic bead based methods is using zirconia beads as DNA extraction matrix. Zirconia beads have many advantages owing to its large surface area and good dispersibility inside the solutions. Furthermore their strong structure allows high physical distraction of samples which provide efficient DNA extraction inside the cells and inactivate the purification prohibitive enzymes. After lysis step, isopropanol was added to dilute the samples and zirconia beads in order to bind DNA to the beads. DNA-zirconia bead complexes are washed with ethanol for contaminants removal and finally DNA is extracted in low salt buffer.[32, 33, 34, 35 13]

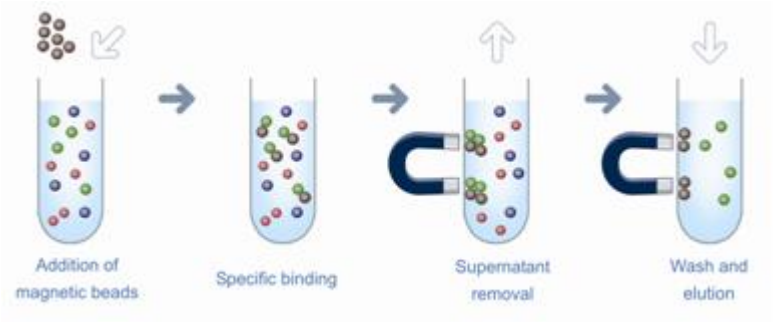

Figure 1.6 –Magnetic particles are rapid, an easy DNA extraction method with easy handling properties.[36]

1.4 Proposal for Innovative DNA Extraction Materials

1.4.1 Challenges of current DNA extraction solid support Materials

The main goal of DNA extraction is to obtain pure DNA molecule without any impurities in the final eluted solution. In order to separate DNA inside the nucleus compartment of the cell, cell lysis is necessary to disrupts the cell structure and inactivate the cellular nucleases like DNase or RNase. For this purpose, salt solutions with high

concentration are applied to bind DNA onto the solid support material, and followed by intensive washing by alcohol based solution to remove other biomolecules on the surface. Finally, DNA can be obtained under low ionic strength solutions. However, at this point the main problem arised after washing and elution steps, as the remaining salt molecules are bonded to the DNA molecule. These salt molecules often bind with other impurities such as protein particles or cell fragments on the surface. In order to continue to downstream process and further analysis, these impurities have to be detached from DNA and the solid support's surface. Additionally, elution removes only 90–95% at the maximum of the DNA from the solid support surface[2, 37]

1.4.2 Graphite oxide based materials

Carbon based materials with various functional groups like carboxylic acids, phenols, peroxides, aldehydes, alcohol etc. can bond organic materials on their surface. Other researchers show that DNA molecules can make strong and stable layer on various carbonaceous materials like glassy carbon, carbon nanotubes, graphene/graphene oxides or pyrolytic graphite. According to these results, carbon substrates are potential candidate substitute material for DNA extraction applications. On the other hand, functional groups on GO surface allows good dispersion and absorption inside polymer matrices and polymers.[38] Additionally, GO shows real molecular level dispersion and distinct layer distribution inside water. Unique properties such as strong structure, flexibility, high interlayer bond properties for filler and matrix structures, low capital costs have supported that GO might be a promising composite material of the future.[39, 40] Considering DNA extraction applications, GO sheets, and silica matrices have similar functional groups on the surface; similar binding mechanism is expected in the presence of chaotropic salts.[41]

1.5 Objective and Content of the thesis

In chapter 1, a general content of this study is to produce an innovative suitable solid

support for DNA extraction applications with a high binding capacity and high elution yield. Theoretically, GO might supreme to the current binding capacity of silica matrices. Cellulose, on the other hand, serves as a robust sustenance material of GO and enhances the overall surface area of GO in order to enhance the binding capacity between GO and DNA. GO, in particular, deliver additional pi stacking force between an aromatic region of GO and nucleobases.

In Chapter 2 Graphite oxide production and DNA characterization methods and devices are explained. Some of the widely used molecular biological applications like Polymerase Chain Reaction (PCR) and agarose gel electrophoresis methods also used in this study so the mechanism and procedure of these methods mentioned briefly. At the end of this chapter the main extraction mechanism of DNA from the conventional solid support surfaces are explained. Chapter 3 includes the preparation of GO/Cellulose beads. ssDNA samples are used to measure the extraction efficiency and the total yield of GO/Cellulose beads. The commercial silica matrix, QIAamp mini spin columns (QIAamp DNA mini kit), were applied as the reference material for the performance of DNA extraction at current state.

Chapter 4 GO/Cellulose beads used for various types of DNA sources used for DNA extraction applications. DNA sources choosed from extraction ability low samples like chewing gum, hair, cigarette bud paper or nails in order to show the value of GO/Cellulose beads for forensic science applications. Chapter 5 presented the modification of GO/Cellulose composite with magnetite particles for Na^+ free DNA extraction applications. Na^+ is causing one of the main problem for extraction systems. Na^+ free systems are required for reliable downstream processes. For comparison, commercial Dynabead magnetic beads and GO/Cellulose beads used to measure the performance.

Chapter 6 summarized the academic significance, general comparison of all surfaces with commercial products and suggesting new proposal for future works.

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Chapter 2

Experimental Setup

Chapter 2 Experimental Setup

2.1 Graphite Oxide Production

Graphite is the starting material to produce graphite oxide (GO). Main source for graphite is well known naturally be formed graphite flake. Until today two main -mechanical and chemical- exfoliation methods are using to prepare graphite oxide samples from graphite. Mechanical methods are mainly focus on to apply physical force in order to separate the graphite layer from each other. The Scotch tape method, sonicators and high temperature ovens are a good example of this method. On the other hand, chemical exfoliation methods are more focusing on intercalation of graphite layers by chemical derivatization, oxidation-reduction, thermal expansion, surfactants or combination of these methods.

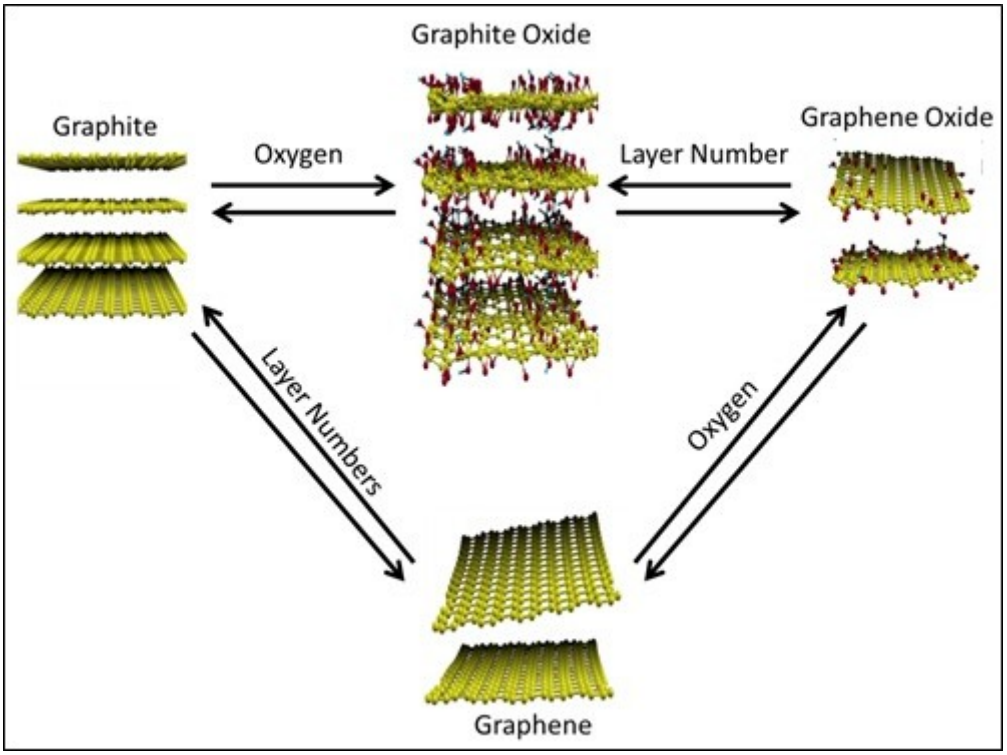

Figure 2.1- Structural diversity between graphite, graphite oxide, graphene oxide and graphene.[1]

The conventional chemical exfoliation method is to use strong oxidizing agents to prepare GO.[2, 3, 4, 5, 6]. Even though the determination of the graphite oxide structure is difficult

after exfoliation, it is very clear that layers are disturbed by various functional groups. This interruption can be observed by an increase of interlayer distance from 0.335 nm for graphite to 0.625 nm for graphite oxide.[7] Brodie introduced the first graphite oxide production by mixing nitric acid, graphite and potassium chlorate together in a same solution. After Brodie, Staudenmaier enhanced this method by mixing sulfuric acid with nitric acid and add them together with potassium chlorate inside graphite containing beaker. Sulfuric acid addition significantly increases the graphite oxide yield inside single reaction vessels.[8] Then Hummers reported the most used technique to produce graphite oxide from graphite. He oxidized graphite with $KMnO_4$ and $NaNO_3$ inside the concentrated H_2SO_4 solution. The biggest disadvantage of these methods is they are generating NO_2, N_2O_4, and/or ClO_2 toxic gases additionally later is become explosive.[9]

2.2 Graphite Oxide / Cellulose Production

Cellulose is the most abundant renewable polymer produced in the biosphere and it is the primary product of photosynthesis. Because of the un-moldable characteristic of cellulose it is very difficult to refabricate like other thermosetting or thermoplastic polymers. Therefore to find an effective and environmental friendly dissolution method for cellulose had a great important for further new materials as renewable and sustainable engineering polymers. At this point NaOH/urea aqueous solution systems are improved in order to dissolve the cellulose without any pretreatment and/or derivatization at low temperatures. This new method founded to be very simple, relatively safe and a low energy procedure. At this study to produce GO/Cellulose beads I used the same aqueous system by modifying with GO powder. Briefly GO powder and the water are mixed together and make a good dispersion. Secondly this GO dispersed solution is cooled down to 0°C and with a good stirring NaOH and urea added to this solution. After that Microcrystalline cellulose is added and stirring continued more ten minutes in order to obtain good homogenous solution. This solution kept at -20°C for 2 hour

and afterwards thaws at room temperature and excluded from a pipette tip in to a coagulation solution to obtain bead shape structure.

2.3 GO/Cellulose/Magnetite

GO/Cellulose/Magnetite composites are produced by adding magnetite powder in to NaOH-urea-GO water system. The same procedure replied and beads with magnetic properties are obtained.

2.4 DNA characterization

2.4.1 Single strand DNA (ssDNA) and double strand DNA (dsDNA) structure and properties

DNA is a particle which is carrying the inherent data that using for improvement, reproduction, functioning of all known alive organism and viruses. DNA is consisting nucleotides called base units. Each based unit is composed of deoxyribose called sugar, phosphate group and nitrogen containing four different (Adenine, Guanine, Cytosine and Thymine) nucleobase structure.

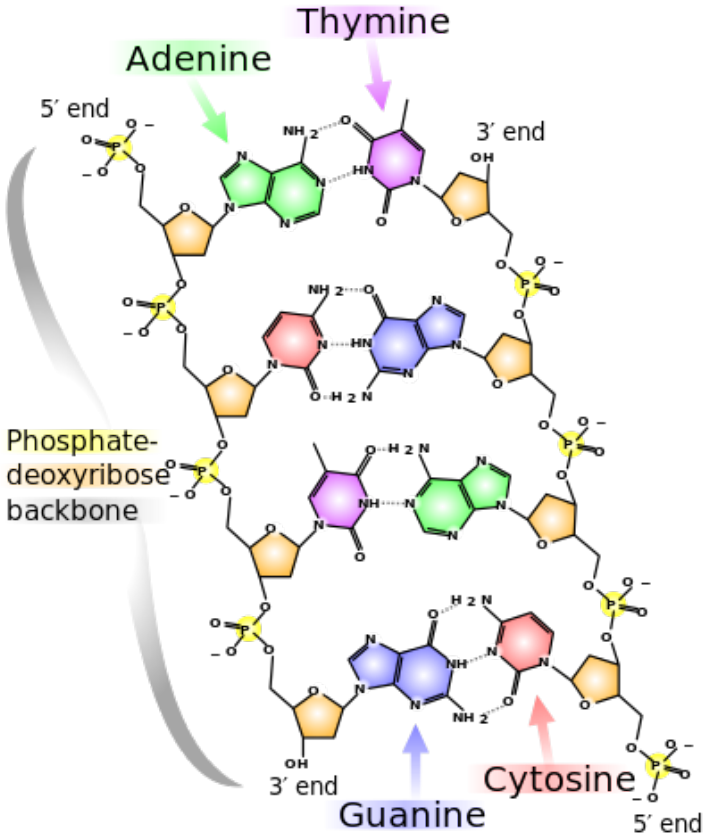

Figure 2.2- DNA molecule structure.[10]

DNA molecule is consisting, base, sugar and a phosphate groups inside double helix structure. Base molecule and sugar connecting each other and creating a nucleoside structure. This nucleoside can bind more than one phosphate group and named as nucleotide. These nucleotides also can be connecting each other with multiple molecules and making polynucleotide like a DNA structure. Covalent bond is occurring between sugar and phosphate groups in order to connect the nucleotide base units each other. Making a sugar-phosphate backbone structure and can build a vertical structure like a strand. These strand came across to each other and bound together in a helical shape with a non-covalently bonds to create dsDNA. On the other hand these strands can come apart from each other by heating, low salt and high pH conditions or produced and used as a ssDNA by the organism. In dsDNA structure each nucleobase on a one strand could match only with a one kind according to their physical structure properties by hydrogen bonding. This physical conformity is called base

pairing which is very important aspect for dsDNA durability. Purines and pyrimidines bind each other by using hydrogen bonds with a various bond numbers. For example, adenine bind to thymine with two hydrogens where guanine bind to cytosine with three hydrogen bonds. Eventually hydrogen bonds are not as strong as covalent bond so by applying force, temperature or pressure strands can break or assemble together more easily.[11, 12, 13, 14, 15, 16, 17, 18]

