Carbon Nanotubes for the Stabilization of Lipid Nanostructured Particles

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Type of Award **Master of Science (by Research)**

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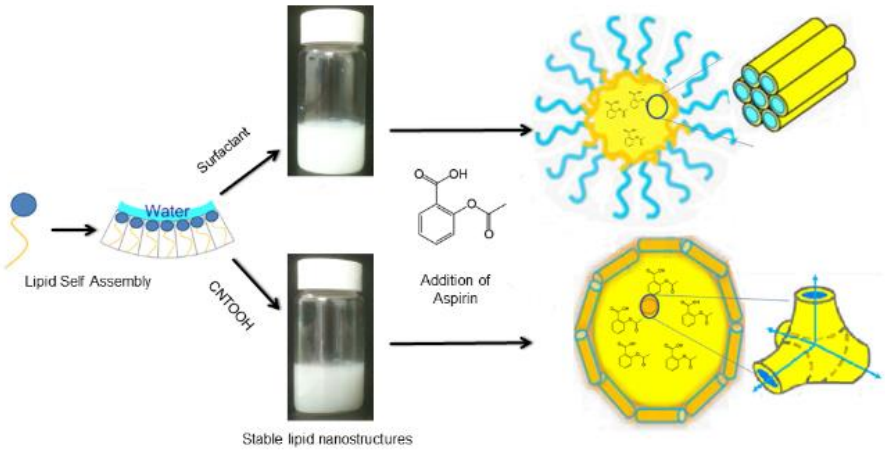
Abstract

Lipid molecules are amphiphilic due to hydrophilic head-group and hydrophobic alkyl chain/s. Lipids are known to self-assemble into remarkable nanostructures in the aqueous medium. In order to enhance their applicability, these nanostructures are further stabilised into nanoparticles using various stabilisers; resultant are the oil-in-water emulsions, which are common in many food and consumer products. Surfactant based stabilisers have been very popular; however, for particular applications the surfactant molecules are not completely beneficial. In this case, the solid materials are employed to obtain surfactant-free emulsions also called Pickering (or Ramsden Pickering) emulsions. Natural and synthetic clays, silica nanoparticles and hydrocolloids have been used for this purpose. Carbon nanotubes (CNTs) have received noteworthy attention due to their unique structural, physical and electrical properties. These properties allow use for a huge range of applications, of which this thesis will outline. In the past, the hydrophobic surface of CNTs have limited their potential in many fields, as well as their tendency to aggregate in most solutions, leading to large amassed particles which prove near-impossible to disperse. The ability to stabilise lipid and CNTs would benefit several areas and applications such as drug delivery and gene therapy. With the techniques in the following report, however, the study has proven that the combination of Dimodan U (DU) and CNTs can form stable nanostructured dispersions, which remain homogeneous for several months. This idea proposes a new way to delivery simple drugs and by tweaking the nanostructure, through alteration of the ratios of CNT to lipid, these systems could be used in targeting different organs.



Graphical Abstract: Internally Nanostructured Lipid Particles Stabilised by Surfactant and Carbon Nanotubes for drug loading.

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List of Abbreviations

CA	Contact Angle
CCD	charged coupled device
CNT	Carbon Nanotube
-COOH	Carboxylic acid functionalization
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DU	Dimodan U
EM	Electromagnet
f-CNT	Functionalized Carbon Nanotubes
FMDV	Foot and Mouth Disease Virus
f-SWCNT	Functionalized Single-walled Carbon Nanotubes
FTA	First Ten Angstroms
GnRH	Gonadotrophin-releasing hormone
MWCNT	Multi-walled Carbon Nanotubes
NanoLCP	Nanostructured Lipid-Carbon Nanotube Particle
O/W	Oil in water
-OH	Hydroxyl functionalization
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
pH	Potential Hydrogen
PL-PEG	Phospholipid-PEG
PS	Phosphatidylserine
SAXS	Small Angle X-ray Scattering
SDS	Sodium dodecyl sulfate
SWCNT	Single-walled Carbon Nanotube
UV-Vis	Ultraviolet-Visible
XRD	X-ray Diffraction

Contents

Chapter 1: Introduction

Understanding the relationship between structure and function of materials is of the utmost importance in the modern scientific community. Nanomedicine is an emerging discipline, likely to revolutionise our lives in general, and health in particular. It defines as the ability to manipulate matter at the nanoscale (0.1 nm to 1000 nm) and has resulted in several new materials, products, and devices that show original and unfamiliar behaviour. Nanomedicine is now a widely used term, which refers to all nanomaterials, nanoparticles, and nano-composites used for biomedical purposes, which implies the medical application of nanotechnology and related research lead in to the designing, testing, and optimizing of the pharmaceutical formulations [1] Nanotechnology is the science of particles in the nanoscale range (1 nanometre to 1 micrometre) having unique properties, which change when the size of the particles as well as the melting point, electronic and optical properties are altered extensively [2,3]

1.1 Carbon Nanotubes

In this nanotechnological development, carbon nanotubes (CNTs) have attracted a great deal of attention. Bacon, in 1950, is recorded for discovering the first CNTs but at that time they were not fully appreciated. It was not until 1991, when Iijima, a Japanese researcher, described the CNTs and proposed them as an exciting new material for drug delivery as novel carrier systems, due to their unique structural properties [4] (Iijima S, 1991). A Carbon Nanotube can be defined as carbon atoms arranged in a series of condensed benzene rings or graphite sheets rolled up into a cylindrical structure. Along with diamond and graphite, these materials belong to the fullerenes family. CNTs are completely insoluble in water and are biologically non-degradable. CNTs can be sub-

classified into Single-walled carbon nanotubes (SWCNTs), and Multi-walled carbon Nanotubes (MWCNTs).

1.2 Properties and Advantages of Carbon Nanotubes

Mehra et al (2008) stated that the increase in the use of CNTs was due to their outstanding mechanical strength, as well as thermal conductivity, biocompatibility and ultra-light weight. These traits, as well as their high surface area available for functionalization, make them extremely competent to use in a huge range of industrial and medicinal sectors. [1]

The same paper, also reported by Jain et al (2007), listed the following as advantaged CNTs may offer over other colloidal systems [3]. These include traits such as biocompatibility and non-immunogenic nature, non-biodegradable, highly elastic nature. Intracellular delivery is also possible, and, on the basis of in-vitro tests, exhibit minimum cytotoxicity. CNT toxicity is a topic fiercely debated, but the study also suggests that 96% is excreted by urine and remaining 4 % by faeces [1]. CNTs have an ordered structure with high aspect ratio and do not break down during processing as carbon fibres do so easily, despite being ultra-light weight. It has open end on both sides, which makes the inner surface accessible and subsequent incorporation of species within nanotubes is particularly easy. Nanotubes have longer inner volume relative to diameter of nanotubes for entrapment. CNTs are able to enter cells by spontaneous mechanism due to its, tubular and nano-needle shape. It has distinct inner and outer surface, which can be differentially modified for chemical or biochemical functionalization.

1.3 Drug Delivery using Carbon Nanotubes

The advances in nanotechnology and nano-medicine have enabled ground-breaking solutions in the field of drug delivery. Whereas conventional chemotherapeutic drugs often cause severe side effects and are not tumour-specific, nanoparticles preferentially accumulate in tumours through the "enhanced permeability and retention" (EPR) effect. In comparison to their small-molecule counterparts, nanoparticle drugs have proved to have higher intratumoural drug concentration and lower normal-tissue concentrations [5]. Additionally, nanoparticles, which contain high surface area-to-volume ratios, can be potentially engineered to carry tumour-targeting molecules, tissue permeation enhancers, two or more types of therapeutics for more efficient cancer treatment [39].

Carbon nanotubes (CNTs) offer opportunities for chemotherapy drug delivery. Structurally, CNTs can be viewed as a tube rolled from layers of graphene sheets. CNTs can then be further sub-categorised depending on the number of sheets. CNTs that have only one layer of graphene are classified as single-walled carbon nanotubes, whereas multi-walled carbon nanotubes are CNTs with multiple layers of graphene. Due to their ordered structure, CNTs show outstanding physical traits, including ultra-high surface area, high aspect ratio, high tensile strength [7,8,9], excellent optical [10], electrical and thermal properties [11,12,13]. It has been found that CNTs easily penetrate all sorts of cells, including cells thought to be hard to transfect [14]. CNTs are being widely explored for potential biological applications because of their size, unique shape, and structure, as well as their physical properties.

CNT toxicity is a field that is heavily under research, both *in vitro* and *in vivo.* Studies have proven that that appropriately functionalized CNT, *e.g*. polyethylene glycol (PEG) functionalized CNT does not cause noticeable toxicity to the treated animals [15]. Bio-distribution of the lipid-polymer, phospholipid-PEG (PL-PEG), functionalized SWCNT

showed that CNTs are safe due to their ease of excretion *via* the biliary and renal pathways after intravenous injection [16,17]. These results have paved the way for applications of CNTs in cancer therapy. Recently, SWCNTs have been used in a variety of biomedical applications extending from cancer drug delivery to tumour imaging and detection [13].

Since CNTs are pre-formed supramolecular nanotubes, the drug loading to this structure can prove to be extremely difficult. SWCNTs can be filled with a large variety of compounds, including organic molecules [14,15] and inorganic materials. [16,17] Chemotherapeutic drugs have been previously loaded into the interior of SWCNTs using a capillarity-induced filling. However, the loading amount is below 5% (w/w) [18]. This being a rather small percentage, researchers have also investigated the loading of small molecule drugs directly onto the CNT surface. It was discovered that pre-functionalized CNTs have large surface areas, which allows for direct attachment to some hydrophobic drugs that contain flat benzene ring structures. One of these studies investigated the adsorption of doxorubicin onto a SWCNT surface [19]. This method could yield a very high loading amount (400% by weight). When the drugs are loaded directly into a CNT, the CNT-coating polymers are freed for conjugation from other functionalities, *e.g.* targeting molecules, antibodies, fluorescence molecules or other drugs for multifunctional delivery [19]. However, drugs such as paclitaxel, with a bulky structure, do not absorb drugs especially well and as a result; the subsequent formulations are not stable.

Drug delivery in conjunction with CNTs is a new technology in the pharmaceutical science. As stated before, CNTs have a great potential in this regards for the delivery of drugs, antigen, vaccines. Several studies have shown the many benefits regarding the use of these materials in a medicinal use.

Liu et al., 2007 studied the arginine-glycine-aspartic acid (RGD) peptide and Polyethylene glycol (PEG) functionalized Single walled carbon Nanotubes (*f*-SWCNTs) on the delivery of doxorubicin, which is an anticancer drug. The in-vitro release profile revealed no toxic effects on the cancer cell, and shows the drug release was pH and diameter dependent. They also reported the CNT-doxorubicin transporter led to targeting and show inhibition of specific cells, which could open a new opportunity in cancer chemotherapy. [20]

A report by Yu et al., 2007 described the releasing and *in-vitro* activity of gonadotrophin-releasing hormone (GnRH) from the modified MWCNTs in cancer cells. GnRH plays a pivotal role in the regulation of the pituitary/gonadal axis and reproduction. This hormone is used as a targeting ligand to its receptors, which are overexpressed in the plasma membrane of different types of cancer cells. This study proved that GnRH-CNTs modify uptake receptors and also aid making GnRH-CNTs toxic to cancer cells. [21]

Pantarotto et al., 2004 reported the delivery of plasmid DNA from the functionalized CNTs for gene therapy. The study revealed the better response and was internalized within the mammalian cells and delivery of the DNA in to the cells. [22] In earlier works by Pantarotto and his team, it was reported the cellular uptake of free peptides and oligodeoxynucleotides is extremely poor. Because of this, conjugation of these molecules onto CNTs surfaces may allow improvements in the delivery of such biological molecules. Functionalized SWCNTs have been the focus of several studies for vaccine delivery, in this case by 1, 3 dipolar cyclo-addition attaching a small peptide sequence for the foot and mouth disease virus (FMDV). SWCNTs-FMDV peptide

complex induced a better antibody response and no cross reactivity was observed *in-vivo* for SWCNTs-FMDV conjugate. [23]

1.4 Functionalization

In covalent functionalization techniques, modifications to the sidewall and to the ends are the two major techniques for the surface alteration of CNTs. Ends and defects are more reactive than sidewall functionalization. In covalent functionalization, more functional groups could be generated at the ends or sidewall of surface of CNTs such as carboxylic acids, ketone, alcohol etc. Additionally, several functional groups could be generated on the sidewall surfaces without loss of Von Hove singularities.

In the non-covalent functionalization techniques various types of surfactants, polymers and biopolymer (nucleic acids and peptides) have been used. Surfactant adsorption at interfaces has been widely studied because of its importance in detergents, lubrication, and colloid stabilization [24]. CNTs are insoluble in most solvents [25] and especially in water, but, with the addition of 1% solution of anionic surfactant SDS, have been reported to form stable black suspensions when sonicated [26]. When SDS is adsorbed onto a surface, it creates a distribution of negative charges that prevents their aggregation and induces stable suspensions in water. Several different possible orientations of the SDS molecules can be envisioned on the surface of CNTs. For example, the SDS molecules can be oriented perpendicularly to the surface of the nanotube, forming a monolayer [27,28]. However, according to previous studies into molecular organization, two other arrangements are possible for the surfactant at the solid-liquid interface. In these cases, the hydrophobic part of the SDS is connected to graphite by weak van der Waals interactions, and the hydrophilic end of the surfactant is oriented toward the aqueous phase, forming half-cylinders on the surface of the graphite

plane [29,30,31,32]. Because CNTs are rolled-up graphene sheets, the SDS molecules may also form similar half-cylinders on the surface of the tubes. These can either form parallel or perpendicularly to the tube axis.

Nanomedicine has also developed in the field of cell biology, with the increased research into nanosized particles such as Liposomes [33] In recent years, Particulate drug carriers including oil-in-water (O/W) emulsions, liposomes, microparticles and nanoparticles based on synthetic polymers or natural macromolecules have been the centre of many studies [34]. The O/W emulsions have been introduced successfully to the clinic for parenteral nutrition in the fifties. Based on these emulsions for parenteral nutrition, drug-containing emulsion formulations have been developed, e.g. containing diazepam and etomidate [35]. For some products, Diazemuls and Diazepam-Lipuro of note, these liposomes are sometimes only used to reduce or eradicate the side effects. In the case of Diazepam, liposome emulsions are used to reduce the pain of injection and inflammation at the injection site. Despite the excellent tolerability of these O/W emulsions the number of products on the market is relatively low, indicating a fairly low success in this research. The main reason for this limited success and the consequent lack of marketable products is the emulsion's instability, which can be caused by the incorporated drug.

1.5 Functionalized Carbon Nanotubes (f-CNTs) for drug delivery

CNTs exhibit unique structural, mechanical, and electrical properties and are intensely studied for possible use in many fields [36,37]. One approach that is currently being considered is the functionalization of their surface. To the current day, there have been several successful strategies using covalent chemistry that have been applied to the sidewall derivatization of CNTs. The single issue only being that this can alter their

inherent properties [38,39]. Thus, the prospect of functionalizing their outer surface in non-covalent ways through the chemical adsorption [40,41]. seems like a very attractive option. *f*-CNTs are useful in multiple ways. For one, it improves the solubility of CNTs, which are completely insoluble in aqueous solutions. It also helps to reduce the aggregation behaviour of individual tubes. It could provide the efficient intracellular uptake and improved efficacy, which enhance the internalization of CNTs within cells due to its nano-needle shapes as well as increasing the possibility of the attachment of various functional groups onto the surface of CNTs.

1.6 Lipids, liposomes and Isasomes

Phospholipid vesicles, rediscovered as 'liposomes' in 1965 by Bangham, found their way to the cosmetic market in 1986 [42] as the anti-aging product Capture (Dior). This helped smooth the way for liposome-based pharmaceutical products. By the beginning of the nineties, the first pharmaceutical products came to the, and included the synthetic lung surfactant Alveofact (Dr Karl Thomae GmbH/Biberach in Germany) for pulmonary instillation, Epi-Pevaryl, a topical product for anti-mycotic therapy (drug: econazole) and other products for intravenous injection (e.g. Ambisome with amphotericin and cytotoxic-containing formulations like Doxil and Daunosome) [34]. This being said, the total number of products on the market is still quite limited. One of the reasons for this–apart from possible technological problems–is the non-availability of a 'cheap' pharmaceutical liposome.

The number of products based on polymeric microparticles on the market is low. After the introduction of the first wave of products, there was only a slight increase in the number of microparticulate products. The situation worsened for polymeric nanoparticles, after more than 30 years of research; this delivery system still practically

does not exist. An exception is the product Abdoscan produced by the company Nycomed, however, this is not a formulation for chronic treatment. Rather, it is a diagnostic agent. [43]

Liposomes are a form of vesicles that consist either of many, few or just one phospholipid bilayers. The polar character of the liposomal core enables polar drug molecules to be encapsulated. Amphiphilic and lipophilic molecules are solubilised within the phospholipid bilayer according to their affinity towards the phospholipids. Liposomes have the advantage of being both non-toxic and biodegradable because they are composed of naturally occurring substances. Materials that are encapsulated within liposomes are perfect for use in drug carrier systems as they are protected to varying extent from immediate dilution or degradation. These materials also have the unique ability to entrap drugs both in an aqueous and a lipid phase, making such delivery systems attractive for hydrophilic and hydrophobic drugs. Recently, high-entrapment efficiencies are becoming more possible, due to advancements in the methods of preparing and formulating liposomes, creating a tremendous pharmaceutical impact. Additionally, such encapsulation has been shown to reduce drug toxicity while retaining or improving the therapeutic efficacy. Several studies have claimed the use of liposomes as drug carriers aid in the treatment of cancer, [34,35] leishmaniasis,[42] metabolic disorders, and fungal diseases.[43]

Making up a large and diverse group of naturally occurring organic compounds, lipids play an essential role in the cell membrane structure. Although there is great structural variety among the lipids, with fats, phospholipids and steroids all being a part of the family, they are related by their insolubility in water, and their solubility in non-polar organic solvents such as ether, chloroform and benzene [44,45]. Phospholipids are one of the major groups of the four membrane lipids. These phospholipids are derived from

the three-carbon alcohol, glycerol, or from sphingosine. The common alcohol components of phospholipids derived from glycerol are called phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI). Due to inexpense and readiness in bulk, phospholipids are very commonly used in the preparation of lipid vesicles and already have very well established and characterized preparation techniques. They occur through an esterification process of one of the primary hydroxyl groups of glycerol to phosphoric acid through its hydroxyl group. The two remaining hydroxyl groups of the glycerol backbone form the non-polar tail end of the lipid by esterification to either saturated or unsaturated fatty acids and form the non-polar tails of the lipid. [45]

Lipids are a naturally occurring surfactant group that form non-covalent aggregates in the presence of an aqueous environment like water [46]. Liposomes are the most known structures composed of lipids curled into spherical shells in an aqueous environment [47]. Very similar to lipid bilayers that form the cell membrane, they share many dimensional, structural and functional properties [48].

Both liposomes and cell membranes can be described by the "fluid mosaic model", which was proposed in 1972 by Singer and Nicholson [49]. The model states that, as the head groups are hydrophilic, they can be left exposed to the aqueous environment while the hydrophobic lipid tails can orientate towards each other. As a result these lipids spontaneously organise in concentric spheres and sheets of bilayers when dispersed in water. However, they may assemble into other types of structures, called meso-structures rather than vesicles under specific conditions [50,51]. The size and shape of the resulting aggregates mostly depend on geometry of the lipid, as well as its chemical properties and the physical conditions (e.g. temperature, pH and salinity). In addition to

these properties, attractive-repulsive forces (DLVO Theory) and thermodynamic interactions have a role in the vesiculation process, due to the amphiphilic and ionic properties of the lipids [52]. The self-assembly of lipids to vesicles relies on two main phenomena, the theory of spontaneous vesiculation and the curvature theory, dominated by the physiochemical properties of the phospholipids such as lipid head group, tail length and saturation level [53].

Isasomes are ordered colloidal systems, i.e., nanometre-sized droplets, which are dispersed and stabilised as aqueous emulsions with self-assembled internal structures. These structures can organise in several different ways: fluid isotropic water-in-oil (W/O) microemulsions (L2 phase), or liquid-crystalline mesophases, including bicontinuous cubic, hexagonal, and discontinuous micellar cubic.