2.5 DNA quantification

2.5.1 Nanodrop and Qubit 2.0

Basically there are two methods to determine the average concentration of DNA inside the solution. One of the methods is measuring UV absorbance ratio of DNA solution and the other one is tagging DNA with fluorescent dye. Each method has its own advantages and their special devices to measure the concentration. For UV absorbance based method device which name is NanoDrop Spectrophotometer, using absorbance ratio of DNA molecule at 260 and 280 nm. A ratio of absorbance at these wavelengths also used to measure the purity of DNA molecule in order to understand the contamination level of the solution. A ratio of ~1.8 is generally accepted as “pure” for DNA. Similarly, absorbance at 230 nm is accepted as being the result of other contamination; therefore, the ratio of A260/A230 is frequently also calculated. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.[19, 20, 21, 22]

For fluorescent tagging method, specific dyes for DNA sample is using. Dyes are having very low emission till creating a dye-DNA complex molecule. After creating complex structure dyes get a rigid shape by intercalation between the bases and become highly fluorescent. The DNA concentration inside the solution can be calculated from the fluorescence dye’s emission signal. The Qubit fluorometer device can collect and calculate the signal strength and translate it into the DNA concentration by comparing DNA standards with

a knowing concentration..[23, 24]

Figure 2.3- Qubit 2.0 and the fluorescent dyes.[25]

Figure 2.4- Nano Drop Spectrometer device. [26]

2.6 DNA characterization

2.6.1 PCR and Gel electrophoresis

After DNA extraction protocols DNA amount could be insufficient or existence of

DNA could be doubtful. In this case there are two widely used methods to multiply DNA amount and proof the existence of DNA. To multiply DNA amount after extraction, DNA polymerase based Polymerase Chain Reaction (PCR) method is using. This method is founded by Kary Mullis in 1980. In this method heat resistant DNA enzymes are playing a key role for reaction mechanism. One of the most used enzyme for these reaction is Taq polymerase which originated from a bacteria named Thermus aquaticus. This enzyme has an ability to create a new DNA strand by using the building blocks and the ssDNA as a template. Throughout the process, DNA is subjected to repeated heating and cooling cycles during which important chemical reactions occur. PCR makes it possible to produce lots of copies of a DNA in a test tube in just a few hours, even with a very small initial amount of DNA.[27, 28, 29]

Figure 2.5- PCR device.[30]

On the other hand in order to separate mixed DNA population or show the existence of DNA inside gel matrix of agarose, Agarose Gel Electrophoresis method is using. This method is based on applying an electric field to move the charged molecules through an agarose matrix. As I know inside the DNA helix structure there are phosphate groups

responsible from the negativity. This situation is causing the movement of DNA molecule inside the electric field through the positively charge anode electrode. This movement inside agarose gel through electric field is named as "biased reptation". Because of phosphate backbone charge the edge of the molecule is move to anode and dragging the DNA The movement of DNA molecule inside the electric field applied agarose gel is depending on some properties of DNA sample like size or structure and the environment properties like agarose gel type, concentration or the buffer that gel is kept inside. Electric field and DNA dye also affecting the performance..[31, 32, 33, 34]

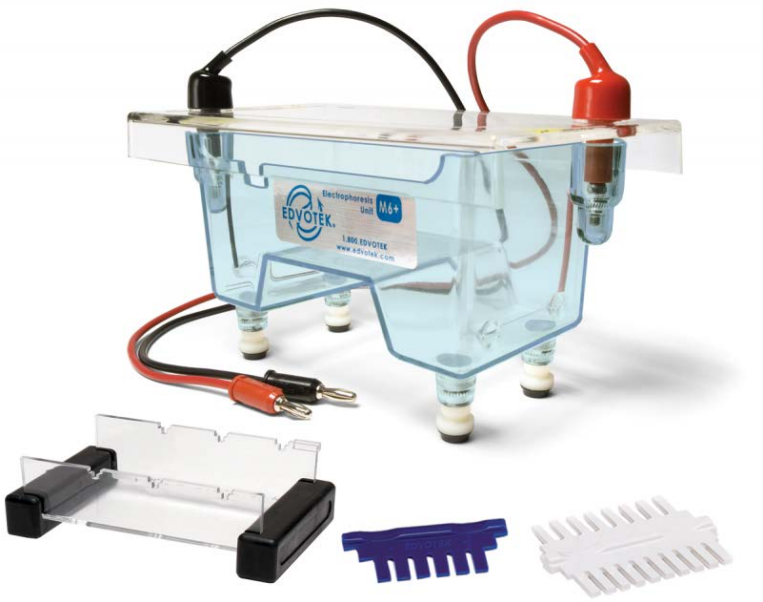

Figure 2.6- Gel electrophoresis experimental setup equipment.[35]

2.7 DNA Extraction Mechanism

Solid support DNA extraction is usually performed by silica spin columns that commercially available on market. As I mentioned previously there are methods and materials like glass particles, diatomaceous earth, anion-exchanges to purify DNA molecule. However using silica spin columns under the centrifugal force is the rapid and easy method compare to other materials.

There are four steps that applied in silica spin column extraction method. These steps are lysis, adsorption of DNA, removal of impurities, and desorption of DNA. At the first step lysis buffer apply to the sample in order to degrade the cell structure and exclude the DNA from nucleus compartment. Secondly, surface condition or the functional groups are converted to particular chemical form with a special solution at a specific pH. After that lysing solution applied and degraded sample will be applying to silica matrices. DNA is going to bind silica surface by of the binding buffer. Next, washing step will apply to remove all contaminants like proteins or salts from the silica surface. Last for the desorption step, AE buffer, distilled H2O can introduce surface in order to release the DNA and collect it. Generally, for desorption and extraction stages further applications are needed.[36, 37].

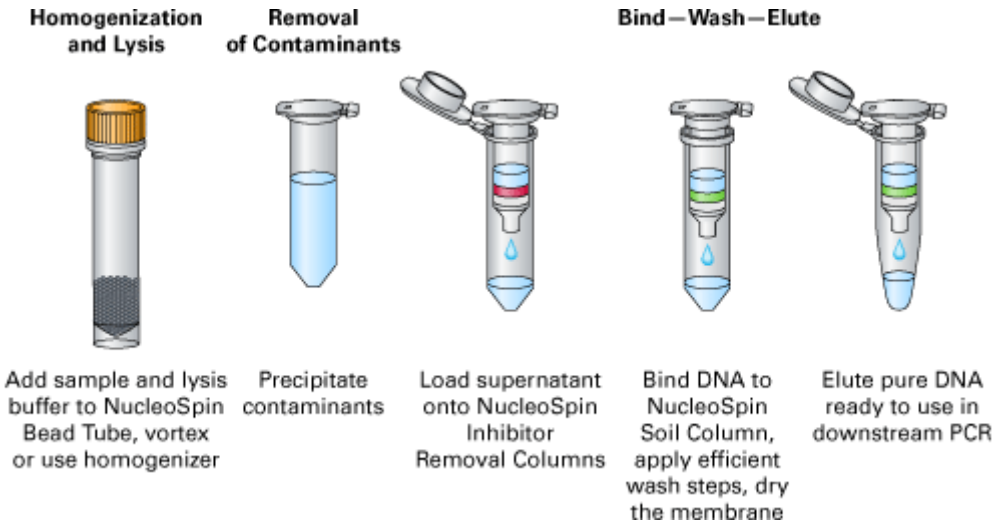

Figure 2.7- DNA extraction steps.[38]

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Chapter 3

Adsorption and Desorption of DNA Tuned by Hydroxyl Groups in Graphite oxide-based Solid Extraction Material

Chapter 3 Adsorption and Desorption of DNA Tuned by Hydroxyl Groups in Graphite oxide-based Solid Extraction Material

3.1 Introduction

The extraction of DNA is the vital technique used in genetic engineering.[1] It is an essential step at diagnostic kits. DNA can be isolated from any biological material such as living or conserved tissues, cells, virus particles, or other samples for analytical or preparative purposes.[2, 3, 4] Silica matrices, glass particles, diatomaceous earth, magnetic beads, anion-exchange carriers and modified polyurethane (PU) sponges are samples which are applied as solid support immobilization technique for DNA. [5, 6, 7, 8] Among these solid supports, silica matrices have been widely used due to their unique properties specific for selective DNA binding. DNA binds to silica through hydrogen-binding interaction under concentrated chaotrophic salt conditions.[9] Sodium has significant part providing cation-bridge that affects the negatively charged oxygen ions in silica under high salt conditions ($pH \leq 7$). When DNA is tightly bounded to the silica matrices, extensive washing by ethanol was followed in order to remove potential contaminants in the sample. Then, the extracted DNA molecules can be collected under low ionic environment ($pH \geq 7$) for DNA extraction by Tris-EDTA solution or distilled H2O.[10] For the binding of DNA, silica surface consists of three types of silanol groups on the surface: isolated, geminal, and vicinal. The surface also contains exposed siloxane bonds (Si-O-Si). The silanols are considered as strong adsorption sites with high polarization, high acidity and high reactivity among the hydroxyl groups.[11, 12, 13, 14, 15, 16] This is causing a strong adhesion effect on the silica surface and decreasing desorption performance of DNA. As a result, it limits the extraction yield during elution process. Generally, the DNA extraction efficiency is less than 95% for silica matrices.[17] In this

research, I have proposed an innovative material, graphite oxide (GO)/Cellulose composite, as an advanced solid support matrices for DNA extraction. DNA can be adsorbed on carbon-based materials through hydrogen-binding interaction under chaotrophic salt conditions.[18] Carbonaceous materials such as glassy carbon (GC), pyrolytic graphite, carbon composites, carbon inks, graphite pencil leads, carbon nanotubes (CNT) or graphene/graphene oxides has been applied as a stable DNA adsorbed layer.[19] GO, in particular, can provide alkyl and phenol C-OH functional groups to bind the nucleobases within DNA.[20, 21, 22] Theoretically, alkyl and phenol (C-OH) groups has lower polarization and reactivity compared to silanol (Si-OH) group. Thus, I expected GO has higher desorption of DNA compared to that of silica surface. In this chapter, I have successfully achieved high extraction yield of pure DNA by innovative cellulose/GO composites.

3.2 Experimental Procedures

3.2.1. Preparation of Graphite Oxide

Graphite Oxide (GO) was prepared from synthetic graphite flake (<20 μm, synthetic, Sigma-Aldrich (Japan)) by improved Marcano's method.[23] In the first step, 360 ml H_2SO_4 and 40 ml H_3PO_4 (Tokyo Chemical Industry, Japan) was added into a mixture of graphite flakes (3.0 g, 1 wt% equivalent) and KMnO (18.0 g, 6 wt equivalent ,Wako Chemicals, Japan). The mixture was heated to 50 °C and stirred for 12 hours. Then, the solution was cooled down to room temperature and poured into 400 mL of ice, mixed with 3mL of 30% H_2O_2 (Kanto Chemical Co. Inc, Japan) and purified by dialysis (Fisherbrand® dialysis tubing MWCO 6000-8000) for 48 hours. After purifying, the solution was filtered by 0.1 μm Omnipore™ membrane filter and dried at room temperature in order to obtain GO powder.

3.2.2. Preparation of Graphite Oxide Cellulose Composite

In this study, GO/Cellulose composites were manufactured by dissolving cellulose and

GO in NaOH–urea–water mixture, followed by coagulating the dissolved GO/Cellulose in nitric acid solution. [24, 25, 26] Adjusted amounts of GO powder (0.2, 1, 2.2 and 4.5 g) was dispersed into 90 mL ultrapure water and placed inside a sonicator (SND Co., Ltd. Japan, Model US-102, ultrasonic power of 100 W, frequency output of 38 kHz) for 15 mins, before cooled to 0 °C inside a refrigerator. In the next step, 6.0 g of NaOH and 4.0 g of urea were added into the frozen suspension, followed by the addition of 4.0 g of microcrystalline cellulose (MCC) (Sigma-Aldrich, Japan) powder. The mixture was gently stirred for 10 mins within an ice-bath and then cooled at the refrigerator to −20 °C for 2 hours. The mixture was stirred at room temperature for 30 mins and extruded through a 1 mL Eppendorf syringe into 10 mL 2M HNO_3. The coagulated composites were remained inside the acid overnight, and then rinsed by tap water for 30 mins followed by distilled water for 15 mins. The synthesized composite was stored in distilled water at room temperature. The total weight % of GO was calculated to be 0.19%, 0.95%, 2% and 4.15%. The resulted composite exhibited spherical shape, and each composite has an average diameter of 2.6 mm.

3.2.3 Characterization of GO/Cellulose Composites

The structural properties of GO were conducted by Raman spectra (excitation wavelength at 785 nm, NRS-3100 instrument, Jasco). The morphology of the GO/Cellulose composites was examined by Hitachi scanning electron microscopy at 5 kV (SEM) (S-4800, Japan). Prior to observation, samples were coated with platinum sputter at 20 °C with 20 mA current for 15 secs.

3.2.4 DNA Extraction by GO/Cellulose Composites

Three key steps were involved in solid-phase DNA extraction: binding/adsorption of DNA to the solid matrices, washing impurities such as proteins and excess salts, and elution /desorption of DNA from the solid matrices. The DNA solution with DNA size ranged from 587 to 831 base pairs (Sigma Company (D7290)) was applied as DNA specimen. A mixture of

saturated, 2M of guanidine hydrochloride GuHCl (Sigma-Aldrich, Japan) in 96% ethanol,[27] 2M NaCl (Sigma-Aldrich, Japan) solution and PB buffer (5M GuHCl in 30% propanol) were used as binding buffers in order to measure the binding capacity of GO/Cellulose composites. For DNA analysis, 200 µl saturated DNA solution (containing 20 µl DNA solutions and 180µl pure water) were mixed with 300 µl binding buffer. Five GO/Cellulose composites were inserted into a 1.5 ml eppendorf tube with a total volume of 500 µL DNA with binding buffer solution and incubated for 10 mins. The solution was carefully taken out by pipette and further washed with 70% ethanol in order to remove the remaining salts from the composite's surface. Then, 500 µL of elution buffer (AE buffer -10mM Tris-HCl, 0.5mM EDTA pH 9.0-) were added into the tubes and incubated for 5 mins. After incubation, the elution was separated from the composites. The quantity and quality of DNA in the elusion were assessed in terms of extraction efficiency and purity, respectively. The commercial silica matrix, QIAamp mini spin columns (QIAamp DNA mini kit), were applied as the reference material for the performance of DNA extraction at current state. The binding, centrifugation and elution processes were performed as described in the DNeasy blood and tissue handbook.[28]

3.2.5 Analysis of the yield and purity of extracted DNA

The purity of the extracted DNA was assessed by the optical intensity in elusion at two different wavelengths, 260 nm and 280 nm, by NanoDrop device. The absorbance ratio between 260 and 280 nm (also denoted as A260/280 nm) should be ranged in between 1.8 to 2.0 for high purity DNA.[29] In order to measure the total yield of DNA purification, last elution solution's volume multiplied with DNA conc. (ng/µL). On the other hand, by dividing the DNA total yield to input volume (total DNA amount [ng]/input DNA volume [µL]) extraction efficiency was calculated. All experiments were repeated three times and all data were replicable.

3.3 Results and Discussion

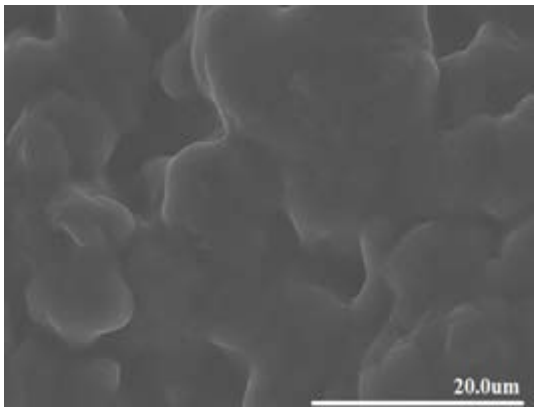
3.3.1 The morphology and composition of GO/Cellulose composites

The GO/Cellulose composites were presented as dark brown spherical structure, where the average diameter was measured with a digital caliper (SK digital calipers, BLD-150, Niigata Seiki Co.,LTD, Japan). The averaged diameter was 2.6 mm with a standard deviation of 0.1387 mm. The morphologies of GO/Cellulose composites were shown in Fig. 1 (a)-(b). The result indicated that the GO/Cellulose composites were relatively homogenous in terms of size. As a side note, the diameter of the composites can be easily controlled by the volume and the tip size of the eppendorf syringe. GO/Cellulose composites were kept in distilled water for long term storage in order to maintain extraction properties. The morphologies of GO/Cellulose composites with various GO wt. % were shown in Fig. 2 (a) - (e). As observed by the SEM images, the existence of GO on the surface was more obvious at higher GO wt. %. Homogeneous dispersion was observed on the surface of the composites in Fig 2(e).

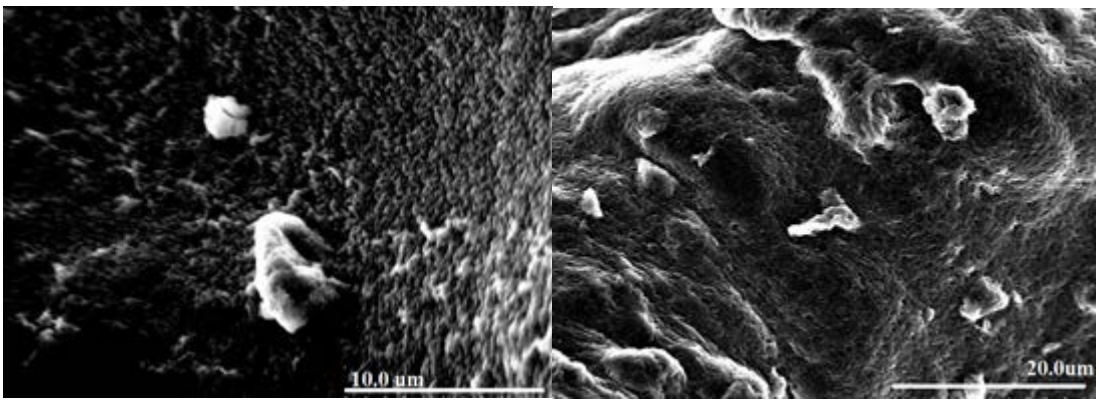
(a) (b)

Figure 3.1- Morphology of (a) graphite oxide /cellulose composite with 4.15 wt. % of GO with an average diameter of 2.6mm, and (b) multiple graphite oxide/ cellulose composites stored in ultra-pure water

(a)



(b) (c)



(d) (e)

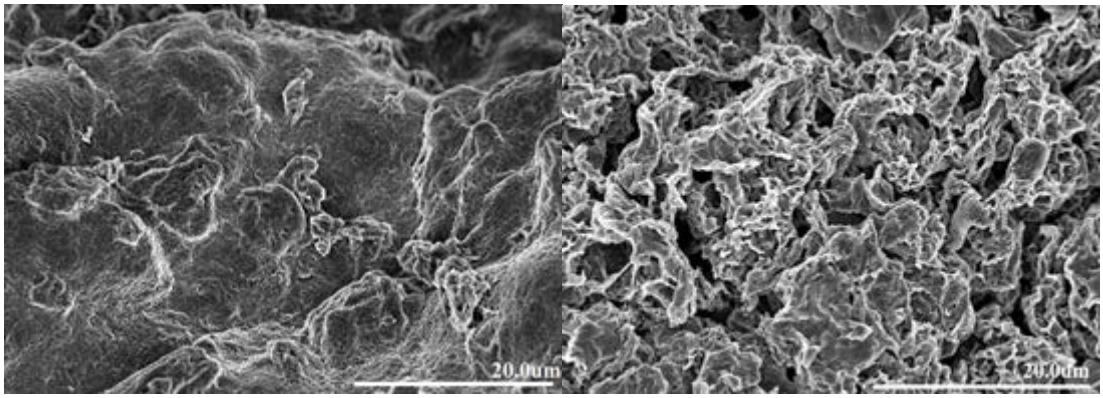


Figure 3.2- SEM images of (a) pure cellulose composite surface, (b) 0.19 wt% GO/cellulose composite surface, (c) 0.95 wt% graphite oxide/cellulose composite surface, (d) 2 wt% graphite oxide/cellulose composite surface, and (e) 4.15 wt% graphite oxide/cellulose composite surface

Raman spectra specific for 4.15 wt. % GO/Celluose composite was shown in Fig. 3. The figure demonstrated two spectral features (D-peak) at 1360 cm^{-1} and (G-peak) 1600 cm^{-1} to prove the existence of GO. The D and G peak position and intensity are commonly used in the classification of graphite or GO.[30, 31, 32] The band intensities which also stated as ratio of the p eak intensities are corresponding to the structural change or disturbance of carbon network. The ID/IG ratio (the ratio of intensity of D peak to the intensity of G peak) was calculated as 1.27 for 4.15 wt. % GO/Celluose composite.[33]

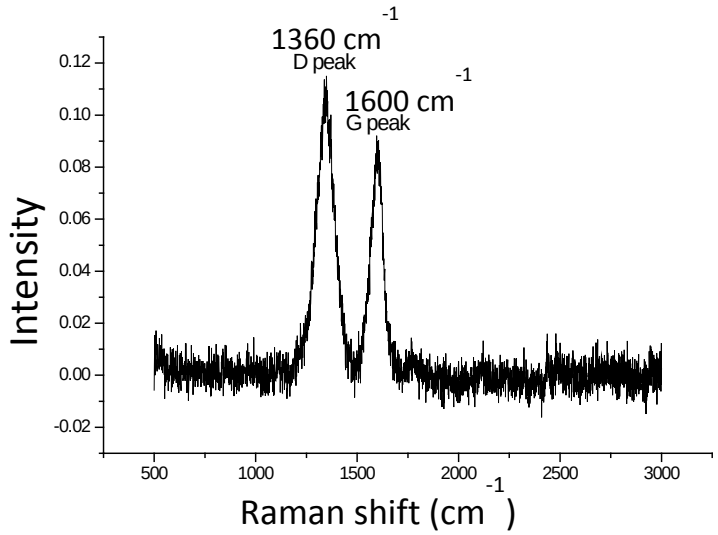


Figure 3.3- Raman spectrum of GO/cellulose composite with 4.15 wt. % of GO

3.3.2 Extraction efficiency and purity of DNA

The extraction efficiency of eluted DNA was highly depended on the wt. % of GO within the composite and the type of binding buffer. From Fig. 4, the total yield and extraction efficiency increased proportionally to the GO wt. %. It was observed that 4.15 wt. % GO/Cellulose composite provided the highest yield and extraction efficiency in all samples.

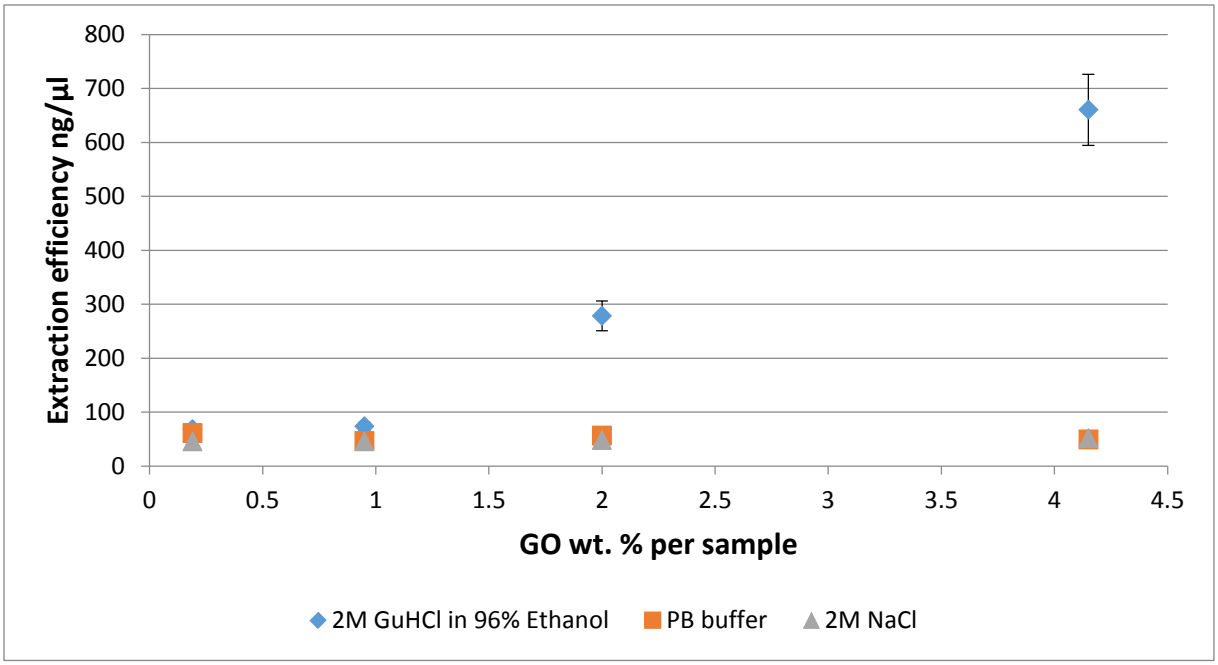


Figure 3.4- Extraction efficiencies of GO/cellulose composite with 0.19 – 4.15 wt.% of graphite oxide in three different types of binding buffer solution. (The standard deviation of 3 samples were within 7 %)

Among the three types of binding buffer, GuHCl buffer obtained the highest yield and extraction efficiency. The DNA extraction performance of 4.15 wt. % GO/Cellulose composite was compared to that of commercial silica-based spin columns (QIAamp Mini Spin Columns). The result showed that the extraction efficiency of 4.15 wt.% GO/Cellulose composite was 660.4 ng/µL, which was 50% higher than that of silica-based spin column (406 ng/µl) when applying GuHCl as binding buffer. Higher percentage of GO/Cellulose composites with 4.5 wt. %, 5.4 wt. % and 6.3 wt. % were also prepared for extraction experiments. However, the binding capacity decreased crucially when the percentage of GO was above 4.15 wt. %. This can be explained by the deformation of the composite. When the composite consisted of more than 4.15 wt. % GO, the amount of cellulose was not enough to act as a solid support for GO powder. As a result, the physical structure of the composite changed drastically and GO could not be bounded to the surface securely. Thus, the total surface area of GO was limited and the binding capacity decreased accordingly. The purity of

extracted DNA were similar between GO/Cellulose composites and silica-based spin column. The absorbance ratios of A260/A280 in all samples were ranged between 1.8 - 2.0, which indicated the both solid extraction materials were capable to eluted high purity of DNA.

3.3.3 The mechanism of adsorption and desorption process between GO and DNA

Several mechanisms have been suggested to play significant roles in DNA-GO adsorption, e.g. hydrogen bonding, π-π stacking and electrostatic forces. First of all, the main adsorption mechanism between DNA interaction and GO is considered to be the π-π stacking forces between the aromatic ring in GO and nucleobases of DNA molecule.[20, 21, 34, 35, 36] Secondly, electrostatic forces are contributing to the adsorption mechanism as well. DNA molecules are negatively charged due to the existence of phosphate backbone structure, thus it has an affinity on positive charged surfaces. The binding buffer (e.g. GuHCl) forms a "cation bridge" between DNA and the OH functional groups (alkyl or phenol C-OH) on GO surface. This cation bridge mechanism enhanced the adsorption between DNA and GO surface. By the addition of elusion buffer, DNA can be desorbed from GO surface by breaking the cation bridge. The major functional groups on GO surface include (1) C-OH groups which bonded to saturated, sp^3 hybridized carbon, and (2) phenol-OH groups which bonded to sp^2 hybridized carbon. These bonds have weaker R−OH connection compared to that of Si-OH, and thus desorption rate of DNA will be higher. As a result, the extraction efficiency increased accordingly.

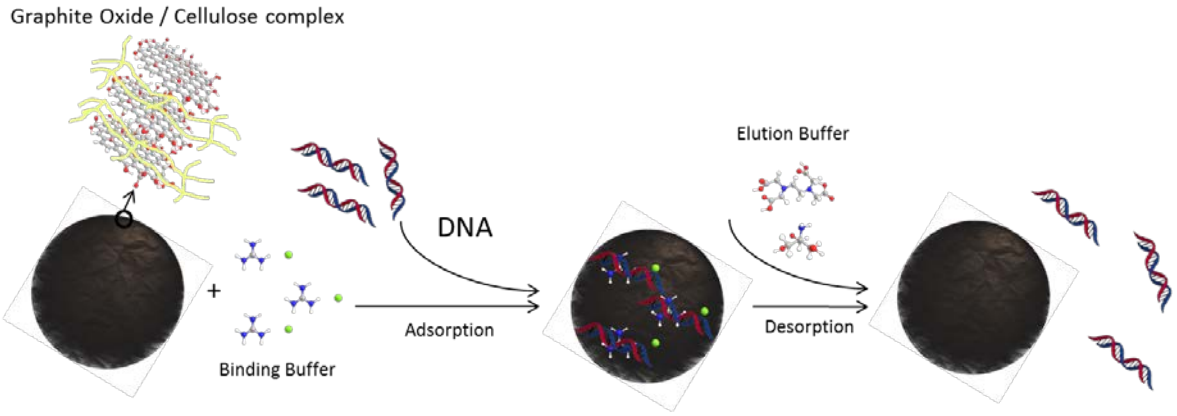


Figure 3.5- The proposed mechanism of graphite oxide/cellulose composite: (1) adsorption of DNA to the composite in the present of binding buffer and, (2) desorption of DNA from the composite in the present elution buffer

3.4 Conclusion

In the present publication, I have demonstrated the preparation of GO/Cellulose composites with designed properties and the composites have been examined as solid-phase DNA extraction material. The GO/Cellulose composites were present as dark brown spherical structure with an average diameter of 2.6 mm. The A260/A280 ratio of all elusion samples were reported between 1.79-1.86, which indicated high quality of DNA was successfully selected. By increasing the GO wt.%, the extraction efficiency of DNA increased proportionally. The highest performance was demonstrated by 4.15 wt. % GO, where the extraction efficiency and DNA purity (A260/A280) reading were, respectively, 660.4 ng/µl and 1.86. The extraction efficiency was 50% higher than that of commercial silica-based material.