Isasomes are usually stabilized by surfactants, such as Pluronic F127, or block copolymers. However, such O/W emulsions are also known to be stabilized by proteins and nanoparticles, the latter are called Ramsden–Pickering or Pickering emulsions. Isasomes and other nano-structured dispersions find applications in a wide range of fields, ranging from pharmaceutics to foods. The internal structure of Isasomes can be tuned by varying the δ value into cubic, hexagonal or even fluid isotropic structures,

Bulk liquid crystalline phases can be detected via rheological techniques thanks to their characteristic viscosity, which can be determined by the amount of dispersed (hydrophobic) phase. This viscosity can be further adjusted by adding hydrogelling agents to the continuous water phase. In this way, the Isasomes can be entrapped into a thermoreversible hydrogel network which may help controlling uptake and release rates by Isasomes.[53]

Chapter 2

Materials

Single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes with -OH functionalization (MWCNT-OH) were obtained from Nanoamor (Houston, USA) whereas –COOH functionalized multi-walled CNTs (MWCNT-COOH) were purchased from Sigma-Aldrich (Athens, Greece). The lipid, Dimodan U/J (DU) was a generous gift from Danisco (Brabrand, Denmark). It is a commercial product containing more than 98% monoglycerides. The triblock copolymer pluronic F127 (PEO_{99}-PPO_{67}-PEO_{99}) was purchased from Sigma-Aldrich, UK. All solutions were prepared with Millipore water.

Methods

2.1 Ultrasonication

The stable nanostructured systems were prepared by Ultrasonication using the ULT065 from Ultrawave on samples made from pre-dispersed solutions of CNTs and Dimodan U (DU) lipid that had been gently heated to slightly above room temperature for ease of use. The lipid was then added to the bottle of the container before adding the CNT-solution and ultrasonicating at 40% amplitude for two minutes without pulsating.

2.2 Dynamic Light Scattering

Dynamic Light Scattering (DLS) is a technique using the intensity of light scattered by the molecule as a measurement of time. When light is scattered by a molecule or particle some of the incident light is scattered. If the molecule was stationary then the amount of light scattered would be a constant. However, since all molecules in solution diffuse with Brownian motion in relation to the detector there will be interference (constructive or destructive) which causes a change in

light intensity. By measuring the time scale of light intensity fluctuations, DLS can provide information regarding the average size, size distribution, and polydispersity of molecules and particles in solution. The faster the particles diffuse, the faster the intensity will change (if the light was bright enough this would be seen as a twinkling effect). The speed of these changes is thus directly related to the motion of the molecule.

Particle sizes in suspension were determined by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano-ZS and Malvern DTS version 5.00 software. Samples were ran 10 times, and an average being taken. This was repeated 5 times instantly after, all using a ZEN0118 cuvette (also from Malvern)

2.3 Small Angle X-ray Diffraction

CNT, lipid and CNT-lipid samples were dehydrated using nitrogen gas followed by vacuum drying in the desiccator for about 20 minutes. XRD measurements were carried out on a Bruker D2 phaser utilising its 0.1mm slit and a 0.1mm knife edge distance, allowing more accurate results. Samples were measured on the D2 phaser, at both low angle as well as higher angle, 1.2-10 degrees and 5-50 degrees, respectively, and analysed on the DIFFRAC.SUITE software. Samples were measured for 20 minutes for both low and high angles.

2.4 Raman Spectroscopy

CNT, lipid and CNT-lipid samples were dehydrated using nitrogen gas followed by vacuum drying in the desiccator for about 20 minutes. Spectroscopic measurements were carried out on a Horiba Jobin-Yvon LabRAM HR800 spectrometer. A 532 nm laser was utilized to collect spectra from 100 – 4000 cm-1 using a grating of 600 g mm-1 and blazed at 750 nm. Spectra were acquired

using x50 long working distance objective with a numerical aperture of 0.50. The confocal hole was set at 100 μm for spectral collections. The detector used was an Andor electromagnet (EM) charged coupled device (CCD). A video camera with the Raman system was used to guide spectral collection. All spectra were collected with sample situation on Calcium Fluoride slides (Crystran, UK). The instrumentation was calibrated before operation to silicon at the spectral line of 520.8 cm-1. Spectra were acquired using the 532 nm laser at 1 % exposure for 10 seconds and accumulated 5 times. Immediate data interrogation and manipulation was carried out on the raw data using the LabSpec 6 spectroscopy software suite (HORIBA Scientific).

2.5 Freeze-drying

In order to measure our samples more easily on Raman and other spectroscopic techniques, samples were freeze-dried using an Alpha 2-4 LD Plus from Christ. This model has vacuum control for optimization/reduction of process times and temperature monitor. 1mL flasks of several samples were placed in the freeze dryer and left for 3 days until all water was removed, leaving a darker 'fluffy' solid, which absorbs water from the air over time, once removed from the freeze dryer.

2.6 Contact Angle

To measure the contact angle of water on the CNT surface, a FTA1000 B Class from First Ten Angstroms (FTA) was utilised. Water was dropped from a dropping pipette onto the flat surface of CNT and pictures were taken upon contact with the surface. The angle of contact was then measured through FTA software to provide information of the hydrophobicity of the sample.

2.7. UV-Vis Spectroscopy

To measure our drug encapsulating samples' drug release, a UV-3600 UV-Vis-NIR Spectrophotometer from Shimadzu was used. Samples were run for 24 hours, measuring once every 30 minutes between 200nm^{-1} and 400 nm^{-1}. Equipped with a high performance double monochromator, ultra-low stray light is achieved at high resolution.

Chapter 3: Nanostructured lipid particles: Preparation, Stability and Characterization

3.1 Isasomes with F127

Isasomes, F127 and lipid in water, were formed via the ultrasonication of lipid in the presence of a solution of Pluronic F127. F127 was used as it is a known stabiliser which is also very common and cheap to use. These isasomes were very stable, opaque and white. The solution of surfactant F127 was prepared by simply stirring 200 mg of surfactant in 100 ml water. 9.5 ml of each of above solutions was then added to 500 mg of molten DU in a glass vial. The mixtures were then subjected to ultrasonication and were allowed to cool down (about 10 minutes) prior to further use.

3.2 Dispersion of Carbon Nanotubes

CNTs, on their own, are not known to disperse for extended periods of time in water. First, powdered CNTs (MWCNT-OH, MWCNT-COOH and SWCNT) were dispersed in 100 ml water using probe ultrasonicator (ULT065 from Ultrawave, Cardiff, UK) at 40% power for 2 minutes without pulse. CNTs were stable on their own (without lipid) during preparation time (for about 20 minutes).

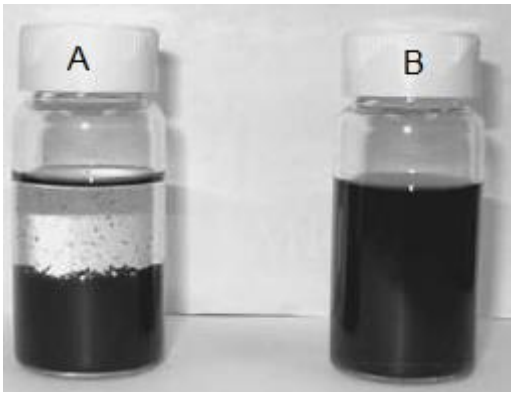

Figure 1: Photographs of nanotubes in Millipore water taken after 30 minutes after A) stirring on a hot plate at room temperature for 2 minutes and B) ultrasonicated at 40% power for 2 minutes without pulse.

3.3 CNT-Lipid Nanostructured Particles with varying F127 and CNT ratios

CNTs and Lipids were put to use together, and were able to form stable emulsions when used in conjunction. A wide range of ratios of F127:CNT were used, from 100:0 to 5:95 and finally using purely CNTs alone to compare how stable the emulsions are. These emulsions proved stable at all ratios between 100:0 and 5:95. There are complications for stabilising with only CNTs, with the complete absence of a stabiliser like F127 which are detailed in future. Despite the difficulties, it is possible to stabilise after optimization steps.

3.4 Characterization of CNT-Lipid Nanostructured Particles

Raman spectroscopy revealed COOH-functionalized CNT-lipid interactions responsible for their mutual stabilization in aqueous solutions **Figure** 2. The CNT spectra contain typical Raman graphite bands; the G band is assigned to the in-plane vibration of ‘C-C bond’, the D band (not shown) is activated by the presence of disorder in carbon systems and the G’ band attributed to the overtone of the D band. Upon interaction and formation of CNT stabilized lipid particles the G and G’ bands display a blue shift (shift

to higher wavenumbers). Table (1) lists the peak centres for characteristic bands from pure CNTs and nanostructured lipid particles containing CNTs. A blue shift, in case of CNTs, is a result of either or a combination of the following: 1) the dispersion of CNTs, as opposed to a bundled state when pure 2) coating of CNTs; Douroumis et al.[54] have shown significant blue shifts when analysing lipid coated SWCNTs and suggest that the presence of functionality affects the symmetric radial vibrations of the hollow cylinders through stiffening which are attributed to the interactions between the CNTs and the lipid molecules 3) subjecting CNTs to a high pressure. [55]

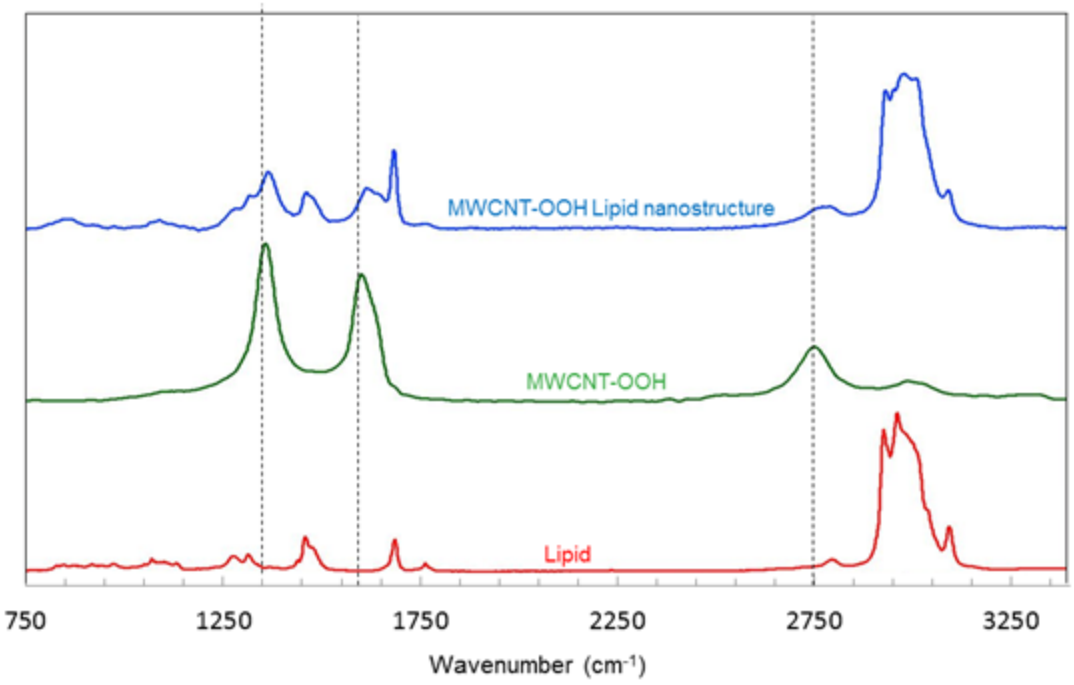

Figure 2: Original spectral plot showing the averaged Lipid, MWCNT-COOH and combined nanostructure. MWCNT-COOH and MWCNT-COOH Lipid nanostructure have both been vector normalised and offset for visual clarity. The dotted lines on the peaks correspond to the Raman Shifts seen in different bands.

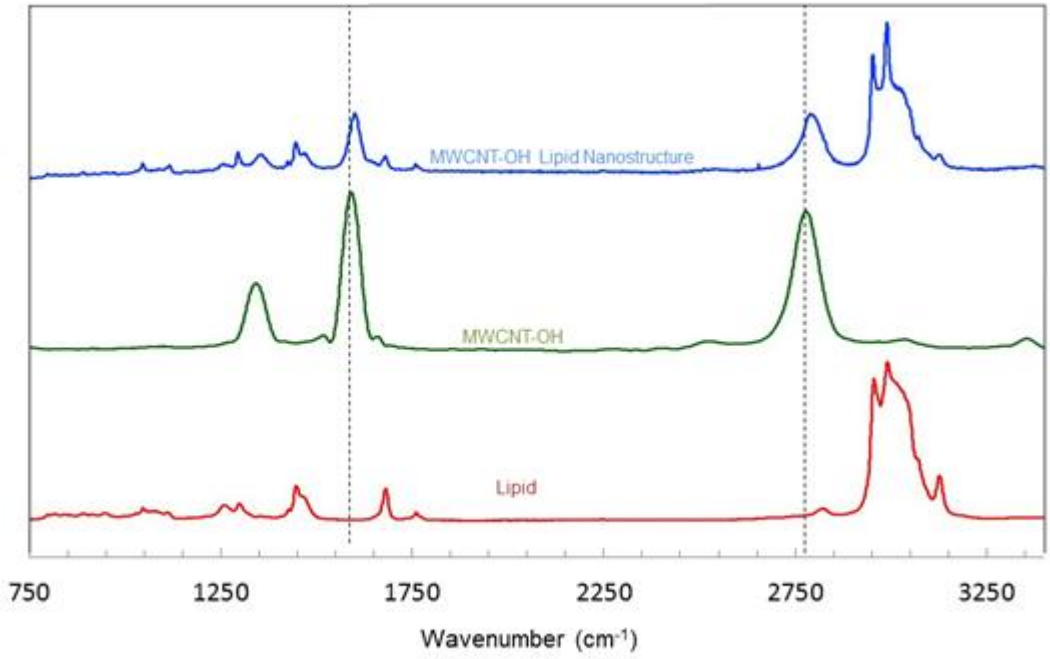

Figure 3: Original spectral plot showing the averaged Lipid, MWCNT-OH and combined nanostructure. MWCNT-OH and MWCNT-OH Lipid nanostructure have both been vector normalised and offset for visual clarity. The dotted lines on the peaks correspond to the Raman Shifts seen in different bands.

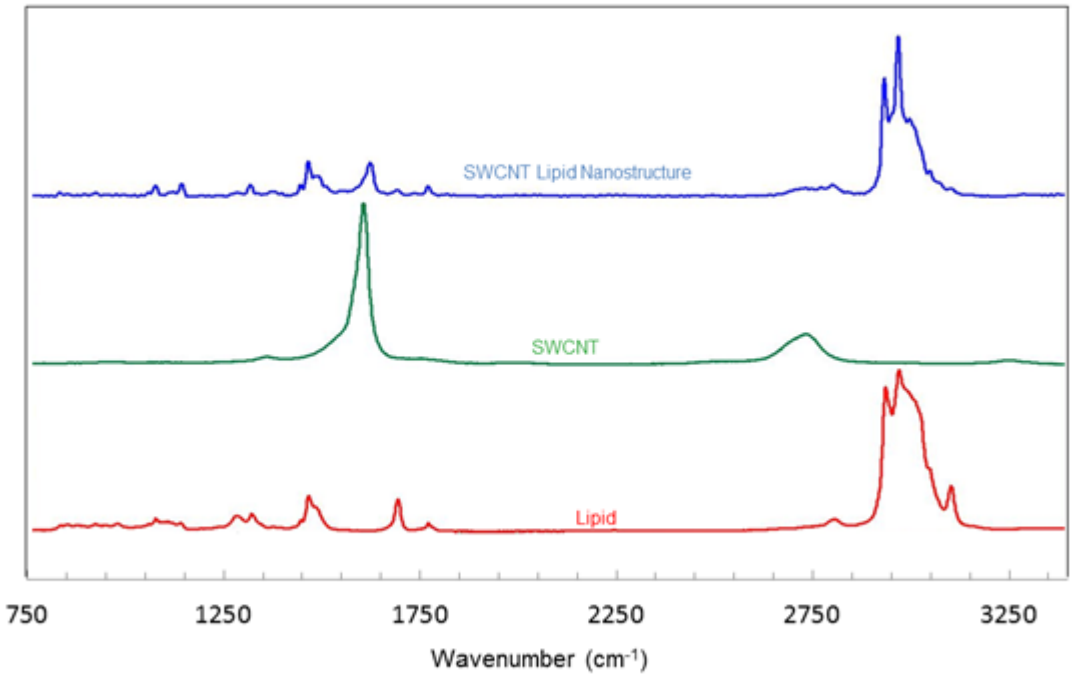

Figure 4: Original spectral plot showing the averaged Lipid, SWCNT and combined nanostructure. SWCNT and SWCNT Lipid nanostructure have both been vector normalised and offset for visual clarity. The dotted lines on the peaks correspond to the Raman Shifts seen in different bands.

Figure 3 displays notable spectral peaks at 1578.23cm^{-1} and 2683.72 cm^{-1}, displaying tell-tale signs of multiple walls in the Carbon Nanotube, as can be seen by comparison to the SWCNT single peak in that area in **Figure** 4. In the Lipid Nanostructure spectra in **Figure** 2, the CNT peaks can be seen, however they appear 21.83 cm^{-1} and 36.36 cm^{-1} shifted, known as a blue shift. This could be caused by Hydrogen bonding affects, which would be more apparent in –OOH than other CNTs. This is backed up by the amount of shift seen, as shorter shifts are seen in MWCNT-OH and shorter still in SWCNT.

CNT Type	Peak centre of CNT (cm^{-1})	Peak centre of CNT-Lipid Nanostructure (cm^{-1})	Wavenumber shifted (cm^{-1})
MWCNT-COOH	1578.23 (G)	1600.05	21.83
	2683.72 (G')	2720.08	36.36
MWCNT-OH	1570.91 (G')	1588.24	17.33
	2687.27 (G')	2698.18	10.91
SWCNT (Pristine)	1570.91 (G)	1585.45	15.44

Table 1: Raman wavenumbers and shift for CNTs and nanostructured lipid particles with CNTs

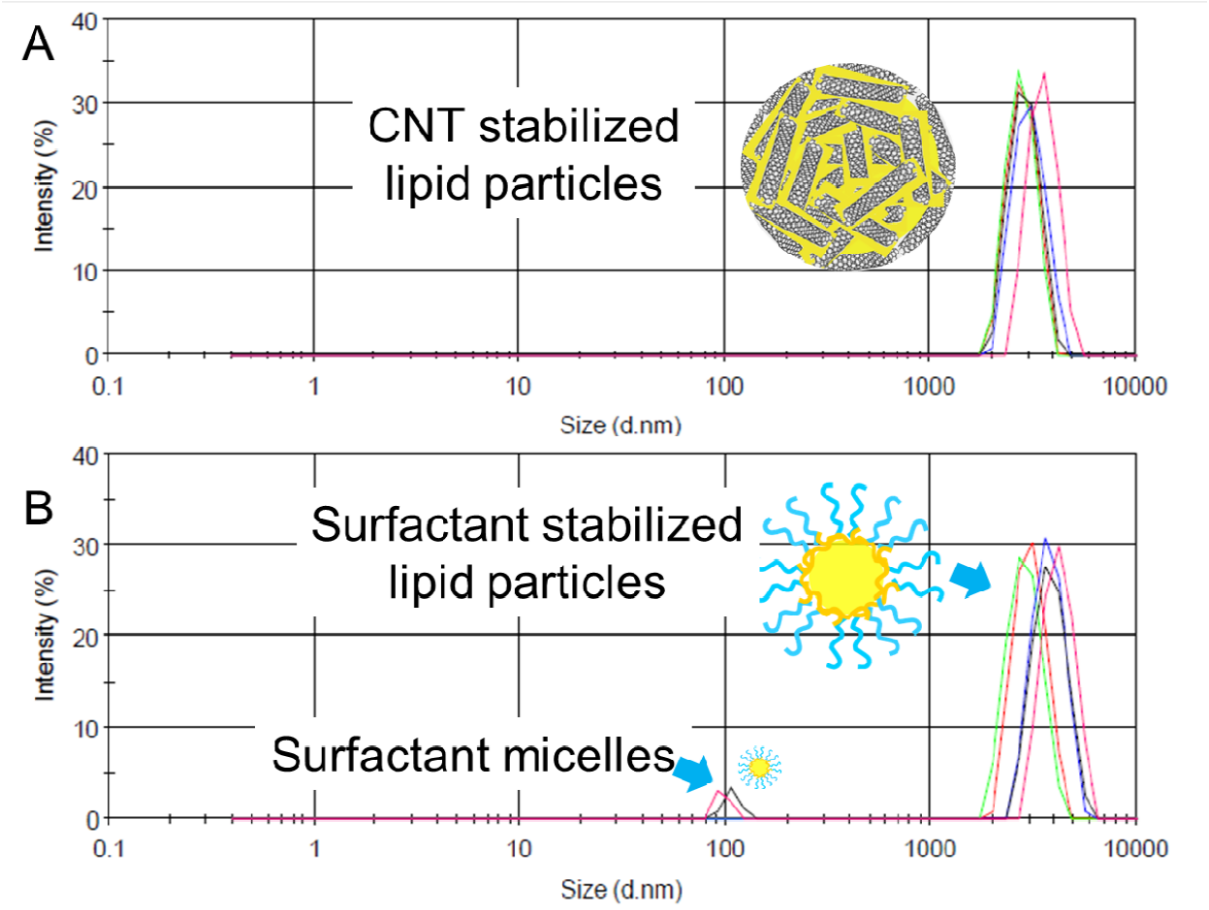

Figure 5: Particle size of nanostructured lipid particles (A) An average particle size of CNT stabilized nanostructured lipid particles was in the range of 2-5 μm and it was comparable to the size of lipid particles stabilized by Pluronic F127 surfactant (B), as determined from dynamic light scattering experiments. An additional peak for surfactant based system could be attributed to surfactant micelles of about 100 nm size. The different colours represent duplicate runs for each sample, for more accurate, repeatable data.