Comparisons of the binding mechanism between the two materials were as follows: In the case of GO based composite, the binding mechanism of DNA was provided by the functional groups such as alkyl-OH and phenol-OH. On the other hand, these binding forces are relatively weak and thus it could be easily broken in the present of elution buffer. In the

case of silica-based material, DNA is attached to silianol group with higher reactivity. As a result, the attraction between silianol group and DNA is rather strong and is difficult to separate from the silica matrices. The extraction efficiency was thus lower compared to that of GO/Cellulose composite. In this paper, I have succeeded to decovered an alternative material for pure DNA extraction with high efficiency. GO/Cellulose composites are safe and low cost materials, which are additional bonuses in terms of handling and processing. I believed that GO/Cellulose composites are highly possible to serve as a new generation DNA extraction solid support, and highly applicable tomanual extraction systems or on-site DNA extraction kits.

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Chapter 4

Innovative Graphite Oxide-Cellulose Based Material Specific for Genomic DNA Extraction

Chapter 4 Innovative Graphite Oxide-Cellulose Based Material Specific for Genomic DNA Extraction

4.1. Introduction

For genomic DNA analysis, the commonly used method is to obtain the DNA from nucleated cells of peripheral blood. [1, 2] This invasive method requires tedious isolation schemes and relatively long total analysis time. [3] Other alternative sources for genomic DNA including human hair, nail clips or tissue samples. However, the purity of DNA is often influenced by the starting material and substrate properties. [4, 5] The ideal genomic DNA extraction material should be capable to isolate high quality of DNA from a wide variety of genomic samples. High extraction efficiency is also required for the application of polymerase chain reaction (PCR). [6, 7] So far, the genomic DNA extraction is still a challenging process.

The extraction of genomic DNA from non-invasive samples have been conducted by various techniques, including phenol-chloroform extraction, silica-coated magnetic beads kits, silica membranes, [8, 9] centrifugal filter devices, [10, 11, 12] and ion exchange resins. Phenol-chloroform extraction, which has been widely used for many years, is particularly useful for high molecular weight DNA extraction. However, the organic reagent is hazardous and the procedures are time consuming and labor intensive. DNA extractions by ion-exchange resins are relatively simple and inexpensive, nevertheless, possible PCR inhibitors are frequently found in the extracted solution. In order to minimize the inhibition, the extract solution requires extra filtering process.[13] Silica-based materials are often applied as solid DNA extraction matrices in laboratory studies. DNA binds to silica membrane in the presence of the chaotrophic salts, and can be extracted by washing with elusion buffer. During the binding process, PCR inhibitors such as polyvalent cations and proteins can be removed during subsequent washes. The extracted DNA is relatively clean and is almost free of PCR

inhibitors. [14, 15, 16] Another problem arises during the elution process. The elution buffer only removes less than 90% of the DNA from the surface of the silica matrices, and thus limited the extraction efficiency of genomic DNA.

Carbon-based material can adsorb DNA through hydrogen-binding interaction in the present of chaotrophic salt. Among different types of carbon materials, graphite oxide (GO) might be a good candidate for DNA extraction applications since GO contains multiple polar functional groups (–O, –OH and –COOH groups) which allows the material to absorb DNA through hydrogen bonding interaction. [17] GO also contains aromatic ring structure which can provide additional π stacking force with the aromatic structure of nucleobases in DNA. [18, 19, 20] Theoretically, the supreme binding capacity of silica matrices might be approached by GO. Our previous work has studied the purity and extraction efficiency of single strand DNA by GO/Cellulose composites. [21] Cellulose served as a strong support material and enhanced the total surface area of GO in order to optimize the binding capacity between GO and DNA. The results showed that the optimum binding capacity was observed at 4.15 wt% of GO. In this study, I applied 4.15 wt% GO/Cellulose beads as a progressive solid support surface for genomic DNA purification. I have successfully demonstrated high purity and extraction yield of various type of genomic DNA for practical application. Also, I was able to show that this innovative GO/Cellulose composite has superior results than that of the commercial silica-based DNA extraction product.

4.2. Experimental

4.2.1. Preparation of Graphite Oxide

Graphite oxide (GO) was prepared from synthetic graphite flake (<20 micron, Sigma-Aldrich (Japan)) by improved Marcano's method. [22] In brief, concentrated H_2SO_4 (360 mL) and H_3PO_4 (40 mL) (Tokyo Chemical Industry, Japan) were added to a mixture of graphite flakes (3.0 g, 1 wt% equivalent) and $KMnO_4$ (18.0 g, 6 wt% equivalent, Wako Chemicals,

Japan). The mixture was heated to 50 °C and stirred for 12 hours. The solution was cooled to room temperature and poured into an ice cooler (400 mL). The solution was mixed with 3 mL of H_2O_2 (Kanto Chemical Co., Inc. Japan) and purified by dialysis (Fisherbrand® dialysis tubing MWCO 6000-8000 USA) for 48 hours. After the purification process, the solution was filtered by 0.1 µm Omnipore™ membrane filter and dried at room temperature (Fig. 1). The dried GO powder was used for the preparation of GO/Cellulose composite.

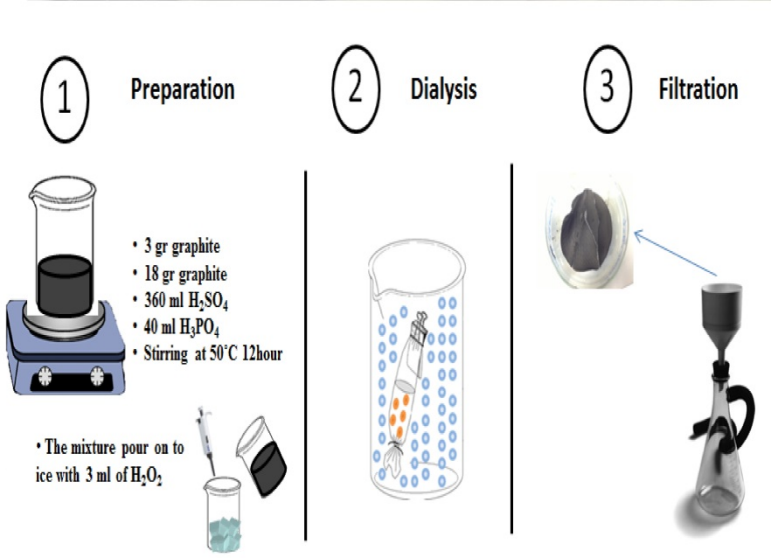


Figure 4.1- Graphite Oxide powder was produced from graphite powder in 3 stages. 1st stage is the chemical exfoliation step where graphite is oxidized by strong acids and oxidizing agent. At the 2nd stage this acidic solution is applied to dialysis for 48 hour in order to increase pH to 7 and remove the salts from solution. After dialysis, as a 3rd stage, solution filtered by vacuum filter and filter cake is dried in the oven.

4.2.2. Preparation of GO/Cellulose Composite

About 4.5 g of GO powder was dispersed into 90 mL ultrapure water and placed inside a sonicator (Model: US-102, SND Co., Ltd. Japan, ultrasonic power of 100 W, frequency output of 38 kHz) for 15 min, before was cooled to 0 °C inside a refrigerator. In the next step, 6.0 g of NaOH and 4.0 g of urea were added into the frozen suspension, followed by the

addition of 4.0 g of microcrystalline cellulose (MCC) (Sigma-Aldrich, Japan) powder. The mixture was gently stirred for 10 min within an ice-bath and then cooled to −20 °C for 2 hours. The mixture was stirred at room temperature for 30 minutes and extruded through a 1 mL Eppendorf syringe into 2 M HNO_3. The coagulated composites were remained inside the acid overnight, and then rinsed by tap water for 30 minutes followed by distilled water for 15 minutes. The synthesized composite was stored in distilled water at room temperature (Fig. 2). The overall mass % of GO was calculated to be 4.15%. The resulted compound exhibited spherical shape, and each composite has an average diameter of 2.6 mm.

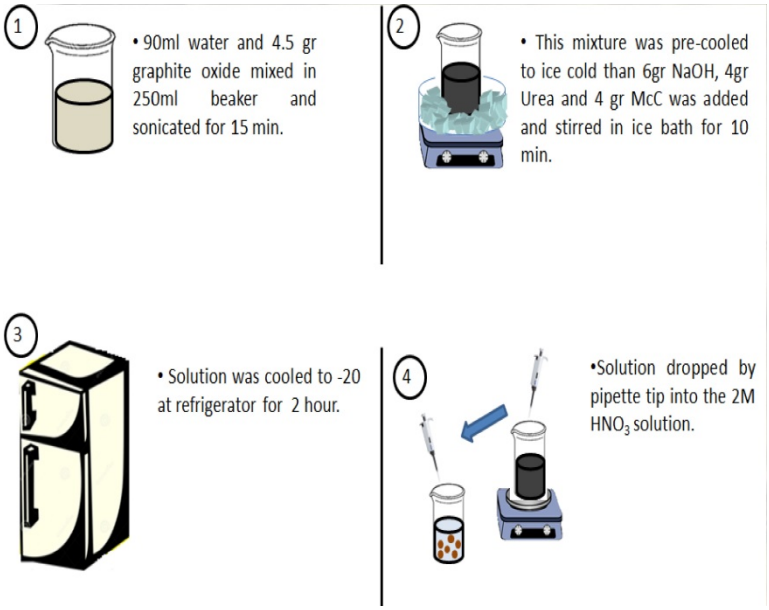


Figure 4.2- NaOH-Urea-Water system was used to dissolve cellulose with GO in order to make GO/Cellulose beads

4.2.3 Characterization of GO/Cellulose Composites

The structural properties of GO were conducted by Raman spectra (excitation wavelength at 785 nm, NRS-3100 instrument, Jasco). The morphology of the GO/Cellulose composites was examined by Hitachi scanning electron microscopy at 5 kV (SEM) (S-4800, Japan). Prior to observation, samples were coated with platinum sputter at 20 °C with 20 mA current for 15 seceonds.

4.2.4 Lysis Processes of Various Types of Genomic DNA Samples

Five different kinds of genomic DNA were applied for DNA extraction in current study. The lysis process is specific for each type of genomic DNA:

1. Three samples of 30 mg of chewed gum were cut into small pieces and add into a 1.5 mL eppendorf centrifuge tube along with 300 µL lysis buffer (Buffer ATL Qiagen cat. No 19076) and 20 µL proteinase K (Qiagen cat. No. 19131). The tube was vortexed and placed into a water bath at 56 °C (as described in the QIAamp DNA Investigator Handbook) for 3–6 hours.

2. Three samples of human hair were cut into 0.5 cm pieces and added into a 1.5 mL eppendorf centrifuge tube along with 300 µL lysis buffer, 20 µL proteinase K and 20 µL 1 M dithiothreitol (DTT-Sigma cat. No. 646563). The tube was vortexed before placing into a water bath at 56 °C for 1–3 hours until the tissue was totally dissolved.

3. Three samples of cigarette bud paper (area~ 0.1 cm^2) were added into a 1.5 mL eppendorf centrifuge tube along with 300 µL lysis buffer and 20 µL proteinase K. The tube was vortexed and placed into a water bath at at 56 °C for 1–2 hours.

4. Three samples of 20 mg nail clips were transferred to a 1.5 mL eppendorf tube with 300 µL lysis buffer, 20 µL of proteinase K and 20 µL 1 M dithiothreitol. The tube was vortexed before placing into a water bath at 56 °C for 1–3 hours.

5. Three samples of chicken breast was used for tissue sample. About 10 mg of chicken breasts was transferred to a 1.5 mL eppendorf tube with 180 µL lysis buffer and 20 µL proteinase K. The tube was vortexed before placing into a water bath at 56 °C for 12 hours.

4.2.5 Genomic DNA Extraction by GO/Cellulose Composite

In this study, Guanidium based chaotropic salts (Sigma G4505 Guanidine hydrochloride/ $NH_2C(=NH)NH_2 \cdot HCl$) were used as the binding buffer. [23] Depending on the type of genomic DNA sample, the pre-extraction procedure was slightly different. For animal tissue samples, 200 μL of the sample solutions were extracted from the eppendorf centrifuge tube after lysis process and mixed with 300 μL of binding buffer in a new 1.5 mL eppendorf tube. For other samples, 300 μL of the sample solutions were extracted from the Eppendorf centrifuge tube after lysis process and mixed with 400 μL binding buffer in a new 1.5 mL Eppendorf tube.

For the assessment of DNA extraction efficiency and purity of GO/Cellulose composites, eight GO/Cellulose composite spheres were applied as the extraction material. The lysis solutions of each sample (a total volume of 500 μL for tissue sample and 700 μL for other samples) was added into a 1.5 mL eppendorf tube containing eight GO/Cellulose composites spheres and incubated for 10 minutes. The solution was carefully taken out by a pipette, where the composites were washed twice with 70% ethanol in order to remove the binding buffer solution from the composite's surface. At last, a total volume of 1000 μL elution buffer were added into the Eppendorf tube and further incubated for 5 minutes. Then, the elution was extracted from the composites by a pipette.

4.2.6 Extraction Efficiency and Purity of the extracted DNA

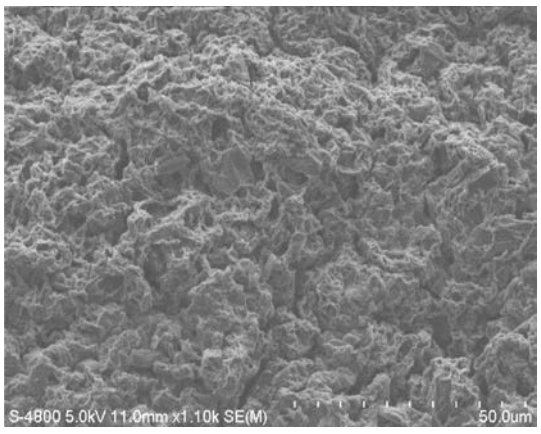
Quantity and quality of DNA in elusion were assessed in terms of extraction efficiency and purity of DNA. The purity of DNA can be measured by the optical density of the elusion at two specific wavelength, 260 nm and 280 nm, by NanoDrop device. The ratio of the absorbance at 260 to 280 nm (also denoted as A260/280 nm) should be ranged between 1.8 and 2.0 for high purity DNA. [24] In order to measure the total yield of DNA purification, last elution solution's volume multiplied with DNA conc. (ng/μL). On the other hand, by dividing the DNA total yield to input volume (total DNA amount [ng]/input DNA volume [μL])

extraction efficiency was calculated. All experiments were repeated three times and all data were replicable. The performance of DNA extraction was compared to that of commercial silica DNA extraction matrix, QIAamp MinElute columns (QIAamp DNA investigator kit). The binding, centrifugation and elution processes were prepared as described in the QIAamp DNA Investigator Handbook.[25]

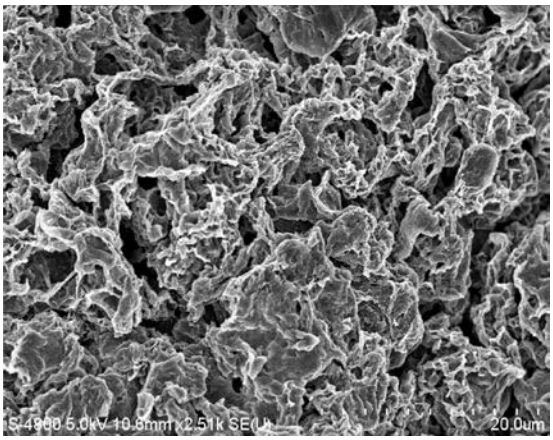
4.3. Results

4.3.1 The Morphology of GO/Cellulose Composites

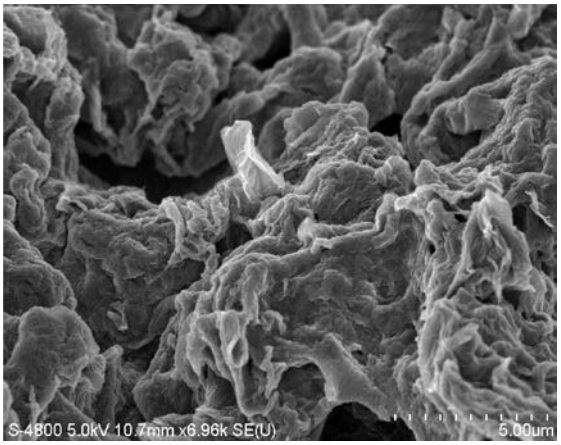
After extrusion, GO/Cellulose composites were presented as dark brown bead shapes, where the average diameter was measured with digital caliper (SK digital calipers, BLD-150, Niigata Seiki Co.,LTD, Japan) as 2.6 mm. Standard deviation of the composite diameter was 0.1387 mm. This result showed that beads with an uniform size and shape of GO/Cellulose composite could be made by a 1 mL eppendorf syringe during the coagulation process. Additionally, the size of the composites can be easily changed by varying the volume and the tip size of Eppendorf syringe. Morphologies of the GO/Cellulose composite were shown in Fig. 3 (a)–(c). From SEM images, homogeneous dispersion was observed on the surface of the composites.



(a)



(b)



(c)

Figure 4.3- Homogenous dispersion of GO and Cellulose were observed on the 4.15% GO/Cellulose beads surface

4.3.2 Raman Spectrometry

Raman spectra of the GO/Cellulose composite were shown in Fig 4. The spectra showed two distinct peaks – Disordered peak (D-peak) and Graphitic peak (G-Peak). The position and intensity ratio are generally used in the classification of diamond-like carbon or amorphous carbon films. [26, 27, 28] These characteristics usually vary based on the type of carbon. Disruption of sp2 bonding and the physical modification inside carbon network are causing because of this. The Raman spectroscopy demonstrated two spectral features, at

around 1360 (D-peak) and 1600 cm^{-1} (G-peak) and the ID/IG ratio (the ratio of intensity of D peak to the intensity of G peak) was calculated to be 1.27 to prove the existence of graphite oxide structure. [29, 30]

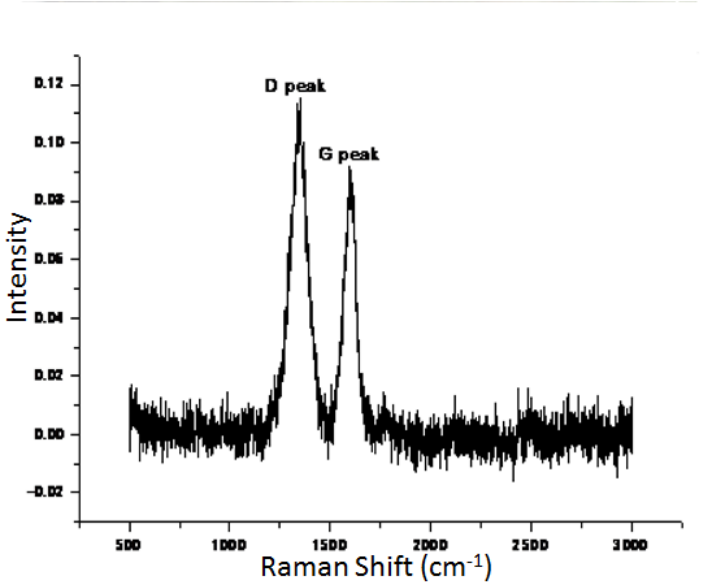


Figure 4.4- From Raman spectrophotometer results D and G bands can be clearly seen. The ratio of these bands (I_D/I_G) indicates the sp^2 structure. I_D/I_G ratio is calculated as 1.27 which proving the GO structure.

4.3.3 Extraction Efficiency and Purity of Genomic DNA

The extraction efficiency and purity of eluted DNA from five different genomic samples were evaluated by NanoDrop spectrophotometer. The commercial silica-based spin columns were used as comparison for DNA extraction performance. The result demonstrated that the GO/Cellulose composite provided a much higher extraction efficiency in all samples. From Fig. 5 and Table 1, the extraction efficiency of animal tissue, chewing gum, cigarette bud, nail clip and hair sample were, respectively, 4, 12, 11.6, 7.3 and 9.8 times higher than that of silica spin column. The A260/A280 absorbance ratios of GO/Cellulose composites were ranged between 1.8 and 2.0 for animal tissue, cigarette bud and nail clip samples, which indicated the extracted elution contained high purity of DNA.

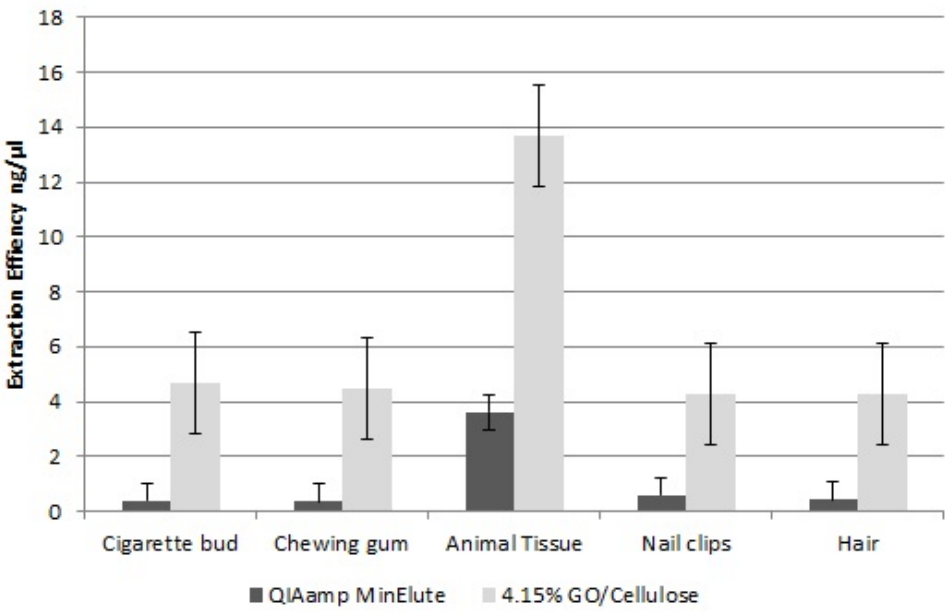


Figure 4.5- 5 different samples were used to compare the extraction efficiency of GO/Cellulose and QIAamp MinElute spin column. GO/Cellulose beads show significantly higher extraction efficiency.

In the case of chewed gum and hair samples, the A260/A280 absorbance ratios were re ported to be 1.6, which indicated a small amount of protein contamination during the elution p rocess. However, the purity of all samples was much higher compared to that of silica spin columns.

Table 4.1- Summary of the total yield, extraction efficiency and DNA purity results from silica based spin colum n (QIAamp MinElute) and GO/Cellulose composites

	Surface	Cigarette bud	Chewing gum	Animal tissue	Nail clips	Hair
Total yield (ng)	QIAamp MinElute	81	74.5	724	117	86
	4.15% GO/Cellulose	1880	1800	2740	1710	1700
Extraction Efficiency (ng/µL)	QIAamp MinElute	0.405	0.3725	3.62	0.585	0.43
	4.15% GO/Cellulose	4.7	4.5	13.7	4.275	4.25
DNA Purity	QIAamp MinElute	2.29	2.81	1.5	6.92	8.37
	4.15% GO/Cellulose	1.82	1.6	2.0	1.92	1.65

4.4. Conclusion

In the present thesis, I have demonstrated the preparation of GO/Cellulose composites for genomic DNA extraction. The GO/Cellulose composites were present as dark brown spherical structure with an average diameter of 2.6 mm. Genomic DNA sources were obtained from various materials including cigarette bud paper, chewed gum, animal tissue, hair sample, and nail clip. The GO/Cellulose composites demonstrated 4 to 12 times higher extraction efficiency compared to that of commercial silica-based spin column. In addition, the extracted DNA from GO/Cellulose composite present a much higher purity.

Carbonaceous materials such as GC, pyrolytic graphite, carbon composites, carbon inks, graphite pencil leads, CNT or graphene/graphite oxides can be modified with a stable DNA adsorbed layer. [31, 32] This modification leads to new DNA/carbon hybrid materials and various applications like biosensor, drug delivery, tissue engineering, enzyme cleavage

protection, etc., in biology and biotechnological studies. [33, 34, 35, 36, 37, 38] However there is no reported research using this interaction on nucleic acid extraction applications. I believe it is one of the first researches in the field of applying carbon based material for DNA extraction.

I have succeeded to decover an innovative and bio-based material for solid phase genomic DNA extraction. The product provided superior performance compared to the current state of art, and it is widely applicable to various types of genomic DNA samples. In terms of handling and processing, the GO/Cellulose composites are safe and low cost materials, which is essential for on-site operation. I believe that GO/Cellulose composites are highly possible to serve as a new generation DNA extraction solid support and further developed as a new material for commercial DNA extraction kits.

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Chapter 5 High Efficiency DNA Extraction by Graphite Oxide Cellulose Magnetite Composite under Na+ Free System

Chapter 5 High Efficiency DNA Extraction by Graphite Oxide Cellulose Magnetite Composite under Na+ Free System

5.1 Introduction

DNA extraction is often applied as an early step in many diagnostic processes, especially in the field of diseases and genetic disorders. DNA have to be isolated, purified, and concentrated from blood or tissue samples. Such sample preparation is commonly accomplished through solid phase extraction, relying on the reversible interactions between nucleic acids and a solid support. Currently under the high salt conditions, naked[1] or coated[2, 3, 4, 5 6, 7] magnetic nanoparticles (MNP) are using for the purification of genomic or plasmid DNA from different biological sources due to its ease handling and magnetic property of the particles[1, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18]. Other commercial DNA extraction method is based on silica systems which apply surface functional groups for purification experiments[19] . The absorption of DNA molecule on solid support is driven through hydrophobic, electrostatic interactions and hydrogen-bonding under concentrated chaotropic salt conditions [20]. In order to increase the extraction efficiency of DNA, chaotropic salts are mostly applied to enhance the binding efficiency by removing the water molecules from the binding surface and inside the DNA molecule. The chaotropic salts also has a key part as providing cation-bridge that attracts the negatively charged oxygen ions in silica under high salt conditions ($pH \leq 7$). DNA is then tightly bound, and followed by extensive washing to remove other contaminants. The purified DNA molecules can be eluted under low ionic strength ($pH \geq 7$) provided by elusion buffer[21] . After elution process it is possible to isolate components of the cell lysate like polysaccharides, phenolic compounds or humic substances[22, 23]. The advantage of high salt condition environment is that it provides decent extraction yield with high purity of DNA. However, the residual chaotropic salt also could remain in the extracted DNA and interfere

with down-stream applications such as polymerase chain reaction (PCR)[24]. For the purpose of limiting the potential interference of PCR process after DNA extraction, the development of DNA extraction solid support without chaotropic salt is essential. Since major binding mechanism of silica surfaces are based on electrostatic force and hydrogen binding with cation bridges, binding of DNA under low salt condition is not applicable to silica surfaces.

Recently, GO[25] based systems have been developed for DNA extraction solid support system as well. The adsorption mechanisms are provided by surface functional OH groups and it is similar to that of silica surface with an additional π-π stacking attraction forces between GO and DNA. On the other hand, oxide or hydroxides of Fe(III) have variable surface charge, mainly depending on the surface density of protons or hydroxyl groups[26] . Fe (III) oxides are positively charged in nature and can interact with anions such as phosphates molecules, which is also the backbone structure of DNA[27]. This interaction is also applicable to DNA since DNA molecule has an affinity to be attracted on positive charged surfaces. In this research, I have proposed an innovative material, graphite oxide (GO)/Cellulose/Magnetite composite, as an advanced solid support matrices for DNA extraction method under low salt condition. Our previous work has studied the purity and extraction efficiency of DNA by GO/Cellulose composites (supplementary data) [25]. Cellulose served as a strong support material and enhanced the total surface area of solid matrices. The results showed that the optimum binding capacity was observed at 4.15 wt% of GO. In this study, I further enhanced the extraction efficiency of the GO based solid support by the addition of various amounts of magnetites/ Fe oxides. The present of magnetites has significantly enhanced the adsorption between the composite and the DNA molecules, where the proposed composites were able to extract DNA without the present of chaotrophic salt. As a result, I have successfully demonstrated high purity and extraction yield of DNA for practical application.