Once stabilised NanoLCPs were established, particle size was measured using a Malvern Zetasizer to see uniformity and to compare to common stabilisers such as Pluronic F127. **Figure** 5a shows DLS data for a Carbon Nanotube stabilised lipid nanostructure whereas **Figure** 5b shows its F127 counterpart, outlining the differences between the two methods of stabilization.

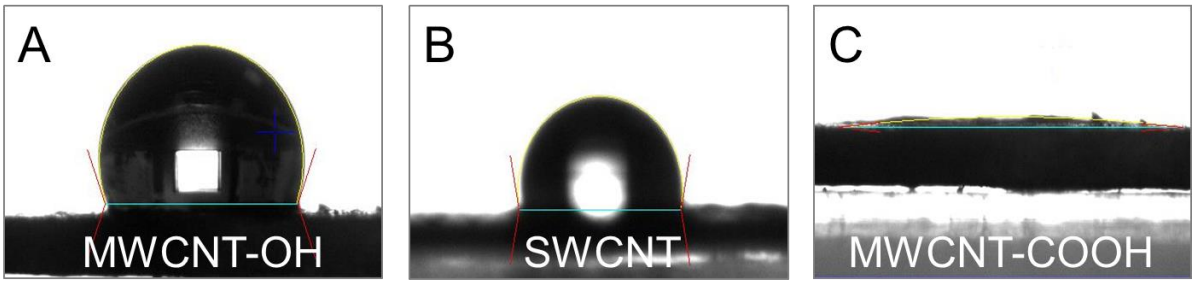

Figure 6: Contact Angle measurements for a) MWCNT-OH b) SWCNT and c) MWCNT-OOH

Contact Angle measurements were performed in an attempt to quantify the wettability of the CNT surface and to ascertain the hydrophobicity or hydrophilicity of the surface, to perhaps determine which CNT is most likely to stabilise best, due to any functionalization on the surface. **Figure** 6 shows the contact angle of each CNT surface and points us to believe that MWCNT-COOH is the most hydrophilic, the contact angle is nil, compared to larger contact angles seen in the MWCNT-OH and SWCNT. These results however, must be taken with a pinch of salt due to the difficulty of making these CNT surfaces completely flat, and not knowing about the surface of the CNT pellet, after being pressed. As well as this, each pellet may have been pressed with a slightly different force which may damage the accuracy of such tests.

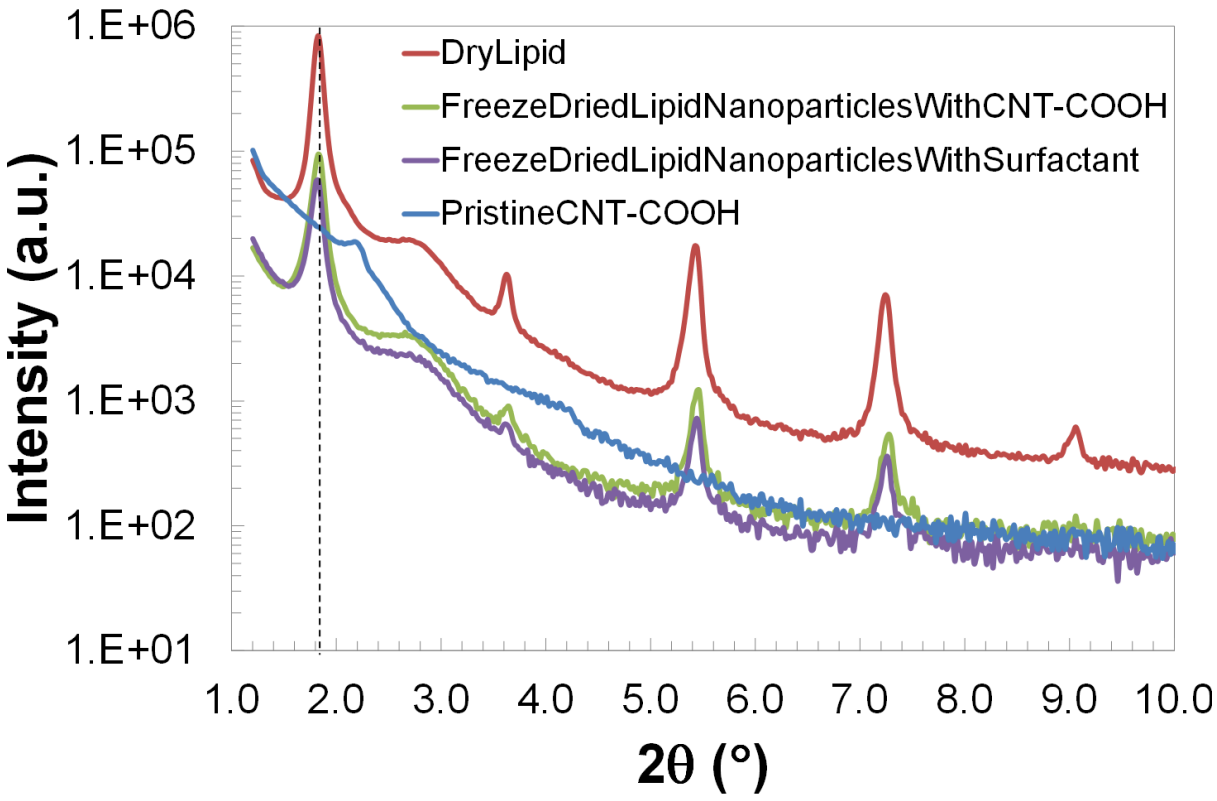

Figure 7: XRD pattern for dry lipid, Pristine MWCNT-COOH, freeze-dried lipid nanostructure with CNT and the same nanostructure, stabilised by F127. The red plot represents the XRD pattern for lipid alone, showing a lamellar pattern. Similar results can be seen for the green and purple lines, representing freeze-dried lipid nanoparticles stabilised with CNT-COOH and F127 surfactant, respectively. The blue line, which shows the plot for CNT-COOH alone, shows no notable peaks. The absence of the lamellar phase is due to Carbon Nanotubes having little diffraction pattern on XRD at this range.

3.5 CNT-Lipid Nanostructured Particle stability

Nanostructured Lipid-CNT particles (NanoLCP) with various concentrations of DU and CNTs, as well as some samples containing Pluronic F127 (a known stabiliser) were prepared via ultrasonication, using various amplitudes (AMPL), pulse settings and also varying time. These settings and concentration ratios vastly affect the stability of the NanoLCP, with some destabilising almost instantly, to some completely stabilising for a period of roughly 3 to 4 months. Once showing signs of destabilization, these NanoLCPs return to their homogeneous state after lightly shaking. **Figure** 8 shows the destabilization and restabilization of such structures, whereas **Figure** 9 highlights a few significant ratios of CNTs, DU and water.

Figure 8: The stability of a CNT-Lipid nanostructured particle over a 3 month period. The ’10 minutes’ sample had been ultrasonicated 10 minutes prior to the photograph. The same sample was then photographed after 2 days, 2 months and 3 months to represent the ongoing stability over several months.

Figure 8 also shows the ease of which these nanostructures can be re-dispersed and regain stability. This could be a very useful property for further use, bringing in the idea of ‘shake-and-use’ nanostructures, adding greatly to their shelf-life.

Chapter 4: Optimization and Generalization

4.1 Optimizing the ratio for MWCNT-COOH

Optimization of these systems is very important in order to reach the full potential of this technique of drug delivery. In order to do this, research had to be done to ensure we had the most stable region. Using Raman Spectroscopy to monitor a range of stable CNT-lipid nanostructures, we can observe any changes in peak heights, or any shifts that take place.

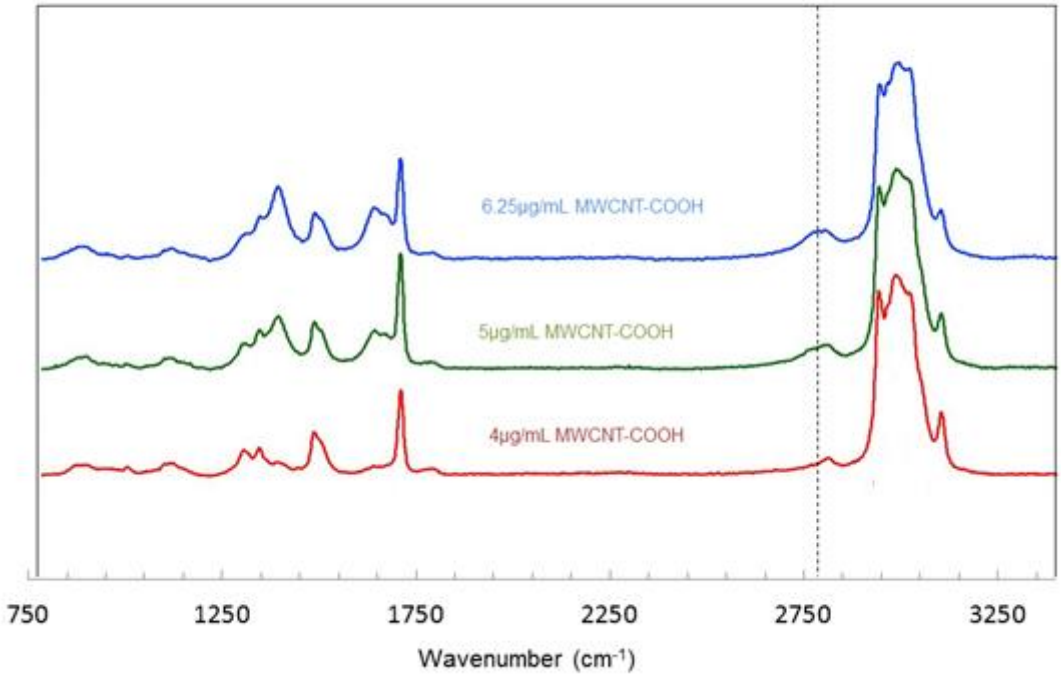

Figure 10: Original spectral plot showing the averaged MWCNT-OOH at different concentrations to show the peak progression. The spectra have been vector normalised and offset for visual clarity. The dotted lines on the peaks correspond to the Raman Shifts seen in different bands.

Figure 10 is a spectral plot showing the stable region of MWCNT-OOH in lipid. As the amount of CNT in the sample increases, certain peaks appear stronger, which again match up to the peaks for native CNTs. This not only shows that there is in fact more CNTs in the nanostructure, but also suggests that stable nanostructures can exist at different ratios. This could prove important if a certain drug needs more or less CNTs than another to be released at the right time. From repeating this for different concentrations of CNTs, **Figure** 9 was produced, showing a rough guideline to the upper and lower limits to the process. 0-2.5ug/mL did not have enough CNTs to stabilise the Lipid for even short lengths of time, whereas the column labelled 'Excess CNTs' showed stable lipid, with aggregated CNTs on the surface, or floating in solution. For MWCNT-OOH, the range of stable concentrations of CNTs are shown between 3 and 5ug/mL.

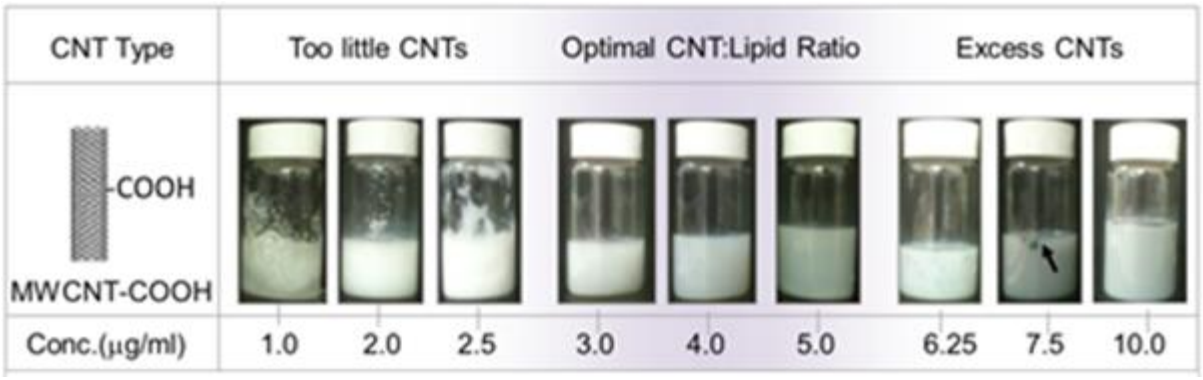

Figure 9: The stable, excess and insufficient zones of CNT:Lipid ratio for MWCNT-OOH

4.2 Optimizing the ratio for differently functionalized CNTs

With this information on MWCNT-OOH, we can expand our study to include different Carbon Nanotubes, namely MWCNT-OH and SWCNT. Using the same ratios as before, MWCNT-OH showed very similar results for stability, as can be seen in **Figure** 12. SWCNT, however, needed far fewer CNTs in order to stabilise. Using roughly 10% of the amount needed for MWCNTs with both –OH and –OOH functionalization.

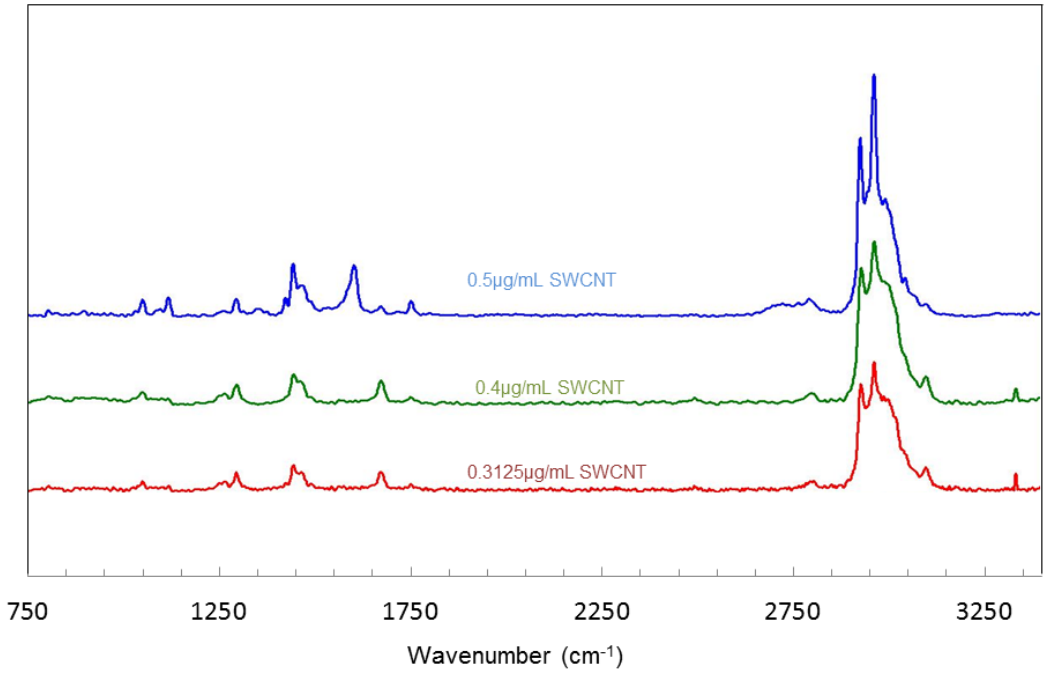

Figure 11: Original spectral plot showing the averaged SWCNT at different concentrations to show the peak progression. The spectra have been vector normalised and offset for visual clarity.

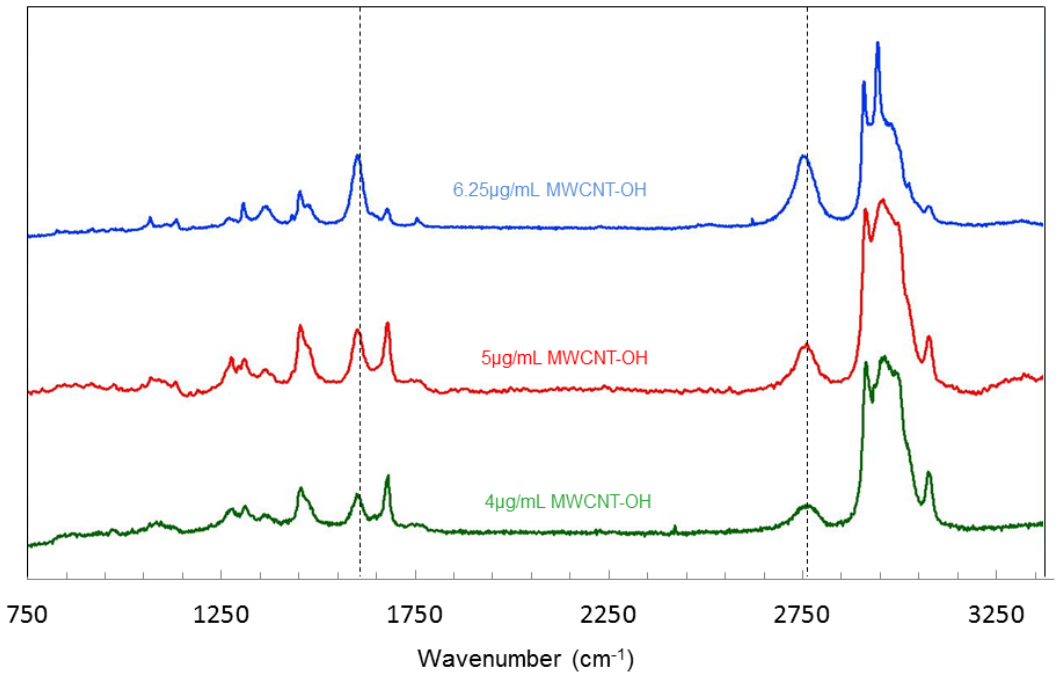

Figure 12: Original spectral plot showing the averaged MWCNT-OH at different concentrations to show the peak progression. The spectra have been vector normalised and offset for visual clarity.