5.2.Experimental Procedures

5.2.1. Preparation of Graphite Oxide

Graphite Oxide (GO) was prepared from synthetic graphite flake (<20 micron, synthetic, Sigma-Aldrich (Japan)) by improved Marcano's method.[28] In the first step, 360 ml H_2SO_4 and 40 ml H_3PO_4 ((Tokyo Chemical Industry, Japan) was added into a mixture of graphite flakes (3.0 g, 1 wt.% equivalent) and $KMnO_4$ (18.0 g, 6 wt.% equivalent ,Wako Chemicals, Japan). The mixture was heated to 50 °C and stirred for 12 hours. Then, the solution was cooled down to room temperature and mixed with 3mL of 30% H_2O_2 (Kanto Chemical Co. Inc, Japan) in a container with 400 mL of ice, and further purified by dialysis (Fisherbrand® dialysis tubing MWCO 6000-8000) for 48 hours. After purifying, the solution was filtered by 0.1 µm Omnipore™ membrane filter and dried at room temperature in order to obtain GO powder.

5.2.2. Preparation of Graphite Oxide/Cellulose/Magnetite Composite

Approximately 4.5 g of GO powder was dispersed into 90 mL ultrapure water by a sonicator (Model: US-102, SND Co., Ltd. Japan, ultrasonic power of 100 W, frequency output of 38 kHz) for 15 min. The solution was cooling down to 0 °C inside a refrigerator. In the next step, 6.0 g of NaOH, 4.0 g of urea and 4.0 g of microcrystalline cellulose (MCC) (Sigma-Aldrich, Japan) powder were added into the frozen suspension, followed by the addition of 0.3, 0.9, 1.5 and 4.5g Fe_3O_4 powder (iolitec nanomaterials, USA). The mixture was gently stirred for 10 min inside an ice-bath and then cooled to −20 °C for 2 hours. The mixture was taken out from the cooler and stirred at room temperature for 30 minutes. The composite was extruded through a 1 mL Eppendorf syringe into 2 M HNO_3. The coagulated composites were left inside the acid overnight, and then rinsed by tap water for 30 minutes followed by distilled water for 15 minutes. The synthesized composite was stored in distilled water at

room temperature. The schematic of the experimental procedures were shown in Fig 1.

The overall mass % of GO was fixed at 4.15%, where the weight percent corresponding to 0.3, 0.9, 1.5 and 4.5 g Fe_3O_4 powder was calculated to be 0.27, 0.82, 1.36, 3.98 wt.%. The samples were denoted as CGM3, CGM9, CGM15 and CGM45, respectively.

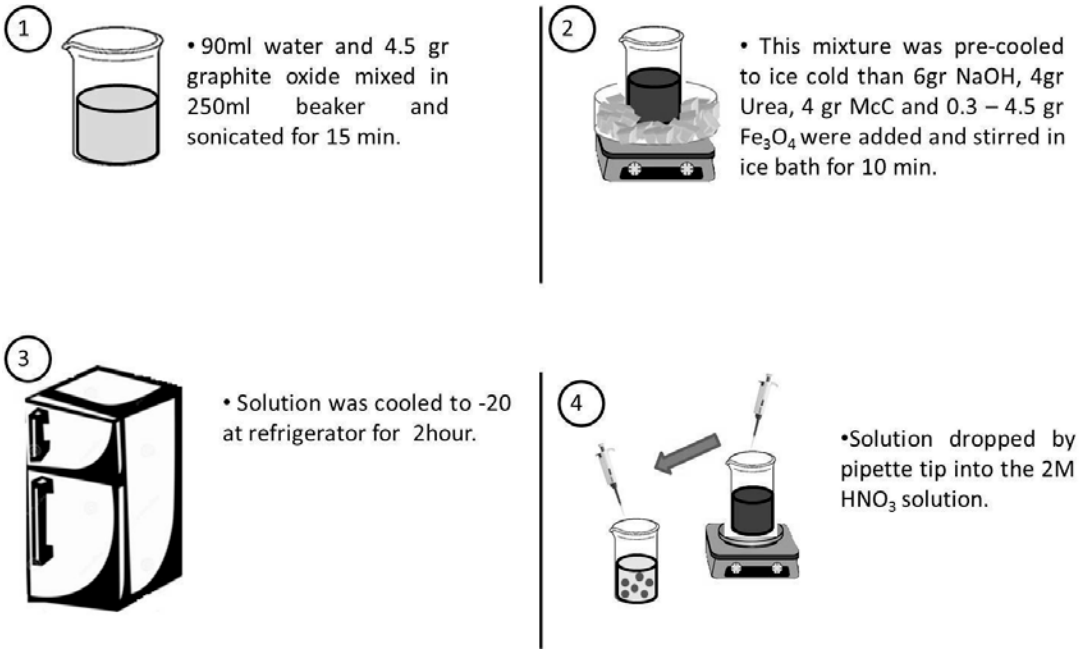


Figure 5.1- Synthesis of Fe_3O_4 GO/Cellulose composites: 1: Mixture of Water and GO powder; 2. Dissolving cellulose with NaOH-Urea-Fe_3O_4-Water mixture; 3. Cooling: Cooling the mixture to -20^{o}C; 4. Coagulation: Dropping the Cellulose/GO mixture into HNO_3.

5.2.3 Characterization of GO/Cellulose/Magnetite Composites

The properties of GO were demonstrated by Raman spectra (excitation wavelength at 785 nm, NRS-3100 instrument, Jasco). The morphology of the GO/Cellulose/Magnetite composites was examined by Hitachi scanning electron microscopy at 5 kV (SEM) (S-4800, Japan). Prior to observation, samples were coated with platinum sputter at 20 °C with 20 mA current for 15 secs.

5.2.4 DNA Extraction by GO/Cellulose Composites

The DNA solution with size ranged from 587 to 831 base pairs (Sigma Company (D7290)) was applied as DNA specimen. 20 µl saturated DNA solution were mixed with 180µl pure water as DNA source. Five GO/Cellulose/Magnetite composites were inserted into a 1.5 ml eppendorf tube with a total volume of 200 µl DNA solution and incubated for 10 mins. The solution was carefully taken out by pipette and further washed with 70% ethanol in order to remove impurities from the composite's surface. Then, 500 µl of elution buffer (AE buffer -10mM Tris-HCl, 0.5mM EDTA pH 9.0-) were added into the tubes and incubated for 5 mins. After incubation, the elution was separated from the composites. Throughout the extraction process, no chaotropic salt was added.

The quantity and quality of DNA in the elusion were assessed in terms of extraction efficiency and purity, respectively. GO/Cellulose composite was applied as the reference material for the performance of DNA extraction. The binding, centrifugation and elution processes were performed as described in our previous research.[25]

5.2.5 Analysis of the yield and purity of extracted DNA

The purity of the extracted DNA was assessed by the optical intensity in elusion at two different wavelengths, 260 nm and 280 nm, by NanoDrop device. The absorbance ratio between 260 and 280 nm (also denoted as A260/280 nm) should be ranged in between 1.8 to 2.0 for high purity DNA.[29] In order to measure the total yield of DNA purification, last elution solution's volume multiplied with DNA conc. (ng/µL). On the other hand, by dividing the DNA total yield to input volume (total DNA amount [ng]/input DNA volume [µL]) extraction efficiency was calculated. All experiments were repeated three times and all data were replicable.

5.3.Results and Discussion

5.3.1 The morphology and composition of GO/Cellulose composites

The GO/Cellulose/Magnetite composites were presented as brown spherical structure, where the average diameter was measured with a digital caliper (SK digital calipers, BLD-150, Niigata Seiki Co.,LTD, Japan). The averaged diameter was 2.6 mm. The result indicated that the GO/cellulose/Magnetite composites were relatively homogenous in terms of size. As a side note, the diameter of the composites can be easily controlled by the volume and the tip size of the Eppendorf syringe. The morphologies of GO/Cellulose/Magnetite composites with various Fe_3O_4 wt. % were shown in Fig. 2. Fig. 2b obviously showed the existence of the magnetic force provided by Fe_3O_4 when a magnet was placed near the composites.

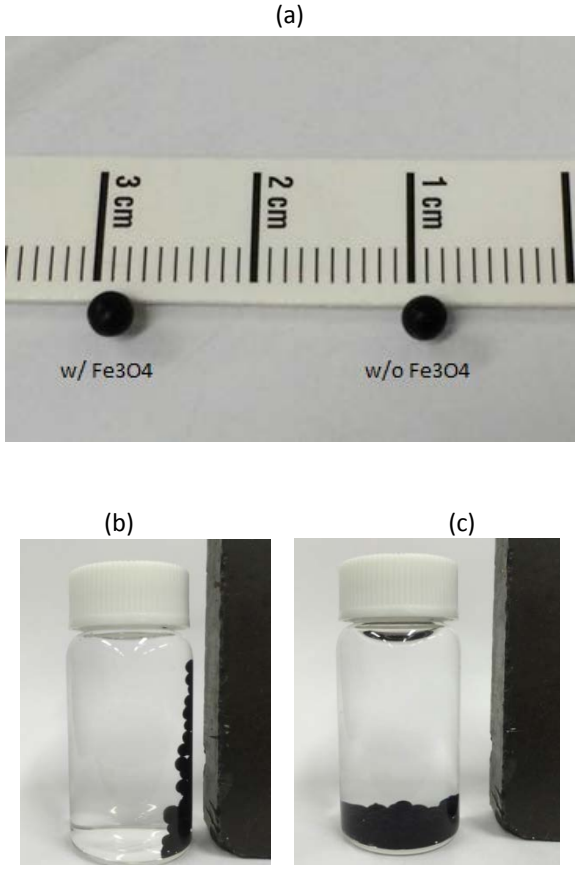
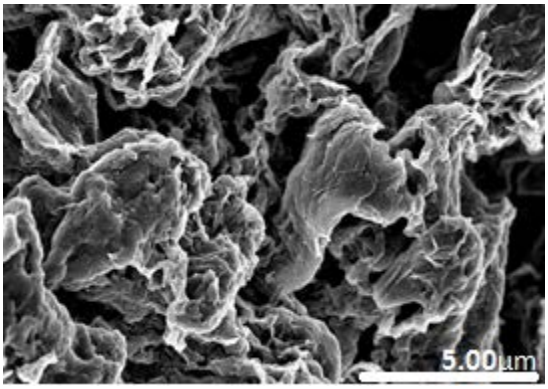
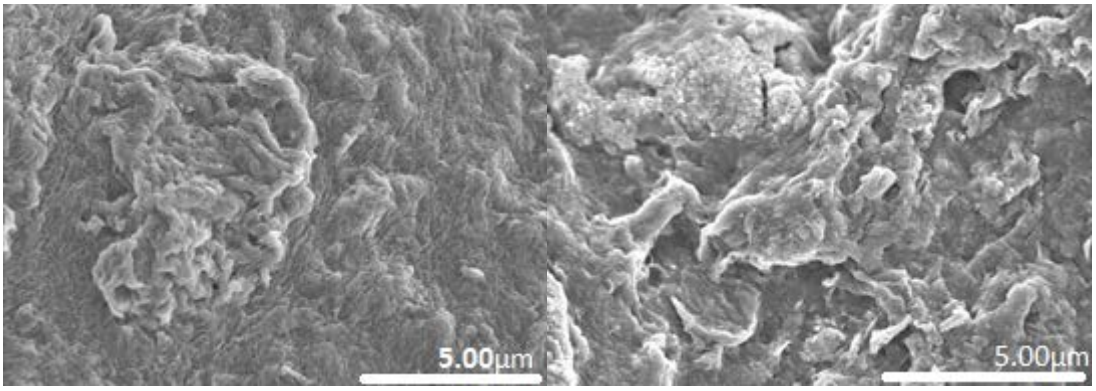


Figure 5.2- Morphology of (a) GO/Cellulose composite with and without Fe_3O_4 at an average diameter of 2.6mm, (b) CGM45 composites in pure water with magnet near to the bottle and (c) CGM0 composites in pure water with magnet near to the bottle

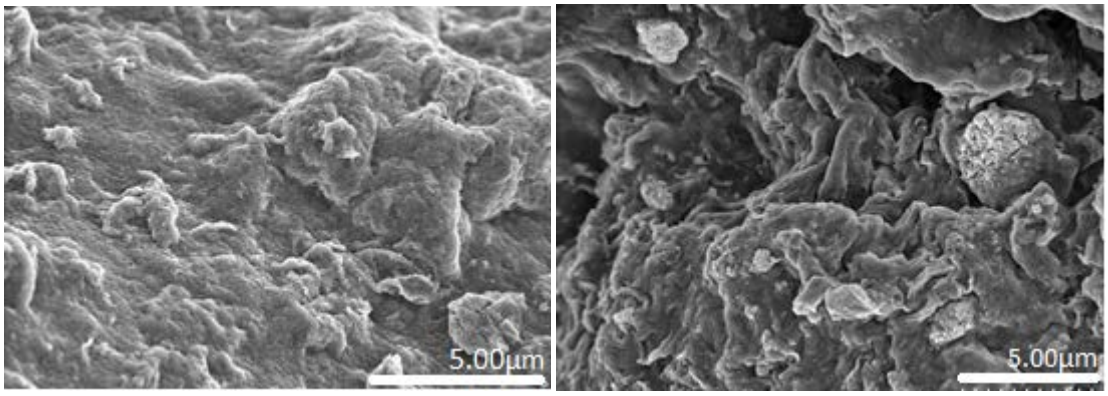
As observed by the SEM and EDX images (in Fig. 3 and Fig. 4), GO and Fe_3O_4 were homogenous dispersion on the surface of the composites.



(a)



(b) (c)



(d) (e)

Figure 5.3- SEM images of (a) CGM0, (b) CGM3, (c) CGM9, (d) CGM15, and (e) CGM45 composite surfaces.

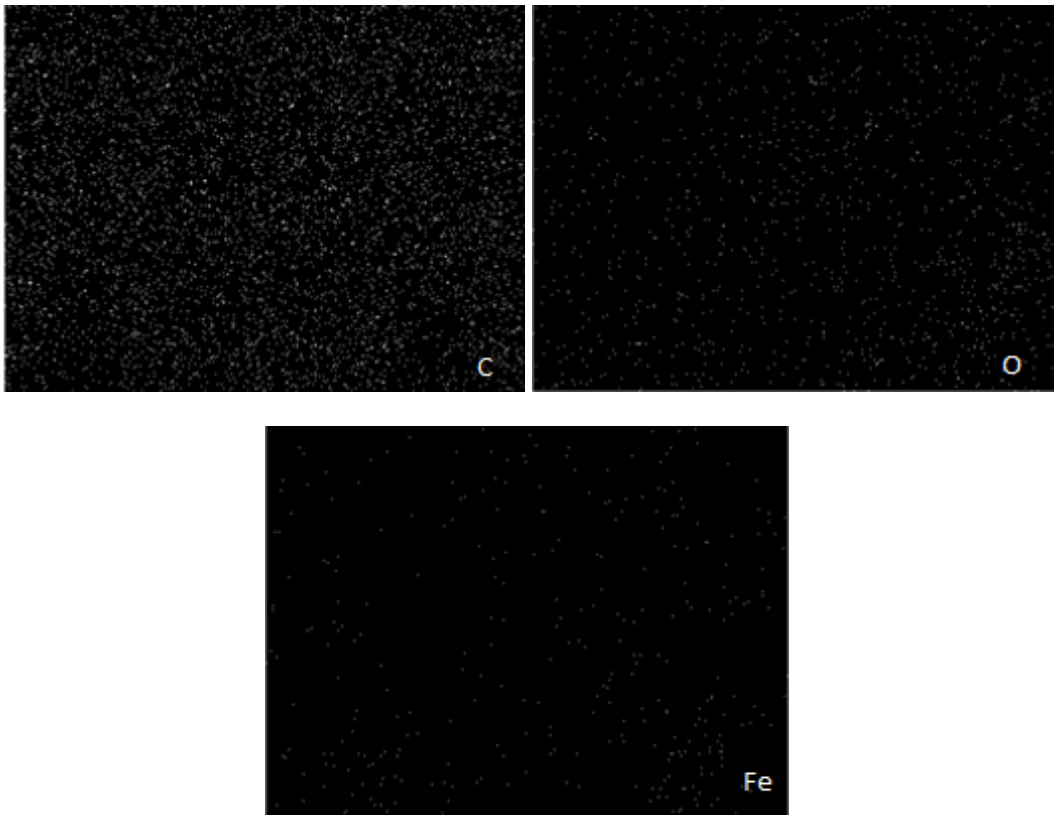


Figure 5.4- EDX spectrum and mapping of C, O and Fe elements corresponding to CGM45 composites surface in Fig. (3e).

5.3.2 Extraction efficiency and purity of DNA

The DNA extraction performance of GO/Cellulose/Magnetite composite was compared to that of GO/Cellulose composite. The extraction efficiency increased from 18.25 to 212.75 ng/μL when 0.27 wt. % of Fe_3O_4 were added into the GO/Cellulose composite. Also, the extraction efficiency of eluted DNA was further enhanced when increasing the wt. % of Fe_3O_4 within the composite. From Fig. 5, the total yield and extraction efficiency of ssDNA increased proportionally to the Fe_3O_4 wt. % concentration. It was observed that 3.98 wt. % Fe_3O_4 containing GO/Cellulose composite provided the highest yield and extraction efficiency in ssDNA samples. The extraction efficiency of 3.98 wt.% Fe_3O_4 containing

GO/Cellulose composite for ssDNA was 338.5 ng/µl, which was 18 times higher than that of pure GO/Cellulose composite (18.25 ng/µl).

The efficiency and purity of extract DNA with various samples were summarized in Table 1. The absorbance ratios of A260/A280 of pure GO/Cellulose, 0.27 and 0.82 wt. % GO/Cellulose/Magnetite were lower than 1.8, which indicated small amount of impurities might be present within the DNA solution. The purity of DNA increased to 1.81 when the ratio of Fe_3O_4 increased to 1.36 and 3.98 wt.% within GO/Cellulose/Magnetite composites. These results indicated that the existence of magnetite was essential for eluting high purity of DNA with higher extraction efficiency.

Table 5.1- Extraction efficiencies and total yields of ssDNA with 0.27 – 3.98 wt% of Fe3O4/graphite oxide/cellulose composite.

Samples Fe3O4	nanodrop ng/ul	260/280	Total yield ng	Extraction effiency ng/µl
CGM0	7.3	1.71	3650	18.25
CGM3	85.1	1.67	42550	212.75
CGM9	110.2	1.65	55100	275.5
CGM15	**119.4**	**1.81**	**59700**	**298.5**
CGM45	135.4	1.81	67700	338.5

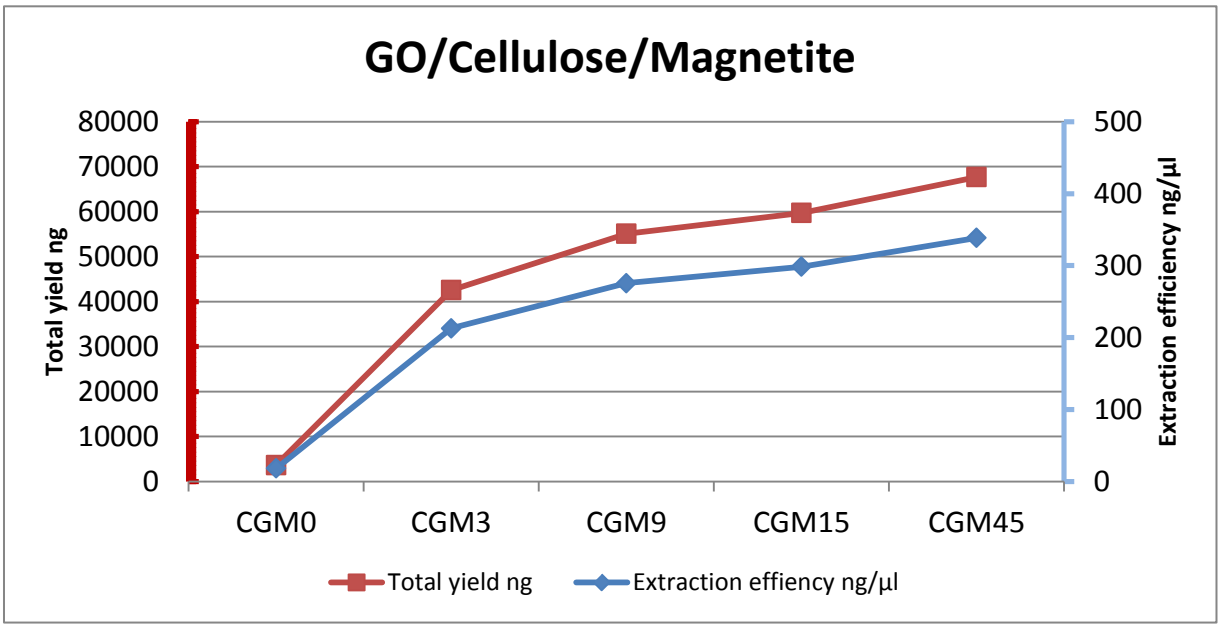


Figure 5.5- Extraction efficiencies and total yields of ssDNA with CGM0 – CGM45 composites.

5.3.3 The mechanism of adsorption and desorption process between GO, magnetite and DNA

Researches showed that only Fe_3O_4 particles have similar adsorption-desorption mechanism and binding capacity (up to 1 μg per 20 mg of sample) compare to silica surfaces under high salt or chaotropic conditions. [15] However, the adsorption of DNA without high salt condition is relied on magnetic properties of magnetite particles. Oxide or hydroxides of Fe(III) have variable surface charge, mainly depending on the surface density of protons or hydroxyl groups[26]. At low ionic strength condition, as I have only applied distilled water and elusion buffer during the extraction process, they are normally positively charged and thus mainly interact with anions such as phosphates molecules[27], of which is also the main composition of the backbone structure in DNA molecule. Several mechanisms have been suggested to play significant roles between GO and DNA adsorption, e.g. hydrogen bonding, π-π stacking and electrostatic forces. Under chaotropic conditions, the major binding mechanisms were provided by hydrogen bonding and electrostatic forces due to the present of Cation Bridge. On the other hand, the major adsorption mechanism between DNA and GO is considered to be the π-π stacking forces between the aromatic ring in GO and nucleobases of DNA molecule[25, 33, 34, 35, 36, 37] when only distilled water and elusion were applied for the extraction process. According to the results, that shown in Fig. 5, the adsorption of pure GO/Cellulose was limited and the addition of Fe3O4 significantly enhanced the interaction between the composite and DNA. As shown at Fig. 6 (a) Fe_3O_4 molecules on the surface of the composite can hold the DNA molecule with magnetic force on DNA's phosphate backbone structure in the same time GO sheets under the Fe_3O_4 molecule increases the adsorption by π-π stacking force where occurs between GO and nucleobase ring structures (Fig.6 (b)). The schematic of proposed binding mechanisms of GO/Cellulose/Magnetite is shown in Fig. 6.

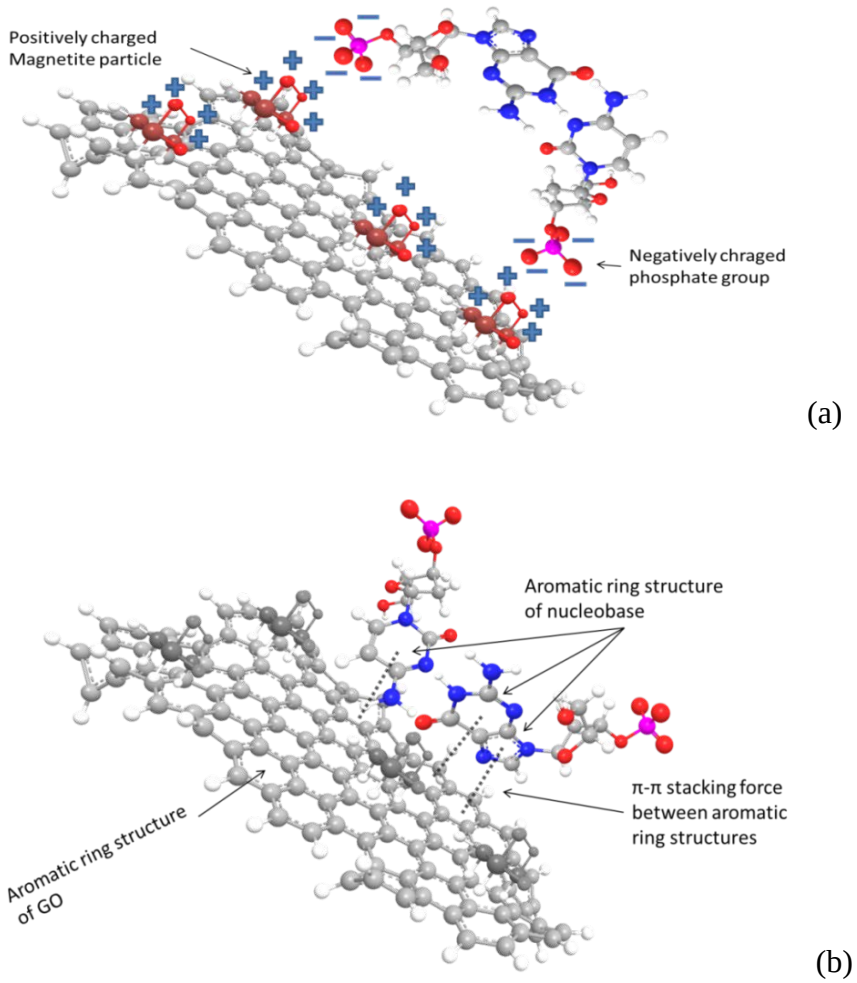


Figure 5.6- Proposed mechanism of the adsorption and desorption of DNA by Fe_3O_4 (a) and GO (b) on Fe_3O_4/ GO/Cellulose Composite.

5.4.Conclusion

In the present publication, I have demonstrated the preparation of GO/Cellulose/Magnetite composites with designed properties and the composites have been examined as solid-phase DNA extraction material without the present of chaotropic salt. The GO/Cellulose/Magnetite composites were present as dark brown spherical structure with an average diameter of 2.6 mm. The extraction of DNA was conducted under pure distilled water followed by elusion buffer. The highest performance was demonstrated by 3.98 wt. % GO/Cellulose/Magnetite, where the extraction efficiency was 18.5 times higher than that of pure GO/Cellulose material without Fe_3O_4. The ratio of A260/A280 were reported 1.36 and

3.98 wt.% GO/Cellulose/Magnetite as 1.81, which indicated high quality of DNA was successfully selected. By increasing the Fe_3O_4 wt.%. The proposed binding mechanisms of GO/Cellulose/Magnetite where as follows: Fe_3O_4 adsorbed the DNA molecule by its phosphate backbone structure with magnetic force where GO increased the adsorption by π-π stacking force. The combination of two different binding mechanisms significantly enhanced the efficiency of extracted DNA under low ionic strength condition. I believed that GO/Cellulose/Magnetite composites are highly possible to serve as a new generation DNA extraction solid support and highly applicable to manual extraction systems or on-site DNA extraction kits. The propsed process is free of chaotropic salt, which can reduce the risk of interference with down-stream PCR applications. In addition, the elimination of chaotropic salt in the process is beneficial in terms of economical aspect and handling.

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Chapter 6

Summary

Chapter 6 Summary

6.1 Summary

The main purpose of this study is to produce an innovative solid support material for DNA extraction applications. First NaOH-urea-water system was proposed in order to dissolve cellulose and graphite oxide in this solution. Afterwards this homogenous solution excluded to coagulation solution and successfully creates bead structure with a diameter of 2.6 mm and a standard deviation of 0.1387 mm. Based on the above method, homogenous composites in terms of size and surface structure were successfully obtained. After producing the composite, they were applied as the extraction solid support for different types of DNA from various sources. The extraction efficiency of each DNA samples were compared to the commercial solid support DNA extraction materials that are available on market. Table 6.1 has summarized the total yield, extraction efficiency and the purity of various type of DNA from different extraction material.