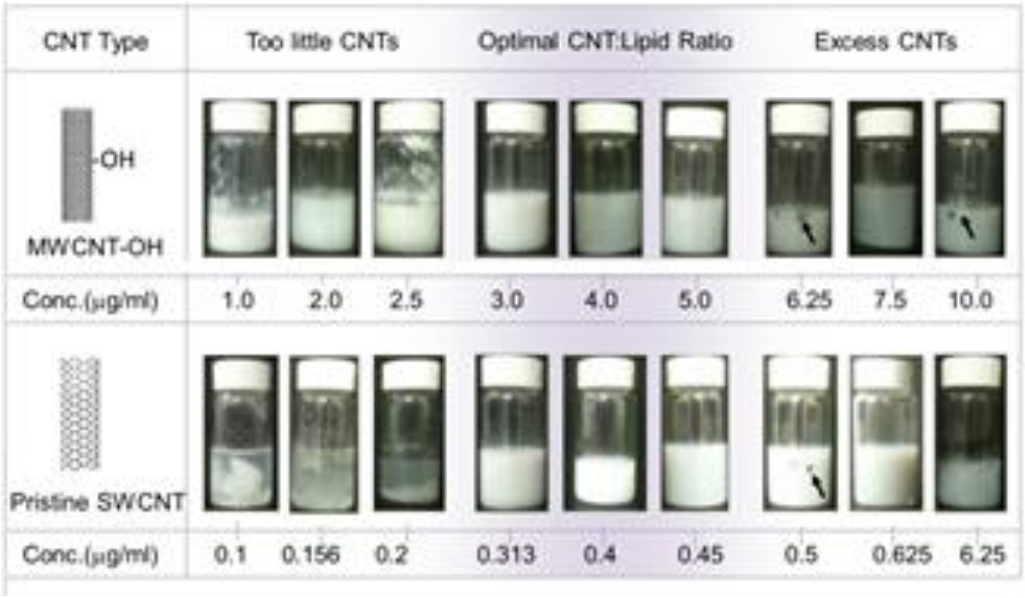

Figure 13: The stable, excess and insufficient zones of CNT:Lipid ratio for MWCNT-OH and SWCNT

4.3 Toxicity

It is likely that occupational and public exposure to CNT-based nanomaterials will increase dramatically in the near future. Hence, it is of the utmost importance to explore the yet almost unknown issue of the toxicity of this new material. The research is lacking, however, there are some reports stating that doses of 400ug/mL are the upper limit of toxicity, and causes cell death [56] 400ug/mL corresponds to roughly 10 million CNTs per cell, a figure this report is far below. As well as this, CNTs have been observed to have enhanced solubility when functionalized with lipids which would make their movement through the human body easier and would also reduce the risk of blockage of vital body organ pathways. Drug encapsulation has been shown to enhance water solubility, better bioavailability, and reduced toxicity. [57]

4.4 Dehydration and re-dispersibility

Figure 14 shows the process of freeze-drying and re-dispersing the CNT-Lipid Nanostructure with the use of a freeze-dryer.

Samples can be subjected to freeze-drying and can be stabilised once more by simply shaking with the equivalent water. The third screw-top vial shows the sample immediately after shaking to re-disperse. A layer of foam can be seen, however disappears when left to settle, leaving the sample completely dispersed and homogeneous, almost identical to the original sample. This is a very useful property, as the freeze-dried samples can easily be stored and re-dispersed when required, for use immediately.

4.5 Fine tuning lipid content

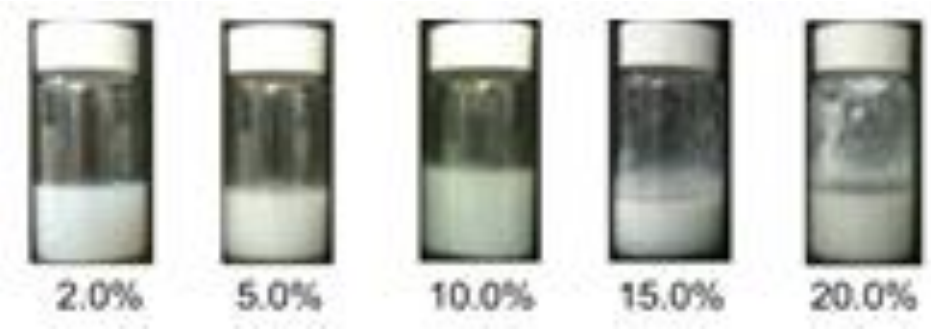

Figure 15 shows the effect percentage of lipid has on the stability of the nanostructure.

Samples containing 2, 5, 10, 15 and 20% lipid content were studied to monitor the limitations and capabilities of the different percentages. 2%, 5% and 10% all easily formed stable CNT-stabilised lipid nanostructures in 10mL. However, 15% and upwards formed very thick unstable emulsion, which could be due to the viscosity affecting the power of the Ultrasonication process. From these results, the attention was kept on 2-10% lipid, with 5% being the main focus to reduce costs and amount of lipid and CNTs needed per sample.

Chapter 5: CNT-stabilized Nanostructured Lipid Particles for Drug Delivery

5.1 Drug loading

Using the table of stable systems reported in the previous chapter, Aspirin can be encapsulated in the nanostructure with a simple method of stirring. 5um of Aspirin is added slowly to the already stabilised structure, shaking the container thoroughly between each miniscule addition.

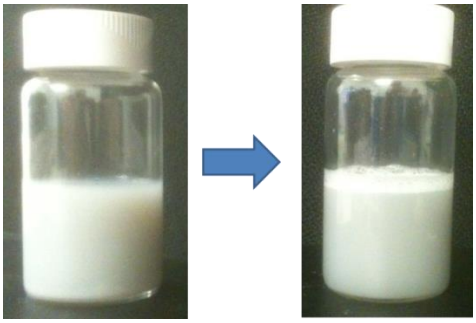

Figure 16: Stable CNT-Lipid nanostructure is shown in the first part of the image. The second showing no difference in stability of nanostructure after stirring.

Figure 10 shows a list of stable CNT-Lipid nanostructured particle ratios, to which Aspirin can be added and simply stirred in, as shown in **Figure** 16. The stability of these new drug-encased nanostructures remain consistently over 2 months, with the only visible difference being a slight colour change, with the new system slightly lighter than its counterpart.

The foam that can be seen in the second image does eventually clear, leaving an almost identical system, when compared to the CNT-Lipid nanostructure without Aspirin.

5.2 Preparing for drug release

To test the drug release aspect of these new structures, a method was devised in order to monitor the sample over a period of several hours. The method, shown in **Figure** 17, involves centrifuging the nanostructures towards the base of the container, before blowing cold air onto the surface to reduce the water content slowly. This was done until a compact and semi-dry layer was present at the base of the cuvette, as can be seen in part D and E. These Aspirin filled nanostructures were taken and tested via Ultraviolet-visible spectroscopy to time the release of the aspirin contained inside. **Figure** 17 shows a diagram of the process used to create our time release experiment, with centrifugation used to pack the drug-filled nanostructure down, followed by the drop-wise addition of Millipore water, not to disturb the surface of the now film-like structure. UV-Vis spectroscopy can then be used to record a spectra every 30 minutes, to continually monitor the release of aspirin from its encasing nanostructure.

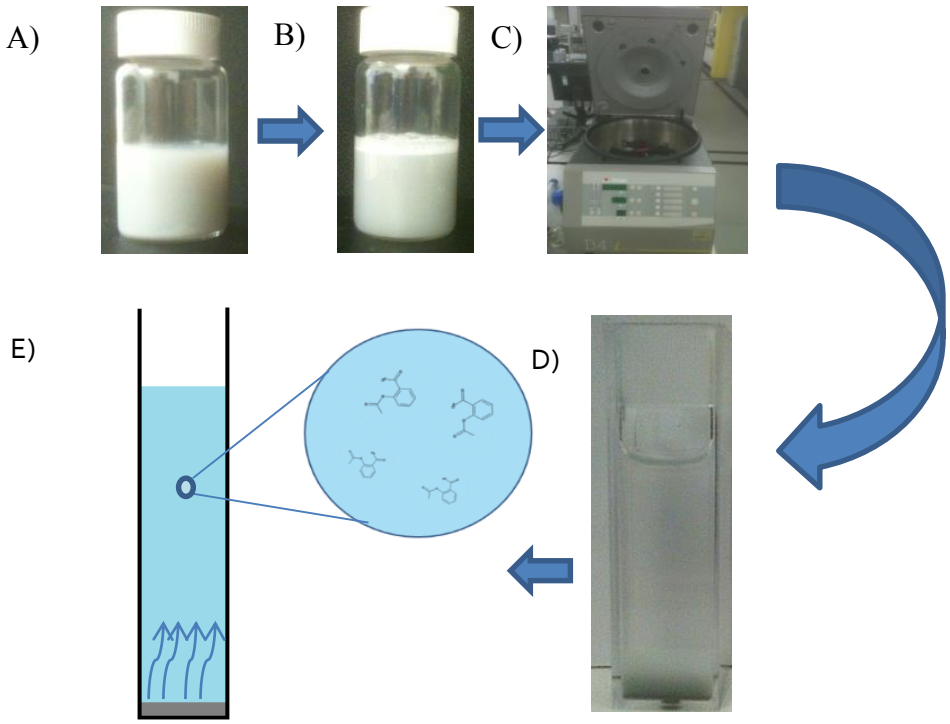

Figure 17: A) Stable CNT-Lipid Nanostructure B) Stable CNT-Lipid Nanostructure with 5mg Aspirin with foam from stirring c) Jouan B4 Centrifuge, used for making the residue in the cuvette. D) Cuvette containing a sediment of aspirin-containing CNT-

lipid Nanostructure beneath an aqueous layer. From this, the top layer is removed and the loaded residue is used for drug release. E) Animated version showing release of Aspirin from Nanostructured sediment.

5.3 Monitoring drug Release with UV-Vis

Using Uv-Vis spectroscopy, we are able to record the spectrum every 30 minutes. These can be plotted together to show any trends in the spectra as shown below in **Figures** 18, 19 and 20.

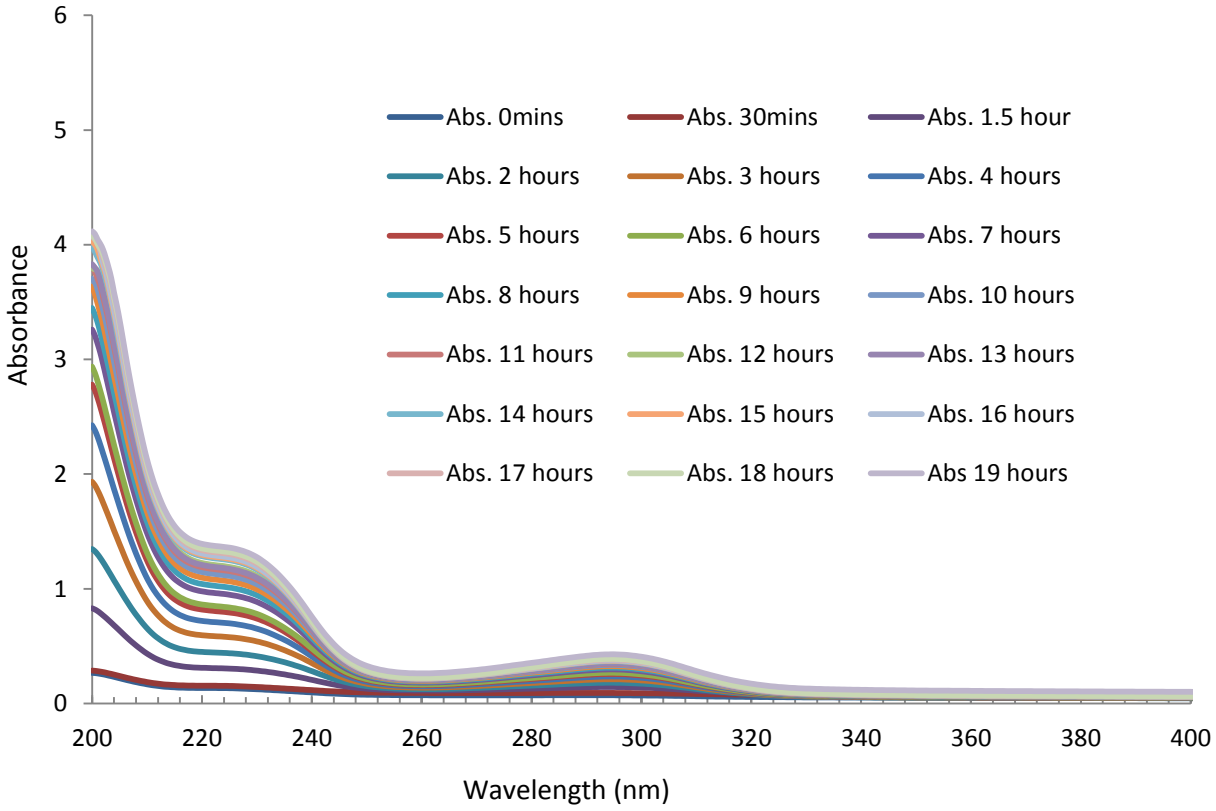

Figure 18: UV Vis plot to show time release of drug, taken every 30 minutes for MWCNT-COOH stabilised lipid nanostructure, containing Aspirin.

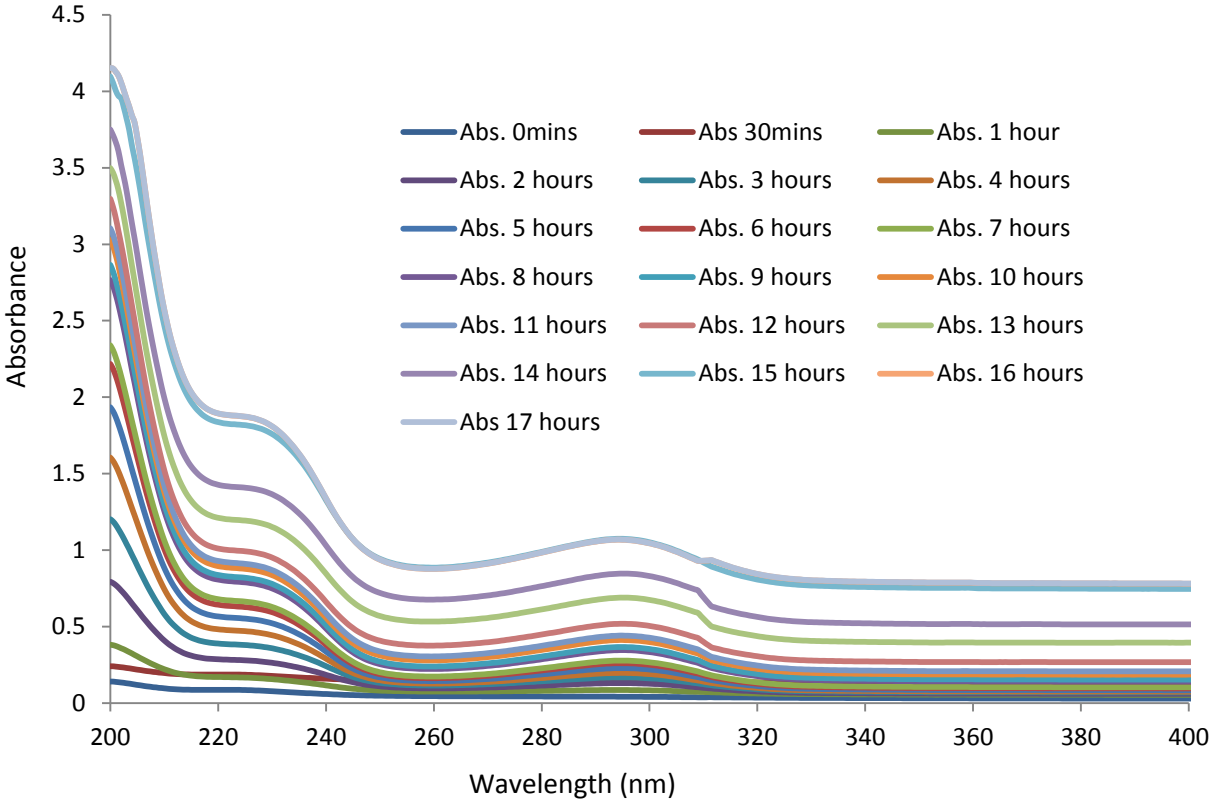

Figure 19: UV Vis plot to show time release of drug, taken every 30 minutes for MWCNT-OH stabilised lipid nanostructure, containing Aspirin.

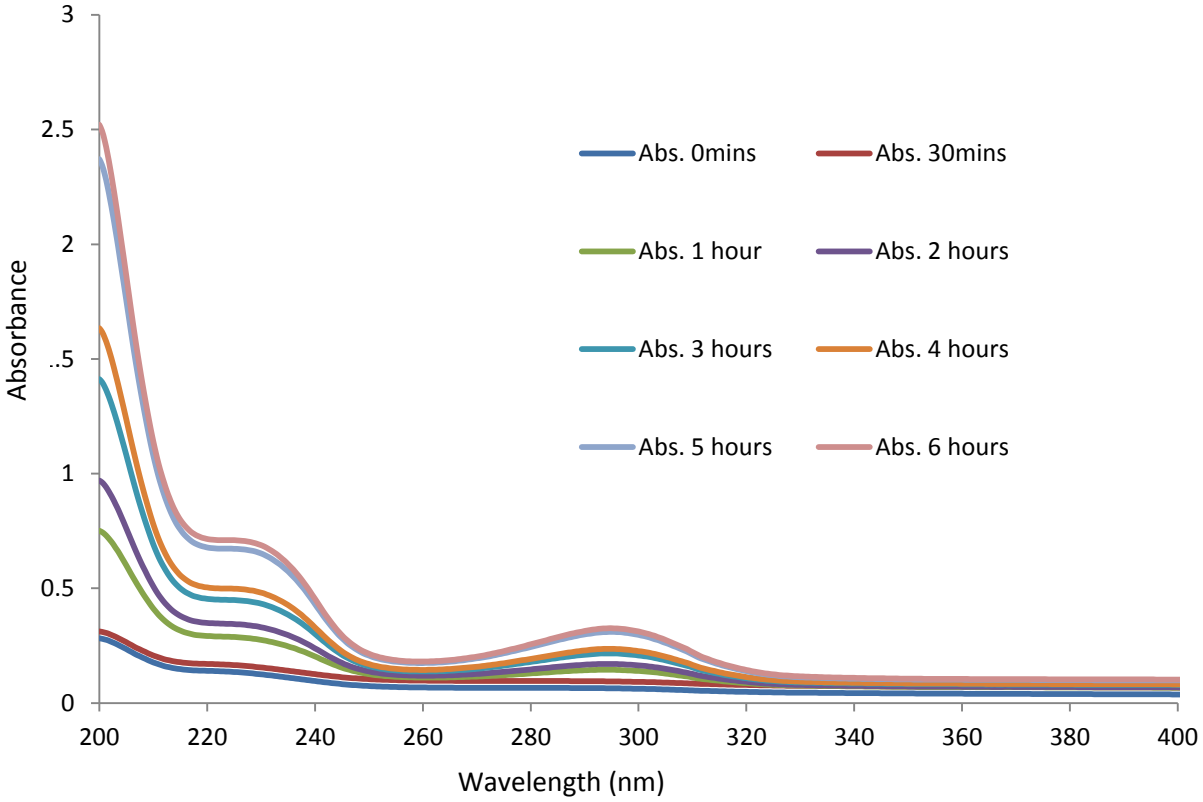

Figure 20: UV Vis plot to show time release of drug, taken every 30 minutes for SWCNT stabilised lipid nanostructure, containing Aspirin.

From these release profiles, measured every 30 minutes, it seems to suggest that there is a larger release of the drug just before it releases fully. This could be due to the breakdown of the structure, releasing Aspirin rapidly through new holes forming in the walls of the encasement. MWCNT-COOH stabilised lipid nanostructures seemed to release most of its drug after around 19 hours, considerably more than the SWCNT which remained stable for only 6 hours.

5.4 Drug Release kinetics

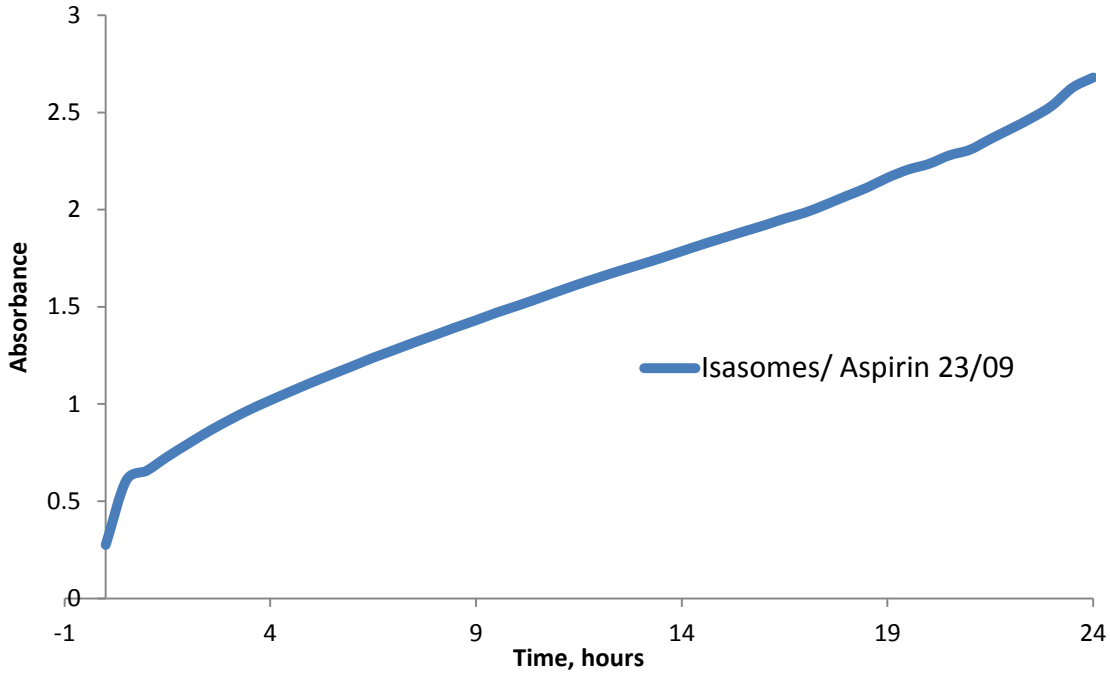

Figure 21: UV Vis plot to show time background, taken every 30 minutes for stabilised lipid nanostructure, containing no CNTs.

Studying the data all at 294nm absorbance shows some interesting results also. **Figures** 22, 23 and 24 show the repeatable results shown by UV-vis of each CNT.

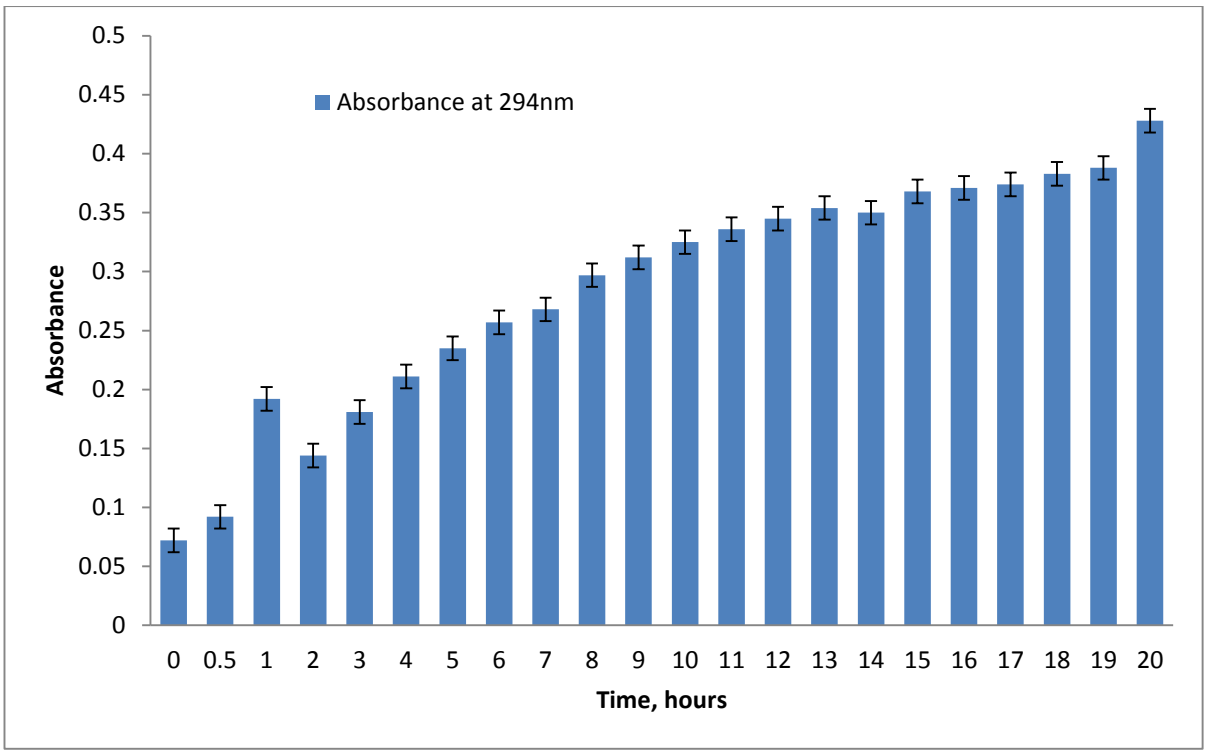

Figure 22: Absorbance at 294nm vs time for MWCNT-COOH

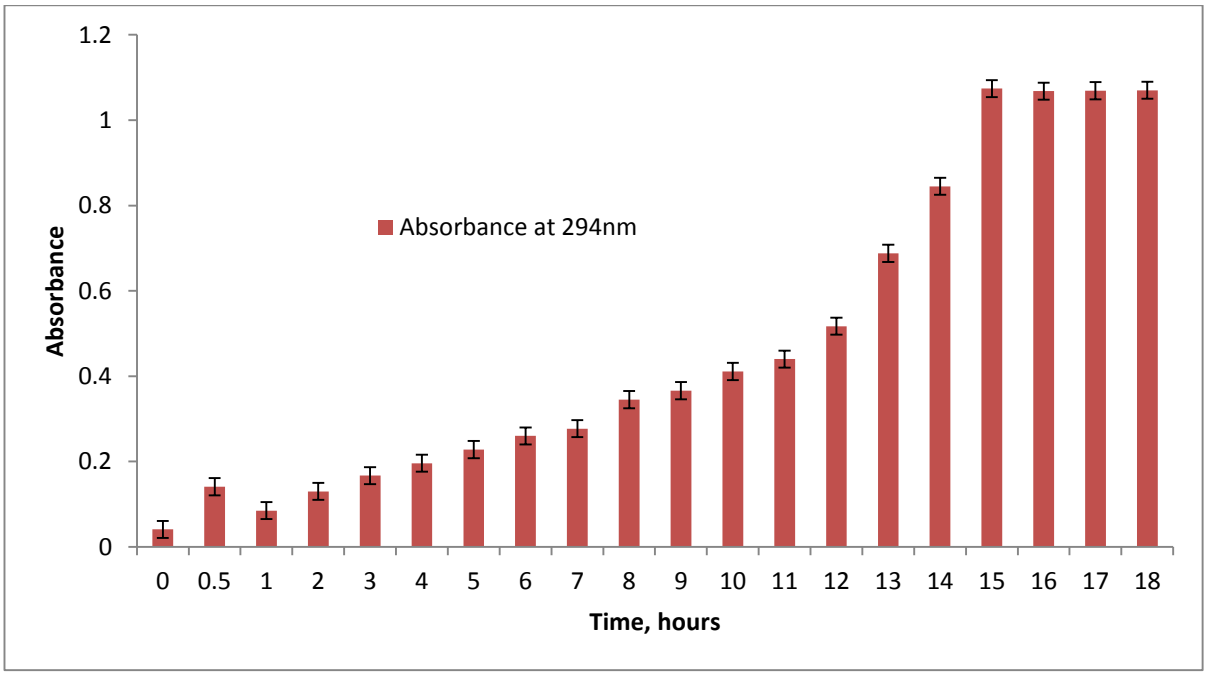

Figure 23: Absorbance at 294nm vs time for MWCNT-OH

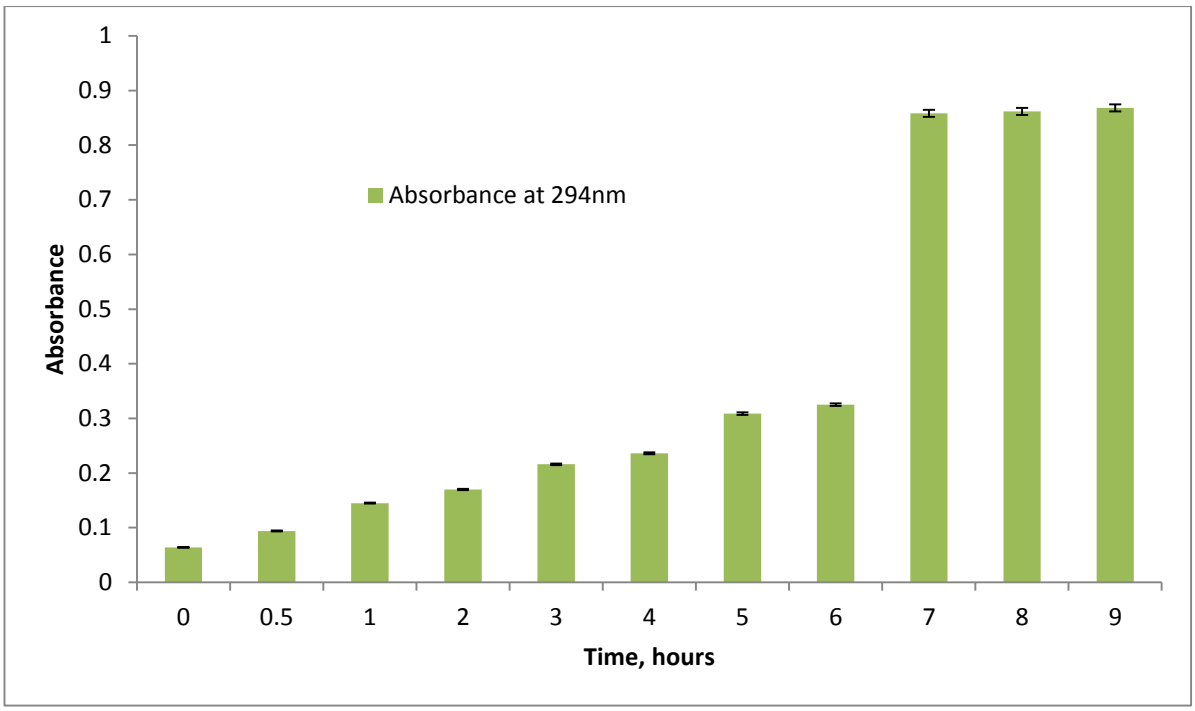

Figure 24: Absorbance at 294nm vs time for SWCNT

These give a good representation of the repeatability of the method, suggesting the uniformity of these release patterns. SWCNT has a much quicker release time when compared to CNT-OOH and CNT-OH, which could be useful in the case of releasing drugs more quickly in the body, rather than the requirement of slower release.

Although this method of Time Release monitoring could be heavily altered by the degradation of these film-like structures, the results from this show very consistent plots, all following a similar release pattern, making it very unlikely that random degradation of these structures is occurring, and making it more of a possibility that aspirin is being released over time in much the same way. Each functionalization CNT had a different release pattern, and released fully over different extents of time, with SWCNT stabilised nanostructures fully releasing the aspirin in 6-8 hours, this being the slowest of the three. CNT-OH stabilised nanostructures taking much longer to release fully at 17 hours and CNT-OOH stabilised nanostructures fully releasing soon after that at 19 hours. These results were repeated and the plots remained the same, allowing us to draw the conclusion that the release times are specific to the functionality of the CNT and further studies could prove these slight structural differences can affect the properties of the nanostructure.

Chapter 6: Conclusions and Perspectives

The field of nanoscience has been witnessing a rapid growth in the last decade. Recently, more and more, the attention of the community of nanoscientists has been focusing on technological applications. Nanotechnology has been emerging as an enabling technology, with high potential impact on virtually all fields of mankind activity (industrial, health-related, biomedical, environmental, economy, politics, etc.), yielding high expectations for a solution to the main needs of society, although having to address open issues with respect to its sustainability and compatibility. The fields of application of the research in nanoscience include aerospace, defense, national security, electronics, biology and medicine. There has been a significant progress in understanding achieved in recent years, both from the theoretical and experimental point of view, along with a strong interest to assess the current state of the art of this fast growing field, stimulating, at the same time, research collaboration among the different stakeholders in the area of nanoscience and the corresponding technological applications, prompting possibly the organization and presentation of joint projects in the near future involving both industry and public research.

In my MSc study, we focused in particular on the biological and medical fields and described current and possible future developments in nanotechnological applications in such areas. Nanostructured, composite materials for drug delivery, biosensors, diagnostics and tumor therapy were reviewed here as examples. Carbon nanotubes were discussed as a primary example of emerging nanomaterials for many of the abovementioned applications. In this thesis, it has been made clear that Carbon Nanotubes, functionalised or not, have the ability to stabilise lipid nanoparticles in Millipore water. Through other simple methods this report has shown how the addition

of drugs such as Aspirin does not affect the overall stability of the system and does have possible uses in the pharmaceutical industry, providing toxicity of nanotubes in the body is not still an ongoing debate, a few years down the line. It can also be said that different functional groups definitely affect the long term stability of the system, and similarly viscosity could also be an issue, due to probe sonication not being able to function properly in thicker solutions.

There are many future studies that could be of interest and benefit to the advancement in this area, due to CNTs fantastic array of properties. Specifically in this field of research, there are certainly more and more calls for toxicity studies into CNTs for pharmaceutical uses. Once a clear limit is given, I can see much more research going into CNTs for these types of uses, and huge advancements in all fields concerned could follow.

If I personally could study further in this topic, I would like to simply repeat my experiments with different lipids, different drugs and many more functionalizations to create a catalogue of drug delivery options, specified to a particular route or organ. There are still thousands of combinations that have not had much attention, for example, the use of different Nanotubes. Using three types of CNTs is barely scraping the surface or the potential of this type of chemistry.

Finally, I would like to see more studies into the hydrophobicity of each functionalised Nanotube, to form a database for the most likely to be able to be stabilised for long periods.

Carbon Nanotubes for Stabilising Lipid Nanostructured Particles

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Lipid Nanostructures Group

Introduction

Lipid molecules are amphiphilic due to hydrophilic head-group and hydrophobic alkyl chain/s. Lipids are known to self-assemble into remarkable nanostructures in the aqueous medium[1]. In order to enhance their applicability, these nanostructures are further stabilised into nanoparticles using various stabilisers; resultant are the oil-in-water (O/W) emulsions, which are common in many food and consumer products [2].

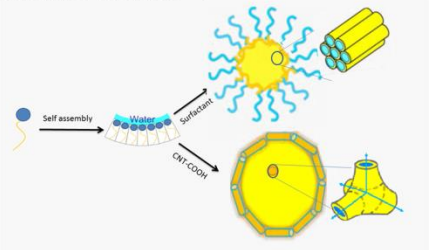

Figure 1: Internally Nanostructured Lipid Particle stabilised by Carbon Nanotubes

Due to there various uses and incredible properties, like strength and flexibility and use in multiple applications, Carbon Nanotubes (CNTs) have attracted tremendous interest in the scientific community, with well over 20000 publications to date. Here, we present how the we have employed Carbon nanotubes (CNTs) to stabilise nanostructured lipid particles. (Fig **1**).

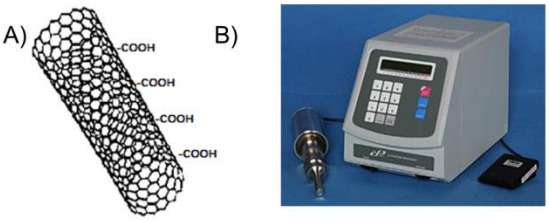

Figure 2: A) CNT with carboxylic acid functionisation B) Ultra sonicator-ULT065

Experimental

Different ratios of functionalised Carbon Nanotubes (Fig **2a**) were dispersed in water with Dimodan U/J lipid, using Ultrasound techniques (Fig **2b**).

Samples were freeze-dried and these systems were characterised using a number of techniques such as Raman Spectroscopy, X-ray diffraction and Contact Angle measurements, whilst DLS was performed on the wet samples, after x1000 times dilution.

Results and discussion

By altering the ratio of CNTs to lipid, whilst remaining in a 10mL solution, we have formed lipid nanostructures that remain stable for several months. These emulsions can be then stabilised again easily, simply by shaking.

Low Angle XRD helped prove Pn3m cubic structure (Fig **3**) of the stabilised nanoparticles whilst blue shifts in Raman peaks ask the question of whether there is Hydrogen Bonding apparent in the case of functionalised nanotubes.

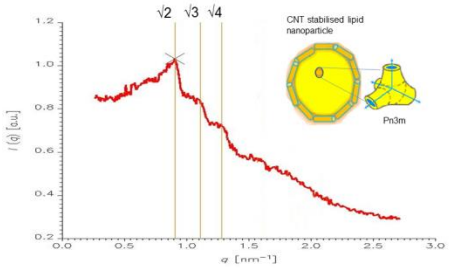

Figure 3: Small Angle X-ray Scattering showing Pn3m cubic phase

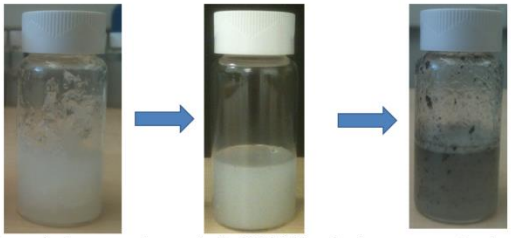

Figure 4: Lower and upper limits A) Lipid forming large aggregates due to no stabilising agent being added. B) The correct ratio of CNTs to lipid C) Excess CNTs aggregrate on the sides and surface on the bottle.

Conclusion and Perspectives

Results demonstrate these studies find very high potential in various biotechnological applications such as drug delivery, as CNT levels are **well below** current toxic limitations for bodily use.

References

1. Kulkarni, C.V., Lipid crystallisation: from self-assembly to hierarchical and biological ordering. Nanoscale, 2012. 4(19): p. 5779-5791.
2. Kulkarni, C.V. and O. Glatter, Hierarchically Organised Systems Based on Liquid Crystalline Phases, in Self-Assembled Supramolecular Architectures: Lyotropic Liquid Crystals, N. Garti, Editor. 2012, John Wiley & Sons, Inc.

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We would like to thank Yogita Patel-Sen, University of Central Lancashire for her support with measurements via Contact Angle. NPG would like to especially show gratitude to Mukta Kulkarni and Ales Iglic for their efforts during his stay at University of Ljubljana. We would also like to acknowledge Danisco for providing us with Dimodan U/J lipid

Functionalised CNT-Lipid Nanostructures as Drug Delivery Carriers

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Introduction

- Amphiphilic nature of lipids arises from their hydrophilic head group and hydrophobic tail/s.
- Lipids are known to self-assemble into remarkable nanostructures in the aqueous medium. [1]
- Kinetic stabilisation of lipid nanostructures into particulate emulsions enhances their applicability, usually done with stabilisers.[2]
- We have used these emulsions for loading and release of various drug molecules.

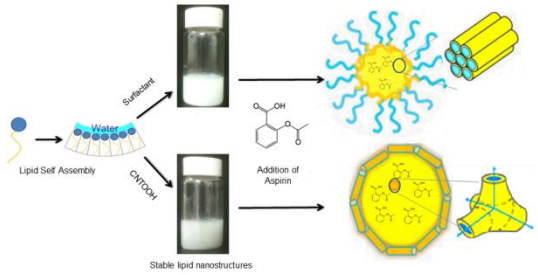

Figure 1: Internally Nanostructured Lipid Particle stabilised by Carbon Nanotubes, encasing Aspirin molecules

Results and Discussion

Aspirin has no effect on the stability of these nanostructures, which in previous works, have been stable for upwards of 3 months[3]. Raman spectroscopy provided us with new questions, with blue shifts in peaks asking whether there is Hydrogen Bonding apparent in the case of functionalised nanotubes (**Figure 3A**).

Experimental

Stabilised CNT-Lipid Nanostructures were made in previous studies, and remained homogeneous for up to 3 months, with different ratios and functionalisations on the CNTs (**Figure 3B**), which were characterised by Raman, Small Angle X-Ray Scattering and Dynamic Light Scattering.