Table-6.1 General comparison of QIAamp mini kit and GO/Cellulose bead with various samples

		ssDNA	Cigarette bud	Chewing gum	Animal Tissue	Nail clips	Hair
Total yield (ng)	QIAamp mini Kit	81200	81	74.5	724	117	86
	GO/Cellulose bead	132080	1880	1800	2740	1710	1700
Extraction Efficiency (ng/µl)	QIAamp mini Kit	660.4	0.405	0.3725	3.62	0.585	0.43
	GO/Cellulose bead	406	4.7	4.5	13.7	4.275	4.25
Purity 260/280nm (nm)	QIAamp mini Kit	1.89	2.29	2.81	1.5	6.92	8.37
	GO/Cellulose bead	1.85	1.82	1.6	2.0	1.92	1.65

After our experiment results it is obvious that GO/Cellulose composite were superior to the commercial silica ssolid support. From the experiments in chapter 3 and 4 single strand DNA (ssDNA), genomic DNA with either long and heavy plasmids or short and light plasmids were applied. GO/Cellulose composites showed higher extraction efficiency, better purity and higher total yield compare to commercial silica surface. On the other hand silica solid support materials require centrifugation or vacuum filtration process for elution step. These applications cause share stress and damage the intra-structure of DNA molecule, where GO/Cellulose beads does not require any post treatment. As it was mentioned in the introproduction, high Salt concentration solutions are widely used to bind the DNA onto the solid support surface. It is an easy and rapid method for extraction applications, however, salts are often remaining on the surface or staying inside the DNA molecule after the elution processes. It is one of the major trouble for downstream process such as PCR or etc. In order to prevent this problem, I have modified the GO/Cellulose beads with magnetite particles. Magnetites can beapplied in extraction applications but due to their low binding capacity and elution difficulties, they are generally mixed with suitable polymers to increase their binding capacity. Starting from this point, another innovative approach was proposed. Magnetites particles can be mixed in GO/Cellulose solution in order to synthesis GO/Cellulose/Magnetite beads for extraction applications in Na^+ free environment. Commercial magnetic bead based DNA extraction kit were compared to the performance of our new material. In chapter 5, ssDNA and genomic DNA samples were both applied as DNA sources. The binding capacity, yield and purity of extracted DNA by GO/Cellulose/Magnetite composites showed superior performance compare to commercial magnetic beads based materials. A brief summary of the above results were conducted in table 6.2.

Table-6.2 General comparison of Dneasy Magnetic bead and GO/Cellulose Magnetice bead with two different type of DNA samples

		ssDNA	Animal Tissue
Total yield ng	Dneasy Magnetic Bead	44	408
	GO/Cellulose/Magnetite bead	67700	219700
Extraction Efficiency ng/µl	Dneasy Magnetic Bead	2.2	2.04
	GO/Cellulose/Magnetite bead	338.5	1098.5
Purity 260/280 nm	Dneasy Magnetic Bead	1.50	1.34
	GO/Cellulose/Magnetite bead	1.81	1.65

DNA interaction with carbon based materials has been known for a very long time. Since the discovery of ssDNA adsorption on the graphene sheets, DNA integrated systems such as biosensors were fabricated. Transportation capability also helped to improve new systems in living cells and in vivo systems. However up to date there was no report or research about applying carbon based material to DNA extraction systems. In this study, I showed that carbon based materials are good candidates to replace silica based materials. Additionally by adding magnetite particles I could create extraction process under Na^+ free environment which is a one of the first application in this research area. For future applications, magnetic composites are also applicable in automatic systems. In the point of this view a new high performance DNA extraction materials are always going to be needed. The DNA extraction automated systems are also hold an important area. In the times of high sample numbers, quick and reliable processes are mostly favored, especially by hospitals or Nucleic acid companies. Because carbon based materials are very open for easy modification,

thus I believed that in the future GO/Cellulose beads materials are going to find application at automated systems.

Achievements

List of Publications

(1) AKCEOGLU, Garbis A., Oi Lun Li, Nagahiro Saito.- Adsorption and Desorption of DNA Tuned by Hydroxyl Groups in Graphite Oxides-based Solid Extraction Material. *Colloids and Surfaces B: Biointerfaces.* 1 December 2015, Volume 136, Pages 1–6

(2) AKCEOGLU, Garbis A., Oi Lun Li, Nagahiro Saito.- Innovative Graphite Oxide-Cellulose based Material Specific for Genomic DNA Extraction. Journal of Minerals, Metals and Materials Society. November 2015, Volume 67, Issue 11, pp 2557-2563

(3) AKCEOGLU, Garbis A., Oi Lun Li, Nagahiro Saito.- High Efficiency DNA Extraction by Graphite Oxide Cellulose Magnetite Composites under Na+ Free system. Journal of Minerals, Metals and Materials Society. October 2015 (Accepted at 22 December 2015)

List of awards

(1) Best Students Poster Rewards: "Graphite Oxide/Cellulose Beads for DNA Extraction Applications", Biomimetic Material Processing (BMMP15) 23 – 26 January, 2015 Nagoya University, Japan

List of Patents

(1) Patent number: C / JP2014 / 0269 Title: "GO/cellulose composite as a solid support material for DNA extraction applications" **Investigators:** Nagahiro SAITO, Oi Lun LI, Garbis Atam AKCEOGLU **Investor:** Nagoya University, Japan

(2) Patent number: PCT / JP2015 / 83438 Title: GO / Cellulose / Magnetite composite as a solid support for DNA extraction applicaitons **Investigators:** Nagahiro SAITO, Oi Lun LI, Garbis Atam AKCEOGLU **Investor:** Nagoya University, Japan

List of presentation in international conferences

2015- International Symposium on Advance Plasma Science (ISPlasma2015) 26 March- 31 March 2015 Nagoya, Japan "Graphite Oxide/Cellulose Beads for DNA Extraction Applications" G. Akceoglu, O.Li, N. Saito

2014- Japan Institute of Metals (JIM2014) 29 – 31 March 2014 Osaka, Japan "Preparing Cellulose/graphene composites by modified marcano's method" G. Akceoglu, O.Li, N. Saito

2013 – Japanese Society of Applied Physics (JSAP-MRS Joint Meeting) 16 – 20 September 2013 Kyoto, Japan "Controlling Oxidation level of Graphene/Graphene Oxide by Solution Plasma from the Exfoliation of Graphite Oxide" G. Akceoglu, O.Li, N. Saito

2013 – International Conference of Surface Engineering (ICSE2013) 18 – 21 November 2013 Busan, Korea "Characterization of Micro Crystalline Cellulose on the surface of Graphene/Graphene Oxide Sheets by Thermal Exfoliation Method" G. Akceoglu, O.Li, N. Saito

2013- European Material Research Society (EMRS2013) 27 – 31 May 2013 Strasbourg, France "Production Improvement and Modification of Graphene Oxide Sheets by Using Solution Plasma Processing for ORR Catalytic Activity" G. Akceoglu, O.Li, N. Saito

2013- International Union of Materials Research Society (IUMRS-ICA) 2013 16-20 December 2013 Bangalore, India "Graphene/Graphene Oxide Production by Solution Plasma in Different Solutions for Potential Applications of Fuel Cells" G. Akceoglu, O.Li, N. Saito

2013- International Symposium on Advance Plasma Science (ISPlasma2013) 28 January- 1 February 2013 Nagoya, Japan "Modification of Graphene Oxide Sheets for ORR Catalytic Activity by Using Solution Plasma Processing" G. Akceoglu, O.Li, N. Saito

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Supplementary Information

Supplementary Information

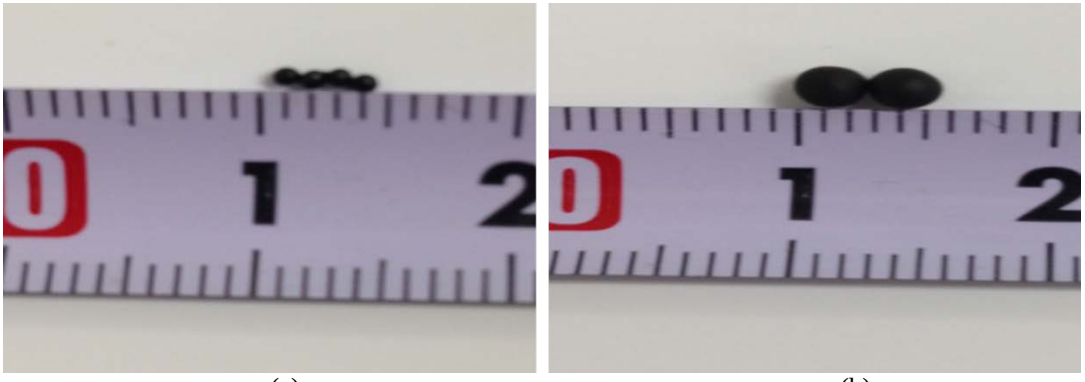
S 1 Binding Efficiency in terms of surface area

S 1.1 Introduction

There are many aspects to affect DNA binding to the matrix at nucleic acid purification applications. These effects can be listed as types of binding and elution buffer solutions, physical properties of DNA molecule, pH of solutions, temperature of environment, surface properties (surface charges, functional groups etc.). Beside these effects, surface area is also an important factor in terms of binding capacity. All materials used in DNA extraction applications have high surface area for increased binding capacity. In our experiments surface area was not the first aim so information about the surface area was incomplete, in this supplementary information we'd like to complete the information about surface area properties.

S 1.2 Experimental

Prepared GO/Cellulose composites have 2 states. They usually kept in distilled water for further experiments and inside liquid solutions; they have soft and comparatively large size (2.6mm diameter). On the other hand if we take composite out of solution and keep, in the room temperature (for one day) or at the oven (for 3 hour at 100°C) composite shrinks and eventually have a small size (0.9 mm diameter) and rigid structure.



(a) (b)

Figure 7.1- Dry state (a) and Liquid state (b) of GO/Cellulose composites

In order to compare silica matrix and GO/Cellulose composite surface area and pore size properties, we used the belsrop device. But because of the GO/Cellulose previously explained properties, when we are using the belsorp device we can only calculate the dry state surface area and the pore size of the bead. From this point we can only estimate an average surface area of the liquid state of the bead composite.

S 1.3 Results

After using belsorp device experimental results showed that dry state of the five GO/Cellulose composite beads have 39.2 m^2/g surface areas where silica matrices have 185.3 m^2/g. As expected silica matrices have six times higher surface area compare to dry state GO/Cellulose composite. After measuring surface area of the GO/Cellulose composite we estimate an average surface area value for the liquid state GO/Cellulose by proportioning to the dry state. For this proportioning first we are thinking that; if these beads were non-porous spheres with a flat surface their surface area are going to be calculated by $4\pi(r)^2$ formula. According to this formula dry state surface area will be 0.03 cm^2 and liquid state surface area is going to be 0.21 cm^2. However because of porous structure and rifts on the surface of dry state belsorp device calculated surface area as $8m^2/g$. If the surface area of dry state (0.03 cm^2) and liquid state (0.21 cm^2) are seven times difference from each other in the same way belsorp results is as expect to be more or less around seven times different. So if dry state has $8m^2/g$ surface area liquid state should be 56 m^2/g.

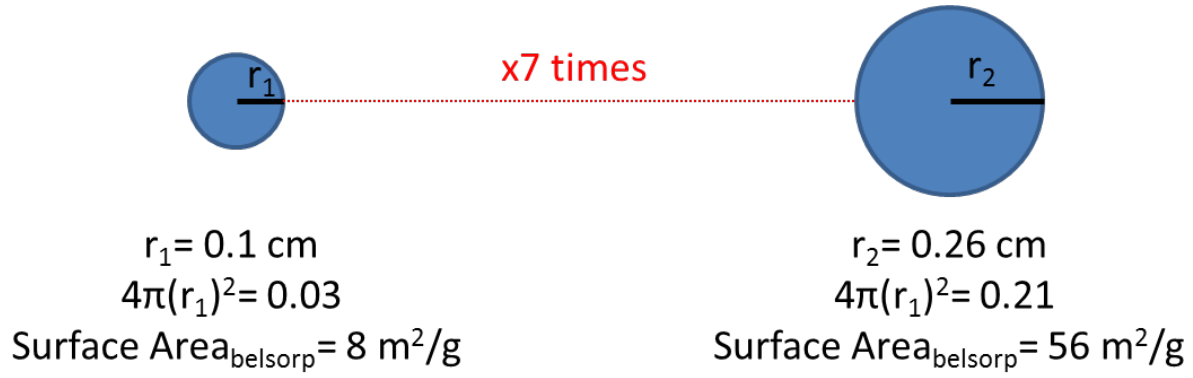


Figure 7.2 Comparison between dry state and the liquid state of GO/Cellulose composite

After calculating surface are of liquid state of GO/Cellulose we can convert extraction efficiency unit from ng/µL to ng/cm^2 unit. For this conversion we are using the total yield of the composite and then divide it to total surface area. For example 4.15 wt. % GO containing composite has 132080 ng total yield of DNA. This amount of DNA is collected from (56 is for one bead surface area for DNa extraction application we are using 5 beads) 280m^2 per gram surface area which equals to 2800000 cm^2 per gram. So by dividing 132080 to 2800000 we can obtain DNA extraction efficiency by ng/cm^2. According to this calculation 4.15 wt. % containing GO composite showed 0.04 ng/cm^2 binding capacity. From this calculation pathway we convert all experimental result data from ng/µL to ng/cm^2.

Sample Name	total yield	ng/cm^2
Cellulose + 0%GO	12850	0.004
Cellulose + 2%GO	13650	0.004
Cellulose + 10%GO	14800	0.005
Cellulose + 22%GO	55700	0.019
Cellulose + 45%GO	132080	0.047
Qiagen mini spin column	81200	0.029

Table S1 As the GO concentration increased inside the composite binding performance per surface area increased proportionally.

	GO/Cellulose composite		Qiagen Investigator Kit	
Source of Genomic DNA	**Total yield (ng)**	**ng/cm^2**	**Total yield (ng)**	**ng/cm^2**
Cigarette bud	1880	0.0004	81	0.00004
Chewing gum	1800	0.0004	74.5	0.00004
Animal Tissue	2740	0.0006	724	0.0003
Nail clips	1710	0.0003	117	0.00006
Hair	1700	0.0003	86	0.00004

Table S2 Forensic DNA samples used as a genomic DNA source for DNA extraction applications. GO/Cellulose composite showed higher extraction efficiency per surface area compare to commercial Qiagen DNa extraction kit.

Sample Name	**total yield**	**ng/cm^2**
CGM0	3650	0.001
CGM3	42550	0.001
CGM9	55100	0.019
CGM15	59700	0.021
CGM45	67700	0.024

Table S3 Under chaotropic saltless DNA extraction systems, by increasing magnetite concentration inside GO/Cellulose composite DNA binding performance increased proportionally.

S 1.4 Conclusions

We prepared GO/Cellulose composites with a bead shape and comparatively large size. At bead shape materials it is a must to have nano size composites for higher surface area properties. In our research our first aim was to show GO effect for DNA binding at different conditions. Further experiments are going to be about decreasing the bead shape into nano size and provide a new material for better DNA extraction applications at manual and automated systems.