To these, 5mg of Aspirin was added, mixed by simple stirring. **Figure 2A** and **2B** shows the stability is unchanged by the addition of aspirin. Samples were then ran for 20+ hours on UV-Vis to profile the time release data (**Figure 3C**)

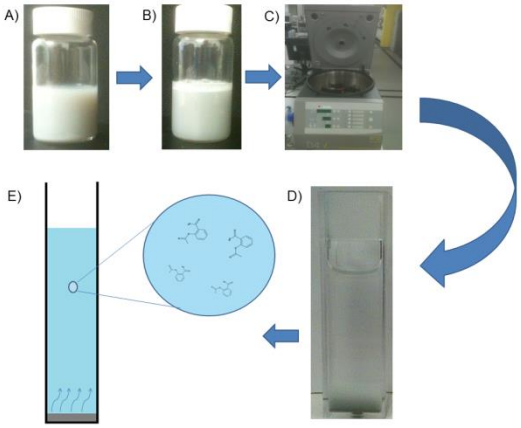

Figure 2: A) Stable CNT-Lipid Nanostructure B) Stable CNT-Lipid Nanostructure with 5mg Aspirin with foam from stirring c) Jouan B4 Centrifuge, used for making the residue in the cuvette. D) Cuvette containing a sediment of aspirin-containing CNT-lipid Nanostructure beneath an aqueous layer. From this, the top layer is removed and the loaded residue is used for drug release. E) Animated version showing release of Aspirin from Nanostructured sediment.

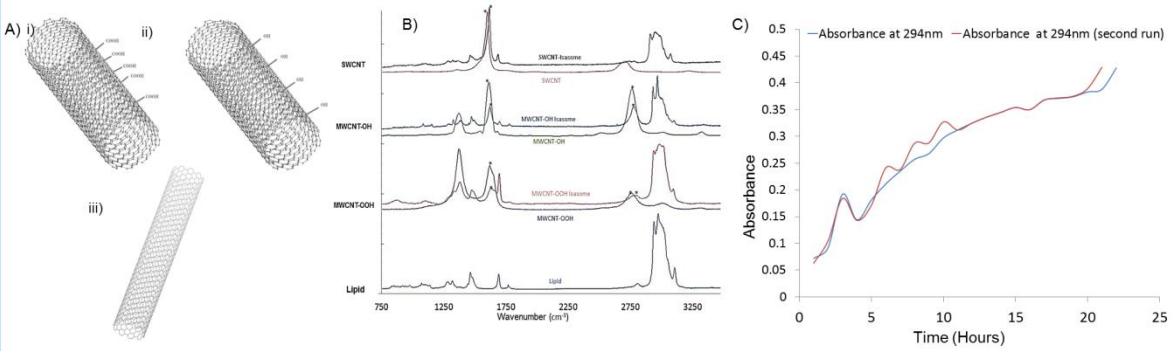

Figure 3: A) Drawn representation of each Carbon Nanotube, namely i) MWCNT-COOH ii) MWCNT-OH and iii) SWCNT. B) Raman Spectroscopy for three different sets of Carbon Nanotubes, showing their presence in a stabilised Lipid-CNT nanostructure C) UV-Vis data showing the reproducibility of drug release data for MWCNT-COOH over the course of 20 hours.

Conclusions and Perspectives

Results demonstrate these studies find very high potential in various biotechnological applications like drug delivery, as CNT levels are well below current toxic limitations for bodily use and, by tweaking the nanostructure, could alter the time release characteristics for more favourable conditions for different drug molecules. UV-Vis gives us a good idea about time release of Aspirin, as seen in **Figure 3C.**

References

1. Kulkarni, C.V., Lipid crystallisation: from self-assembly to hierarchical and biological ordering. Nanoscale, 2012. 4(19): p. 5779-5791.
2. Kulkarni, C.V. and O. Glatter, Hierarchically Organised Systems Based on Liquid Crystalline Phases, in Self-Assembled Supramolecular Architectures: Lyotropic Liquid Crystals, N. Garti, Editor. 2012, John Wiley & Sons, Inc.
3. Nicholas P. Gaunt, Matthew J. Baker, Gary Bond, Matija Tomsic and Chandrashekhar V. Kulkarni*, **Poster Presented** in Macro Group UK Young Researchers' Meeting, Durham University 24-25 July 2014.
4. Gaunt, N.P, Baker, M.J., Bond, G., Tomsic, M. and Kulkarni, C.V., Carbon Nanotubes for Stabilising Lipid Nanostructured Particles, **Under Preparation**

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Carbon Nanotubes for Stabilization of Nanostructured Lipid Particles

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Carbon nanotubes (CNTs) are increasingly studied for innovative biotechnological applications particularly where they are combined with essential biological materials like lipids. Lipids have been used earlier for enhancing the dispersibility of CNTs in aqueous solutions. Here we report a novel application of CNTs for stabilization of internally self-assembled nanostructured lipid particles of 2-5 μm size. Single-walled (pristine) as well as -OH and -COOH functionalized multi-walled CNTs were employed to produce nanostructured emulsions which stayed stable for months and could be re-dispersed after complete dehydration. Concentrations of CNTs employed for stabilization were very low; moreover CNTs were well-decorated with lipid molecules. These features contribute towards reducing their toxicity and improving biocompatibility for biomedical and pharmaceutical applications. Our approach paves the way for future development of combination therapies employing both CNTs and nanostructured lipid self-assembly together as carriers of different drugs.

Introduction

Carbon nanotubes (CNTs), owing to their special properties such as small size and ability to bind with a broad range of molecules, are exploited in many biomedical and pharmaceutical applications[1-5] such as drug delivery,[6, 7] gene delivery,[8] cancer therapy,[6, 9] medical imaging[10] and tissue engineering scaffolds.[11] However, in vivo use of CNTs is still a task to be accomplished due to their debatable toxicity issues;[8] nevertheless some of the recent research in this direction is believed to be promising.[1, 2, 12] Although CNTs are yet to be approved by FDA (Food and Drug Administration) for safe use in humans, their pre-clinical applications as biomaterials have been extremely effective.[12] Many studies performed on animal models endorse CNTs being safe for living organisms provided that they are functionalized properly and more importantly, the method and site of use are appropriate.[12] The functionalization is very important aspect in determining the biological toxicity of CNTs in almost all of the aforementioned applications, for which the CNT surface needs to be modified by non-covalent or covalent interactions using various molecules like e.g. lipids, proteins, surfactants, drugs, polymers, peptides, nucleic acids and metal nanoparticles. Some of these molecules not only interact but also self-organize on CNT surfaces.[13, 14] Amphiphilic molecules, like lipids, are among such molecules which form self-assembled nanostructures when in solvents.[15] However, with CNTs they self-organize in such a way that the non-polar part is shielded from polar medium via hydrophobic interactions with CNT surface while the hydrophilic part faces polar solvent medium, which is usually water based.[10, 13, 16-20]

The type of self-assembled nanostructure, also called liquid crystalline lipid phases interacting with carbon nanotubes could differ from lipid to lipid[21] as well as with physicochemical conditions.[22] Usually the lamellar nanostructure (Fig. 1) is most commonly observed which mimics plasma membrane whereas hexagonal and cubic types of nanostructures resemble complex intracellular biomembranes.[15, 23] Lipid nanostructures are also used for many applications, for example, for separation of biomolecules,[24] protein crystallization,[25] synthesis of metal nanoparticles[26] and electrophoresis gels for amphiphilic molecules.[24] However, in some cases their applicability is hampered by their extremely high viscosity and inconsistent domain consistency. These problems are resolved by dispersing them into submicron (typical diameter 200-400 nm) or micron sized colloidal particles with large quantities of water (about 80-95%).[27, 28] The process involves kinetic stabilization of lipid nanostructures using some energy input and stabilizer molecules like surfactants, polymers, hydrocolloids or biomolecules resulting into oil-in-water (O/W) emulsions.[28, 29] (Note: Here 'oil' phase refers to lipid chains region.) The original lipid self-assembly is usually preserved inside dispersed particles and the overall viscosity is reduced by a few orders of magnitudes thereby enhancing their applicability.[29, 30] Major application of internally self-assembled lipid particles is for 'drug delivery' because of the possibility of loading them with hydrophilic, hydrophobic and amphiphilic molecules.[31-33]

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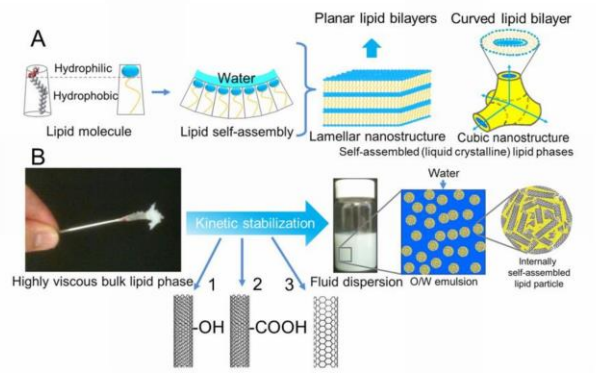

Fig.1 CNT Stabilized Internally Self-assembled Lipid Particles. (A) In presence of water, amphiphilic lipid molecules self-assemble into various liquid crystalline phases such as lamellar and cubic nanostructures. (B) Highly viscous lipid phases like cubic nanostructures are kinetically stabilized (using ultrasound energy and CNT stabilizer) into fluid dispersion. Pristine single-walled (SW) and functionalized (-OH and –COOH) multi-walled (MW) CNTs were used to stabilize the oil-in-water (Pickering) emulsion.

In this work we exploit CNTs as novel stabilizers for nanostructured lipid particles as depicted in Fig.1. Use of CNTs is expected to be significantly advantageous in stabilization of such systems because of their unique surface properties that facilitate extensive functionalization. Presence of lipid also overcame the destabilization of CNTs in aqueous solution which was otherwise occurring in minutes after their dispersion without lipid molecules. As mentioned earlier, CNTs themselves are promising candidates for drug delivery and other biomedical applications.[12] Combining them with nanostructured lipid particles that could be also loaded with drugs may open up many new pathways for example, for combination therapy or polytherapy i.e. for delivering multiple drugs or a drug and other inhibitory molecules at the same time at the same target. Such therapies are required mainly against multidrug resisting macromolecules or pathogens.[34, 35] The concentration of CNTs required to stabilize an emulsion (per unit volume of water) is extremely low (as low as 0.3 μg/ml). Furthermore, the biocompatibility of CNTs is expected to be greatly enhanced due to decoration with lipid molecules. These features can be attributed to a very low to negligible in vivo toxicity of CNT stabilized lipid nanoparticles for biomedical and pharmaceutical applications.

Results and Discussion

Preparation of CNT-stabilized Nanostructured Lipid Particles

Powdered forms of -COOH functionalized multi-walled (MW) CNTS, -OH functionalized MWCNTs and pristine single-walled (SW) CNTs were pre-dispersed in water by probe ultrasonicator followed by addition of molten Dimodan U (DU). The DU is a commercial lipid mainly consisting of monoglycerides and it forms a bicontinuous cubic Pn3m phase (crystallographic space group 224) in excess water (at room temperature).[36] The Pn3m phase exhibits 3-dimensionally folded curved bilayer draped over double diamond type mathematical minimal surface[15] separating two continuous water networks (Fig. 1A). The 'bicontinuous' feature of this nanostructure facilitates reconstitution and practically uninterrupted diffusion of amphiphilic molecules like membrane proteins providing them native like environment.[37] Each DU-CNT mixture in water was subjected to further ultrasonication in order to break large and inconsistent lipid domains formed during the hydration of a lipid. Dispersing the bulk lipid in this manner apparently speeds up the process of lipid hydration and thus equilibrium formation of self-assembled nanostructures, which otherwise requires considerably long time (could be one or many days) and/or freeze-thaw cycling. While the bulk lipid phase breaks into particles, the lipid coated CNTs are presumed to form a shell around them (Fig. 1B). The interfacial stabilization avoids further aggregation of both, nanostructured lipid particles as well as CNTs. Such kinetic stabilization can be called a Pickering[38] (due to the use of solid particles) emulsion of oil-in-

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water (O/W) type, where lipid forms an 'oil phase' while 'excess water' forms a continuous emulsion medium.[28] Such an aqueous medium and the hydrophobic lipid core of similar lipid particles (stabilized by surfactants) are shown to provide right environments for incorporation of hydrophilic and hydrophobic active pharmaceutical agents, respectively.[32, 33] Moreover, the amphiphilic lipid bilayer fabricating the cubic nanostructure is useful for reconstitution of membrane proteins.[39, 40] Dynamic light scattering studies confirmed that CNT stabilized nanostructured lipid particles were comparable in size (2-5 μm) with those stabilized by conventional surfactant stabilizer-pluronic F127 (Fig. 2).

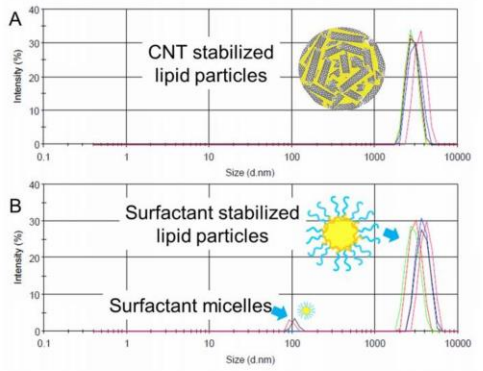

Fig.2 Particle size of nanostructured lipid particles (A) An average particle size of CNT stabilized nanostructured lipid particles was in the range of 2-5 μm and it was comparable to the size of lipid particles stabilized by pluronic F127 surfactant (B), as determined from dynamic light scattering experiments. An additional peak for surfactant based system could be attributed to surfactant micelles of about 100 nm size.

Lipid Decoration on CNTs Revealed by Raman Spectroscopy

Raman spectroscopy revealed CNT-lipid interactions responsible for their mutual stabilization in aqueous solutions (Fig. 3). The CNT spectra contain typical Raman graphite bands; the G band is assigned to the in-plane vibration of 'C-C bond', the D band (not shown) is activated by the presence of disorder in carbon systems and the G' band attributed to the overtone of the D band.[41] Upon interaction and formation of CNT stabilized lipid particles the G and G' bands display a blue shift (shift to higher wavenumbers). Table 1 lists the peak centers for characteristic bands from pure CNTs and nanostructured lipid particles containing CNTs. A blue shift, in case of CNTs, is a result of either or a combination of the following: 1) the dispersion of CNTs, as opposed to a bundled state when pure[41] 2) coating of CNTs; Douroumis et al.[42] have shown significant blue shifts when analysing lipid coated SWCNTs and suggest that the presence of functionality affects the symmetric radial vibrations of the hollow cylinders through stiffening which are attributed to the interactions between the CNTs and the lipid molecules 3) subjecting CNTs to a high pressure.[43]

Significant blue shifts (Table 1) observed in all spectra of lipid nanoparticles containing CNTs provide an evidence for 'dispersed' rather than 'bundled' CNTs. Hydrophobic interactions between CNT surface and alkyl chains of lipid molecules further contribute to the blue shift. Moreover, CNTs are exerted with a greater pressure due to increase in the overall viscosity of lipid particles because of dehydration. Decrease in relative intensities of typical CNT bands (as compared to uncoated CNTs) and emergence of prominent lipid signals also prove the fact that CNTs were decorated with lipid molecules.

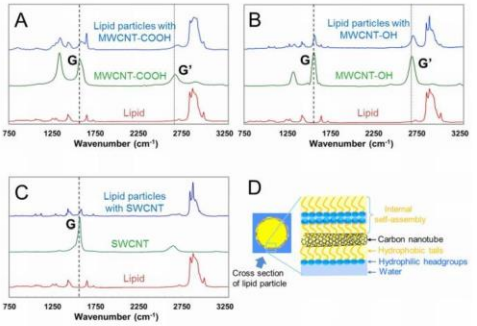

Fig 3 Raman spectra elucidating lipid decoration on CNTs Raman spectra of (A) pure lipid, MWCNT-COOH and CNT stabilized lipid nanoparticles containing 5.0 μg/ml MWCNT-COOH, (B) pure lipid, -OH functionalized multi-walled carbon nanotubes (MWCNT-OH) and lipid nanoparticles containing 5.0 μg/ml MWCNT-OH, and (C) pure lipid, single-walled carbon nanotubes (SWNT) and lipid nanoparticles containing 0.3125 μg/ml SWCNT (Other concentrations' Raman spectra are shown in ESI Fig. S1). All curves represent an average of ten spectra where intensities, in arbitrary units (labels not shown) are plotted along y-axis. Blue shift in typical G and G' bands of CNTs represent the interaction between CNT surface and lipid molecules. Lipid decoration over CNTs was also depicted by decrease in relative intensities of CNT peaks and appearance of lipid signals which are visible from blue curves. Dashed (G band) and dotted (G' band) lines are used to guide the eyes. (D) Schematic diagram of lipid self-assembling on CNT surface. Alkyl chains of lipid molecules interact with hydrophobic CNT surface whereas the hydrophilic lipid headgroups face aqueous region. Interior of particles is filled with self-assembled lipid nanostructure as indicated by the cross section of lipid particle (yellow).

CNT Type	Peak Centre of CNT (cm^{-1})	Peak Centre of CNT-Lipid Nanostructure (cm^{-1})	Wavenumber Shifted (cm^{-1})
MWCNT-COOH	1578.23 (G) 2683.72 (G')	1600.05 2720.08	21.83 36.36
MWCNT-OH	1570.91 (G) 2687.27 (G')	1588.24 2698.18	17.33 10.91
Pristine SWCNT	1570.91 (G)	1585.45	15.44

Table 1 Raman wavenumbers and shift for CNTs and nanostructured lipid particles with CNTs.

Optimum CNT:Lipid Ratio and Stability of Nanostructured Lipid Particles

As discussed earlier, interface of lipid particles is stabilized by a layer of CNTs enclosing self-assembled nanostructures at the core. However, we observed that there is a particular range of

CNT:lipid ratio in which they result in stable emulsions; ratios higher than this cause aggregation of CNTs as shown in Fig. 4 (arrows indicate visible aggregates of excess CNTs). Similarly lower ratios do not lead to stable emulsions because of an insufficient number of CNTs in the solution. Best working concentrations for MWCNT-COOH and MWCNT-OH were found to be between 3 to 5 μg/ml whereas for SWCNT they were in the range of 0.3-0.45 μg/ml. Previous research on the toxicity of pristine MWCNTs demonstrates that 40 μg/ml dosage should have no toxicity on human T lymphocytes,[44] while the research on pristine SWCNTs states that 7.5 μg/ml dosage causes only 10% decrease in cell proliferation and

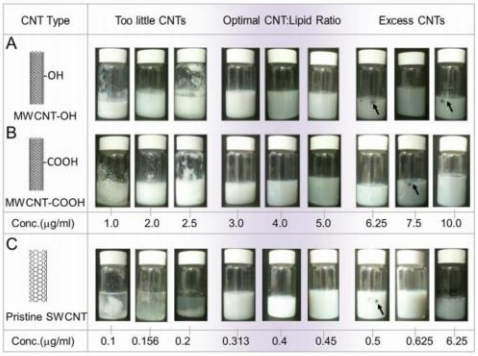

Fig. 4 Optimum CNT concentrations for stable emulsions Stable emulsions (shown by purple shade) were obtained in the region of 3.0-5.0 μg/ml for MWCNT–OH as in (A) and MWCNT–COOH as in (B). The concentration of SWCNTs for stable emulsions as in (C) was about an order of magnitude less than above numbers. It was also possible to get stable emulsions at concentrations very near to these ranges (as indicated by color gradient). For lower concentrations of CNTs, large domains of lipid were seen as there were too little CNTs per lipid whereas for higher concentrations there were too many CNTs which tend to aggregate; some of these are indicated by black arrows.

activity in Mesothelioma cell line MSTO-211H.45 CNT concentrations employed for stabilizing nanostructured lipid particles, in this work are well below (by an order of magnitude) these toxicity limits. Moreover, the functionalization (in case of MWCNTs) and coating with lipid molecules are expected to enhance biocompatibility and reduce the likelihood of a toxic effect.

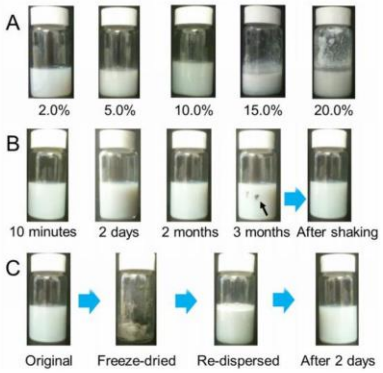

Fig. 5 Stability and regeneration of lipid particulate emulsions (A) Concentration of dispersed phase, mainly lipid could be tuned from 2 % to 20 %. At 20 % the emulsions become thick due to higher lipid material above which it was hard to prepare them using the ultrasonication method. (B) CNT stabilized nanoparticulate emulsions were stable for about 2 months, after which a few CNT aggregates were observed. It was, however possible to re-disperse them merely upon gentle shaking. (C) Emulsions could be completely dehydrated and dispersed again. There was a little foam on the top of re-dispersed emulsion which settled in about 2 days resulting into the stable emulsion. Above studies correspondingly demonstrate interesting properties of CNT stabilized emulsions as, capability of uptaking higher quantities of functional material (e.g. active molecules like drugs), shelf-life and practicability of storage/transport.

We were able to stabilize as low as 2 % dispersed phase emulsions using the same lipid:CNTs ratio (Fig. 5A) used for preparing all above emulsions (5 % dispersed phase). To another end, emulsions up to 20 % lipid material were also prepared by maintaining CNT:lipid ratio constant (Fig. 5A). This wide range of nanostructured lipid particle concentrations facilitates their loading with extensively varied quantities of drug molecules. CNT stabilized emulsions were stable for about 2 months after which CNTs start aggregating, but they were well dispersed again with only gentle shaking (Fig. 5B). Emulsions were also capable of retaining their stability after complete dehydration via freeze-drying followed by rehydration (Fig. 5C). The dehydrated lipid nanoparticles exhibited lamellar nanostructure similar to the dry lipid at ambient temperatures (see small angles x-ray scattering patterns in ESI Fig. S2).

Experimental

Materials

Single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes with -OH functionalization (MWCNT-OH) were obtained from Nanoamor (Houston, USA) whereas –COOH functionalized multi-walled CNTs (MWCNT-COOH) were purchased from Sigma-Aldrich (Athens, Greece). The lipid, Dimodan U/J (DU) was a generous gift from Danisco (Brabrand, Denmark). DU is a low-cost commercial product, as compared to its pure lipid analogues, containing more than 98% mono-glycerides. The triblock copolymer pluronic F127

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(PEO_{99}-PPO_{67}-PEO_{99}) was purchased from Sigma-Aldrich, UK. All solutions were prepared with Millipore water.

Preparation of Nanostructured Lipid Particles

First, powdered CNTs (MWCNT-OH, MWCNT-COOH and SWCNT) were dispersed in 100 ml water using probe ultrasonicator (ULT065 from Ultrawave, Cardiff, UK) at 40% power for 2 minutes without pulse. CNTs were stable on their own (without lipid) during preparation time (for about 20 minutes). The solution of surfactant F127 was prepared by simply stirring 200 mg of surfactant in 100 ml water. 9.5 ml of each of above solutions was then added to 500 mg of molten DU in a glass vial. The mixtures were then subjected to ultrasonication with same parameters as CNT dispersions. Emulsions were allowed to cool down (about 10 minutes) prior to further use.

Particle Size Measurement Using Dynamic Light Scattering

Particle sizes in dispersions were determined by using the Zetasizer Nano-ZS dynamic light scattering (DLS) equipment (from Malvern Instruments, UK). The data, in the form of particle size distribution was analyzed using Malvern DTS version 5.0. Each sample was tested 10 times and 5 such repeats were performed to get final plots.

Raman Spectroscopy on Lipid-CNT Samples

CNT, lipid and CNT-lipid samples were dehydrated using nitrogen gas followed by vacuum drying in the desiccator for about 20 minutes. Spectroscopic measurements were carried out on a Horiba Jobin-Yvon LabRAM HR800 spectrometer. A 532 nm laser was utilized to collect spectra from 100 – 4000 cm^{-1} using a grating of 600 g mm^{-1} and blazed at 750 nm. Spectra were acquired using x50 long working distance objective with a numerical aperture of 0.50. The confocal hole was set at 100 μm for spectral collections. The detector used was an Andor electromagnet (EM) charged coupled device (CCD). A video camera with the Raman system was used to guide spectral collection. All spectra were collected with sample situation on Calcium Fluoride slides (Crystran, UK). The instrumentation was calibrated before operation to silicon at the spectral line of 520.8 cm^{-1}. Spectra were acquired using the 532 nm laser at 1 % exposure for 10 seconds and accumulated 5 times. Immediate data interrogation and manipulation was carried out on the raw data using the LabSpec 6 spectroscopy software suite (HORIBA Scientific).

Conclusions and Perspectives

To conclude, we were able to produce nanostructured lipid particles with internal self-assembly using various CNTs as emulsion stabilizers. In the past, there have been several studies on using lipids or surfactants for dispersion and functionalization of CNTs, however this is the first report showing a range of CNTs for stabilization of lipid based emulsions. Requirement of very low levels of CNTs is promising for in vivo applications as the amount of CNTs administered into the body is an order of magnitude below toxicity limits revealed by the current literature, in addition, the biocompatibility of CNTs is greatly improved due to their coating with lipid molecules. Furthermore, owing to the possibility of loading functional molecules within lipid self-assembly as well as on CNT surface, the CNT stabilized lipid particles exhibit an enormous potential in emerging areas of pharmaceutical and biomedical sciences with special relevance to the development of combination therapies against major diseases. Fundamental properties of these emulsions are that they exhibit good kinetic stability; moreover, they can be regenerated after total dehydration of constituents. This allows for easy storage and transport of stabilized emulsions for use in harsh environments such as developing countries or forward based military hospitals.

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Notes and references

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Electronic Supplementary Information (ESI) available: Details of materials and methods, concentration series studies with Raman spectroscopy and small angle x-ray diffraction pattern for dry lipid and dehydrated CNT-lipid particles support the article. See DOI: 10.1039/b000000x/

1. W. Shao, P. Arghya, M. Yiyong, L. Rodes and S. Prakash, Carbon Nanotubes for Use in Medicine: Potentials and Limitations, 2013.
2. H. He, L. A. Pham-Huy, P. Dramou, D. Xiao, P. Zuo and C. Pham-Huy, BioMed Research International, 2013, **2013**, 12.
3. N. Gulati and H. Gupte, Crit Rev Ther Drug, 2012, **29**, 65-88.
4. M. Prato, K. Kostarelos and A. Bianco, Acc Chem Res, 2008, **41**, 60-68.
5. W. Yang, P. Thordarson, J. J. Gooding, S. P. Ringer and F. Braet, Nanotechnology, 2007, **18**, 412001.
6. M. Foldvari and M. Bagonluri, Nanomedicine: Nanotechnology, Biology and Medicine, 2008, **4**, 183-200.
7. M. Foldvari and M. Bagonluri, Nanomedicine: Nanotechnology, Biology and Medicine, 2008, **4**, 173-182.
8. C. P. Firme Iii and P. R. Bandaru, Nanomedicine: Nanotechnology, Biology and Medicine, 2010, **6**, 245-256.
9. N. W. S. Kam, M. O'Connell, J. A. Wisdom and H. Dai, Proceedings Of The National Academy Of Sciences Of The United States Of America, 2005, **102**, 11600-11605.

10. C. Richard, F. Balavoine, P. Schultz, T. W. Ebbesen and C. Mioskowski, Science, 2003, **300**, 775-778.
11. C. E. Ashley, E. C. Carnes, G. K. Phillips, D. Padilla, P. N. Durfee, P. A. Brown, T. N. Hanna, J. Liu, B. Phillips, M. B. Carter, N. J. Carroll, X. Jiang, D. R. Dunphy, C. L. Willman, D. N. Petsev, D. G. Evans, A. N. Parikh, B. Chackerian, W. Wharton, D. S. Peabody and C. J. Brinker, Nat Mater, 2011, **10**, 389-397.
12. N. Saito, H. Haniu, Y. Usui, K. Aoki, K. Hara, S. Takanashi, M. Shimizu, N. Narita, M. Okamoto, S. Kobayashi, H. Nomura, H. Kato, N. Nishimura, S. Taruta and M. Endo, Chemical Reviews, 2014, **114**, 6040-6079.
13. Y. Wu, J. S. Hudson, Q. Lu, J. M. Moore, A. S. Mount, A. M. Rao, E. Alexov and P. C. Ke, Journal of Physical Chemistry B, 2006, **110**, 2475-2478.
14. G. Liu and Y. Lin, J Nanosci Nanotechnol, 2006, **6**, 948-953.
15. C. V. Kulkarni, Nanoscale, 2012, **4**, 5779-5791.
16. E. Contal, A. Morère, C. d. Thauvin, A. l. Perino, S. p. Meunier, C. Mioskowski and A. Wagner, The Journal of Physical Chemistry B, 2010, **114**, 5718-5722.
17. E. J. Wallace and S. P. S. Mark, Nanotechnology, 2009, **20**, 045101.
18. A. A. Kapralov, W. H. Feng, A. A. Amoscato, N. Yanamala, K. Balasubramanian, D. E. Winnica, E. R. Kisin, G. P. Kotchey, P. P. Gou, L. J. Sparvero, P. Ray, R. K. Mallampalli, J. Klein-Seetharaman, B. Fadeel, A. Star, A. A. Shvedova and V. E. Kagan, Acs Nano, 2012, **6**, 4147-4156.
19. N. Patra and P. Kral, J Am Chem Soc, 2011, **133**, 6146-6149.
20. R. Qiao and P. C. Ke, Journal of the American Chemical Society, 2006, **128**, 13656-13657.
21. C. Paukner, K. K. K. Koziol and C. V. Kulkarni, Nanoscale, 2013, **5**, 8992-9000.
22. C. V. Kulkarni, W. Wachter, G. R. Iglesias, S. Engelskirchen and S. Ahualli, Phys Chem Chem Phys, 2011, **13**, 3004-3021.
23. Z. Almsherqi, F. Margadant and Y. Deng, in Advances in Planar Lipid Bilayers and Liposomes, Elsevier, 2010, vol. 12, ch. 4, pp. 79-99.
24. Application: US Pat., US2001/0025791A1, 2001.
25. C. V. Kulkarni, in Advances in Planar Lipid Bilayers and Liposomes, ed. A. Iglic, Academic Press, 2010, vol. 12, ch. 9, pp. 237-272.
26. Y. Song, R. M. Dorin, R. M. Garcia, Y. B. Jiang, H. Wang, P. Li, Y. Qiu, F. van Swol, J. E. Miller and J. A. Shelnutt, J Am Chem Soc, 2008, **130**, 12602-12603.
27. J. Gustafsson, H. Ljusberg-Wahren, M. Almgren and K. Larsson, Langmuir, 1996, **12**, 4611-4613.
28. C. V. Kulkarni, in Encyclopaedia of Biophysics, ed. G. C. Roberts, Springer Varlag., 2012.
29. A. Yaghmur and O. Glatter, Adv Colloid Interface Sci, 2009, **147-148**, 333-342.
30. C. V. Kulkarni and O. Glatter, in Self-Assembled Supramolecular Architectures: Lyotropic Liquid Crystals, ed. N. Garti, John Wiley & Sons, Inc., 2012, ch. 6.
31. S. B. Rizwan, B. J. Boyd, T. Rades and S. Hook, Expert Opin Drug Del, 2010, **7**, 1133-1144.
32. T. H. Nguyen, T. Hanley, C. J. Porter, I. Larson and B. J. Boyd, J Pharm Pharmacol, 2010, **62**, 844-855.
33. K. W. Lee, T. H. Nguyen, T. Hanley and B. J. Boyd, Int J Pharm, 2009, **365**, 190-199.
34. R. J. Worthington and C. Melander, Trends in Biotechnology, 2013, **31**, 177-184.
35. H. Araoka, Nihon rinsho. Japanese journal of clinical medicine, 2012, **70**, 305-310.
36. R. Mezzenga, C. Meyer, C. Servais, A. I. Romoscanu, L. Sagalowicz and R. C. Hayward, Langmuir, 2005, **21**, 3322.
37. E. M. Landau and J. P. Rosenbusch, PNAS, 1996, **93**, 14532-14535.
38. S. U. Pickering, J. Chem. Soc., 1907, **91**, 2001.
39. M. Caffrey, Annual Review of Biophysics, 2009, **38**, 29-51.
40. C. V. Kulkarni, A. M. Seddon, O. Ces and R. H. Templer, Soft Matter, 2010, **6**, 4339 - 4341.
41. L. Bokobza and J. Zhang, Express Polymer Letters, 2012, **6**, 601.
42. D. Douroumis, D. G. Fatouros, N. Bouropoulos, K. Papagelis and D. Tasis, Int J Nanomedicine, 2007, **2**, 761-766.
43. Q. Zhao and H. D. Wagner, Philosophical transactions. Series A, Mathematical, physical, and engineering sciences, 2004, **362**, 2407-2424.
44. M. Bottini, S. Bruckner, K. Nika, N. Bottini, S. Bellucci, A. Magrini, A. Bergamaschi and T. Mustelin, Toxicology Letters, 2006, **160**, 121-126.
45. P. Wick, P. Manser, L. K. Limbach, U. Dettlaff-Weglikowska, F. Krumeich, S. Roth, W. J. Stark and A. Bruinink, Toxicology Letters, 2007, **168**, 121-131.

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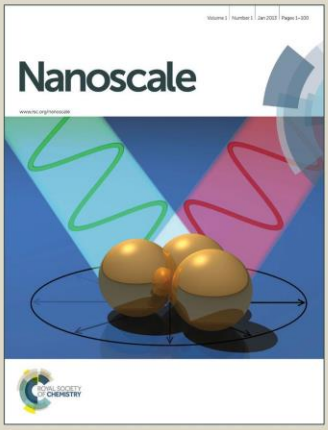


Publications

1. Gaunt, N.P., Baker, M.J., Bond, G., Tomsic, M., & Kulkarni, C.V. (2014, 24-25 July) *Carbon Nanotubes for Stabilising Lipid Nanostructured Particles.* Poster presented at Macro Group Young Researchers' Meeting, Durham University, UK
2. Gaunt, N.P., Baker, M.J., Patil-Sen, Y., & Kulkarni, C.V. (2014, 1-3 September) *Functionalised CNT-Lipid Nanostructures as Drug Delivery Carriers.* Poster presented at Faraday Discussion 173, Royal Society of Chemistry, London, UK
3. Gaunt, N.P., Patil-Sen, Y., Baker, M.J., & Kulkarni, C.V. *Carbon Nanotubes for Stabilization of Nanostructured Lipid Particles* Paper accepted to Nanoscale
4. Gaunt, N.P., Patil-Sen, Y., Baker, M.J., & Kulkarni, C.V. *Functionalised CNT-Lipid Nanostructures as Drug Delivery Carriers.* Paper in progress
5. Moinuddin, Z., Patil-Sen, Y., Gaunt, N.P., Kulkarni, C.V. (2014, 24-25 July) *Encapsulation of Functional Molecules in Hydrogels* Poster presented at Macro Group Young Researchers' Meeting, Durham University, UK

References

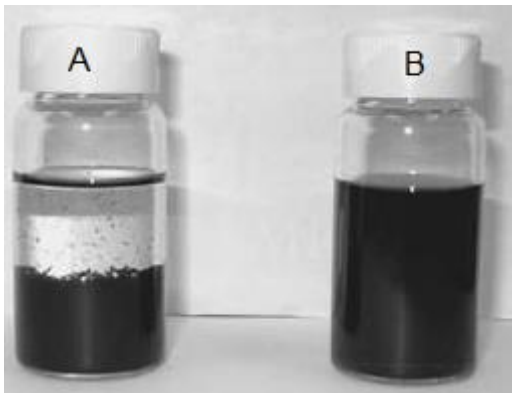
1. N.K. Mehra A.K. Jain, N. Lodhi, V. Dubey, D. Mishra, R. Raj. et al. Challenges in the use of carbon nanotubes in biomedical applications. Crit Rev Ther Drug Carr Syst 2008; 25(2): 169-6.
2. Martin C R, Kohli P. The emerging field of nanotube biotechnology. Nat Rev Drug Discov 2003; 2:29–7.
3. Jain AK, Mehra NK, Lodhi N, Dubey V, Mishra D, Jain NK. Carbon nanotubes and their toxicity. Nanotoxicol 2007; 1(3):167.
4. Iijima S. Helical microtubules of graphite carbon. Nature 1991; 354: 56-58.
5. I. Ojima, Guided molecular missiles for tumor-targeting chemotherapy–case studies using the second-generation taxoids as warheads, Acc Chem Res, 41 (2008), pp. 108–119
6. M. Ferrari, Cancer nanotechnology: opportunities and challenges, Nat Rev Cancer, 5 (2005), pp. 161–171
7. S.S. Xie, W.Z. Li, Z.W. Pan, B.H. Chang, L.F. Sun, Mechanical and physical properties on carbon nanotube, J Phys Chem Solids, 61 (2000), pp. 1153–1158
8. R.J. Chen, Y. Zhang, D. Wang, H. Dai, Noncovalent sidewall functionalization of single-walled carbon nanotubes for protein immobilization, J Am Chem Soc, 123 (2001), pp. 3838–3839
9. Y. Liu, D.C. Wu, W.D. Zhang, X. Jiang, C.B. He, T.S. Chung et al., Polyethylenimine-grafted multiwalled carbon nanotubes for secure noncovalent immobilization and efficient delivery of DNA, Angew Chem Int Ed Engl, 44 (2005), pp. 4782–4785
10. Z. Chen, X. Zhang, R. Yang, Z. Zhu, Y. Chen, W. Tan, Single-walled carbon nanotubes as optical materials for biosensing, Nanoscale, 3 (2011), pp. 1949–1956
11. D. Ostling, D. Tomanek, A. Rosen, Electronic structure of single-wall, multiwall, and filled carbon nanotubes, Phys Rev B, 55 (1997), pp. 13980–13988
12. M.F. Lin, D.S. Chuu, K.W.K. Shung, Thermal conductance and the Peltier coefficient of carbon nanotubes, Phys Rev B, 53 (1996), pp. 11186–11192
13. Z. Liu, S.M. Tabakman, Z. Chen, H. Dai, Preparation of carbon nanotube bioconjugates for biomedical applications, Nat Protoc, 4 (2009), pp. 1372–1382
14. Z. Liu, K. Yanagi, K. Suenaga, H. Kataura, S. Iijima, Imaging the dynamic behaviour of individual retinal chromophores confined inside carbon nanotubes, Nat Nanotechnol, 2 (2007), pp. 422–425
15. M. Koshino, T. Tanaka, N. Solin, K. Suenaga, H. Isobe, E. Nakamura, Imaging of single organic molecules in motion, Science, 316 (2007), p. 853
16. R.R. Meyer, J. Sloan, R.E. Dunin-Borkowski, A.I. Kirkland, M.C. Novotny, S.R. Bailey et al., Discrete atom imaging of one-dimensional crystals formed within single-walled carbon nanotubes, Science, 289 (2000), pp. 1324–1327

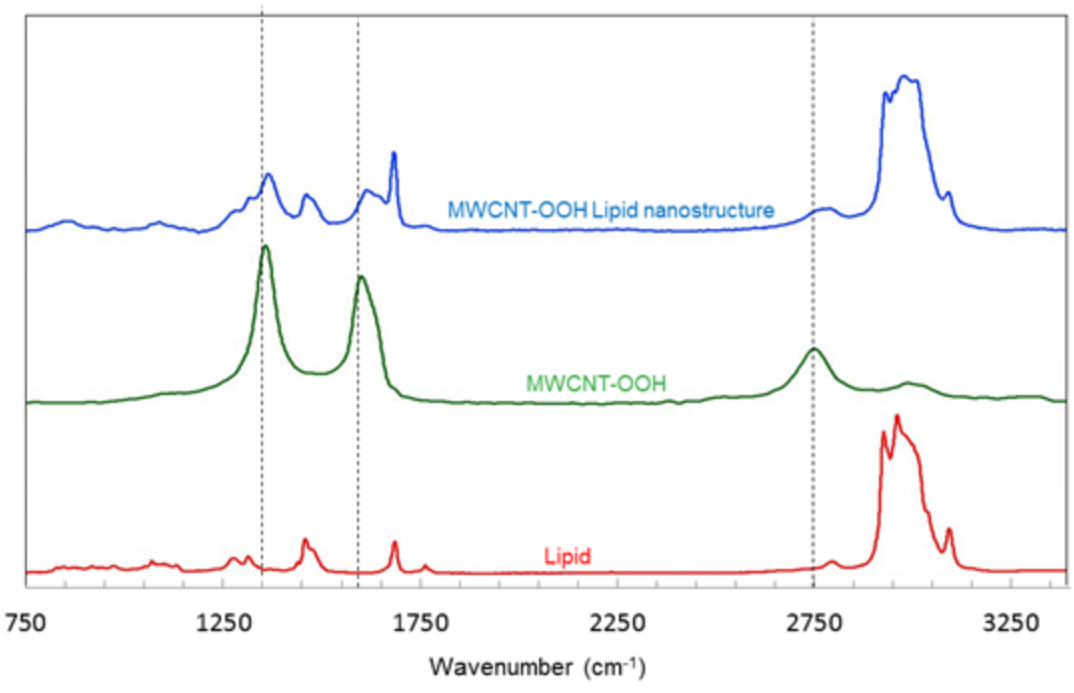
17. S.Y. Hong, G. Tobias, K.T. Al-Jamal, B. Ballesteros, H. Ali-Boucetta, S. Lozano-Perez et al., Filled and glycosylated carbon nanotubes for in vivo radioemitter localization and imaging, Nat Mater, 9 (2010), pp. 485–490
18. S. Hampel, D. Kunze, D. Haase, K. Kramer, M. Rauschenbach, M. Ritschel et al.,Carbon nanotubes filled with a chemotherapeutic agent: a nanocarrier mediates inhibition of tumor cell growth, Nanomedicine (Lond), 3 (2008), pp. 175–182
19. B.S.Wong, et al.,Carbon nanotubes for delivery of small molecule drugs, Adv. Drug Deliv. Rev.(2013), http://dx.doi.org/10.1016/j.addr.2013.08.005
20. Z. Liu, X. Sun, N. Nakayama-Ratchford, H. Dai, Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery, ACS Nano, 1 (2007), pp. 50–56
21. Yu BZ, Yang JS, Li WX. In vitro capability of multi-walled carbon Nanotubes modified with gonadotrophin releasing hormone on killing cancer cells. Carbon 2007;45(10):1921–7.
22. Pantarotto D, Briand JP, Prato M, Bianco A. Translocation of bioactive peptides across cell membrane by carbon nanotubes. Chem Commun. 2004;(1):16–17.
23. Pantarotto D, Partidos CD, Hoebke J, Brown F, Kramer E, Briand JP, Muller S, Prato M. Immunizations with peptide-functionalized carbon nanotubes enhances virus-specific neutralising antibody response. Chem Biol 2003;10:961–6.
24. G. R. Dieckmann et al., J. Am. Chem. Soc. 125, 1770 (2003).
25. C. Zettlemoyer, J. Colloid Interface Sci. 28, 343 (1968).
26. Journet et al., Nature 388, 756 (1997).
27. J.-M. Bonard et al., Adv. Mater. 9, 827 (1997).
28. M. Burghard, G. Duesberg, G. Philipp, J. Muster, S. Roth, Adv. Mater. 10, 584(1998).
29. M. J. O'Connell et al., Science 297, 593 (2002).
30. S. Manne, J. P. Cleveland, H. E. Gaub, G. D. Stucky, P. K. Hansma, Langmuir 10, 4409 (1994).
31. S. Manne, H. E. Gaub, Science 270, 1480 (1995).
32. A. Aksay et al., Science 273, 892 (1996).
33. Dubey V, Mishra D, Dutta T, Nahar M, Saraf D K, Jain NK. Dermal and transdermal delivery of an anti-psoriatic agent via ethanolic liposome. J Control Rel 2007; 123(2): 148-54.
34. R.H. Müller, G.E. Hildebrand (Eds.), Pharmazeutische Technologie: Moderne Arzneiformen, Lehrbuch für Studierende der Pharmazie–Nachschlagewerk für Apotheker in Offizin, Krankenhaus und Forschung (2. Erweiterte Aufl.), Wissenschaftliche Verlagsgesellschaft, Stuttgart (1998)

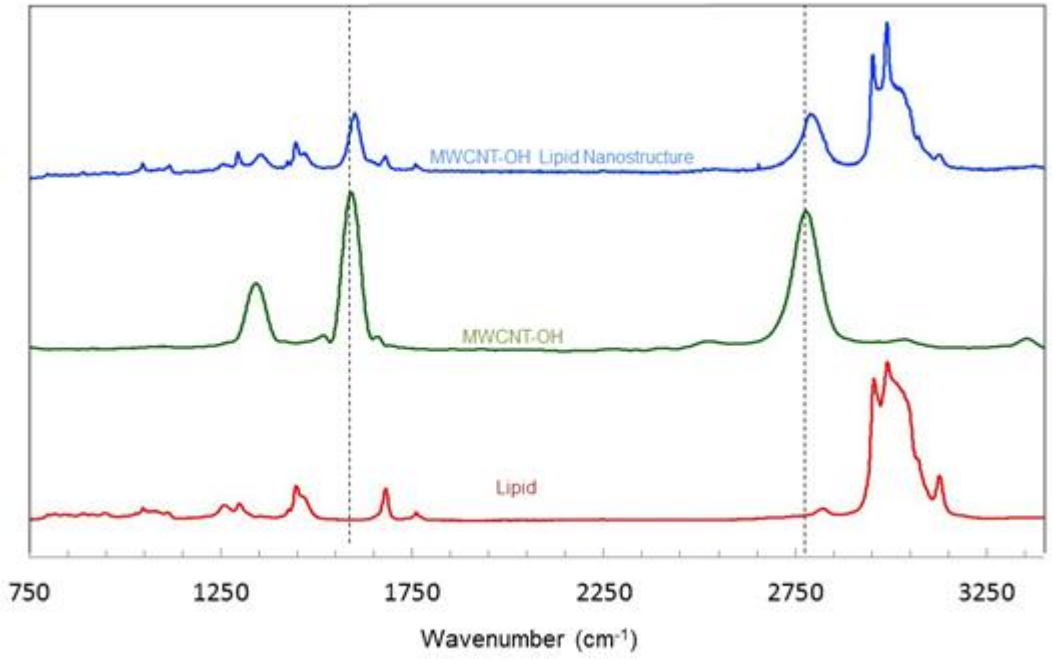
35. J. Schmitt, Parenterale Fettemulsionen als Arzneistoffträger,R.H. Müller, G.E. Hildebrand (Eds.), Pharmazeutische Technologie: Moderne Arzneiformen, Wissenschaftliche Verlagsgesellschaft, Stuttgart (1998), pp. 189–194
36. Liu X, Sun X, Nakayama-Ratchford N, Dai H. Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery. ACS Nano 2007;1(1):50–6.
37. P. M. Ajayan, Chem. Rev. 99, 1787 (1999).
38. M. S. Dresselhaus, G. Dresselhaus, P. C. Eklund, Science of Fullerenes and Carbon Nanotubes(Academic Press, San Diego, CA, 1996).
39. J. Chen et al., Science 282, 95 (1998).
40. J. L. Bahr et al., J. Am. Chem. Soc. 123, 6536 (2001).
41. F. Balavoine et al., Angew. Chem. Int. Ed. 38, 1912 (1999).
42. J.E. Diederichs, R.H. Müller, Liposome in Kosmetika und Arzneimitteln, Pharm. Ind., 56 (1994), pp. 267–275
43. A. Fahr, T. Kissel, Mikropartikel und Implantate: Arzneiformen zur parenteralen Applikation, R.H. Müller, G.E. Hildebrand (Eds.), Pharmazeutische Technologie: Moderne Arzneiformen, Wissenschaftliche Verlagsgesellschaft, Stuttgart (1998), pp. 243–258
44. Roberts, M.P., and Gabriel, N.E. "Unilamellar lipid vesicles and method for their formation." asssachusettes Institute of Technology, U.S. Pat. 4921706, 1991.
45. Segota, S., and Tezak D. "Spontaneous formation of vesicles." Advances in Colloid and Interface Science 121 (2006): 51-75
46. Tomohiro, I., Shoko, Y. and Masahiko, A. "Interactions between Lipid and its Substances in Bilayer Membrane." Adv. in Planar Lipid Bilayer and Liposomes 4 (2006): 191-227.
47. Vamvakaki, V., and Chaniotakis, N.A. "Pesticide detection with a liposome-based nano-biosensor." Biosensors and Bioelectronics 22 (2007): 2848–2853.
48. Bangham, A. "The First Description of Liposomes." Current Contents 13 (1989).
49. Yeagle, PL. "Lipid regulation of cell membrane structure and function." FASEB J. 3 (1989): 1833-1842.
50. Boyd, B.B., S.B. Rizwan, Y.-D. Dong, S. Hook, and T. Rades. "The self-assembled liquid crystalline nanoparticles imaged in three dimensions: hexosomes are not necessarily flat hexoganal prisms." Langmuir 23(25) (2007): 12461–12464.
51. Mouritsen, O. G. " In Life-As a matter of Fat." Springer-Verlag, Berlin, Heidelberg, Germany, 2005.
52. Crommelin, D. J., and H. Schreier. "Liposomes." In Colloidal Drug Systems, 73-199. New York: Kreuter, J.; Dekker, M., 1994.

53. Kulkarni, C. V., Mezzenga, R., Glatter. O., “Water-in-oil nanostructured emulsions: towards the structural hierarchy of liquid crystalline materials” Soft Matter, 2010,6, 5615–5624
54. D. Douroumis, D. G. Fatouros, N. Bouropoulos, K. Papagelis and D. Tasis, Int J Nanomedicine, 2007, 2, 761-766
55. Q. Zhao and H.D. Wagner, Philosophical transactions. Series A. Mathematical, physical, and engineering sciences, 2004, 362, 2407-2424
56. S. Bellucci, Carbon Nanotubes Toxicity, Nanoparticles and Nanodevices in Biological Applications, 2009,7, 47-67
57. O. Agrawal, R. Brahme, M. Faria, S. Shidhaye, Nanotechnology in cancer: A clinical review, Journal of Applied Pharmaceutical Science 2011, 3, 25-29

Appendix


A
B


MWCNT-OOH Lipid nanostructure
MWCNT-OOH
Lipid
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)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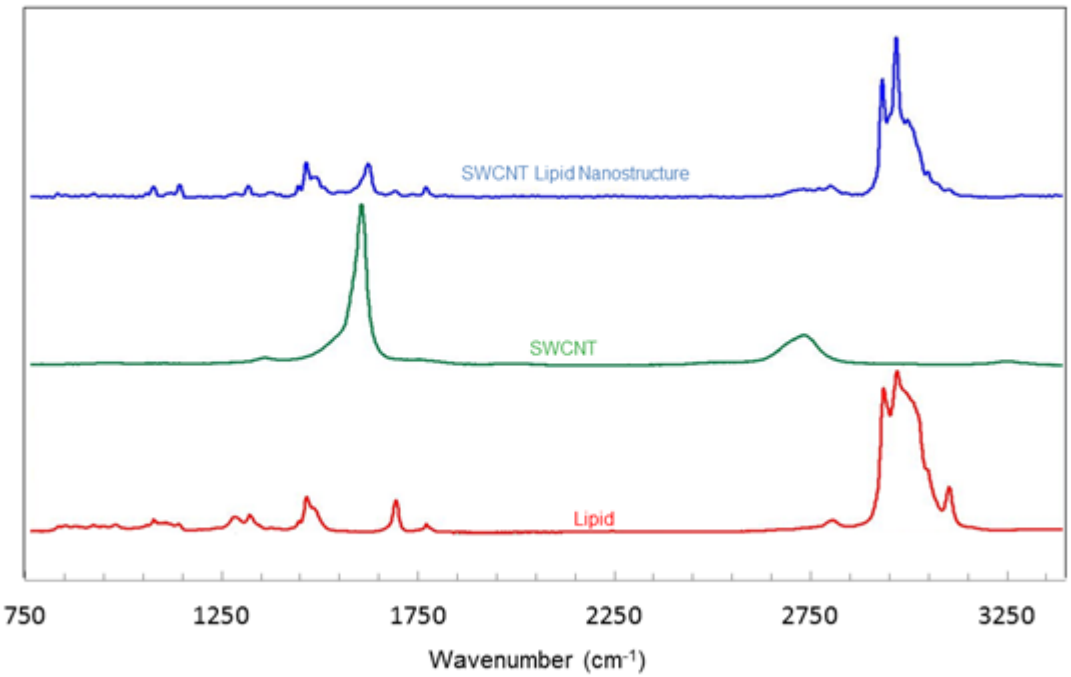
MWCNT-OH
Lipid
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)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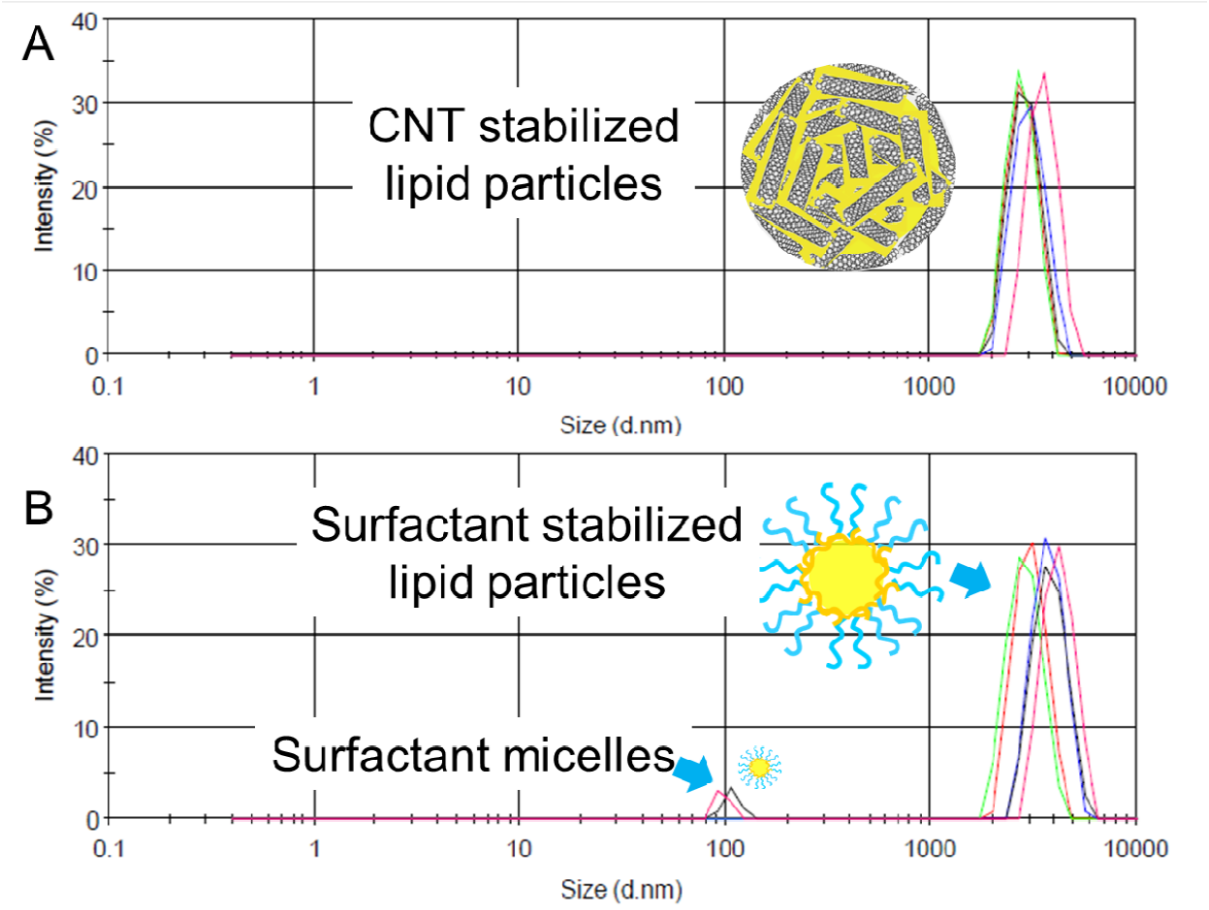
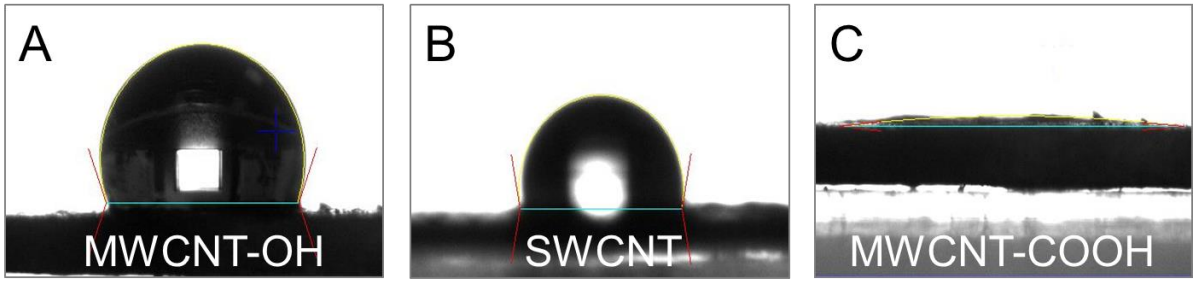
SWCNT
Lipid
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)


A
CNT stabilized
lipid particles
Intensity (%)
40
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)
B
Surfactant stabilized
lipid particles
Surfactant micelles
Intensity (%)
40
30
20
10
0
0.1
1
10
100
1000
10000
Size (d.nm)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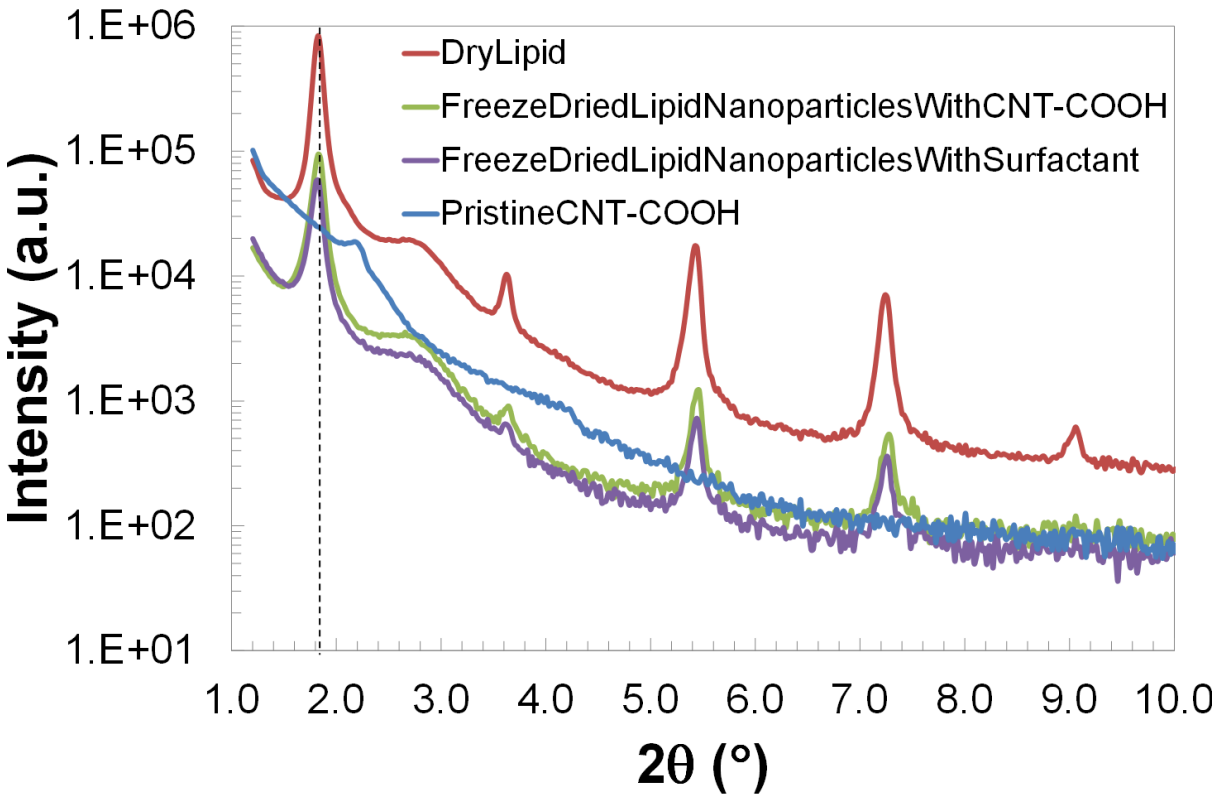
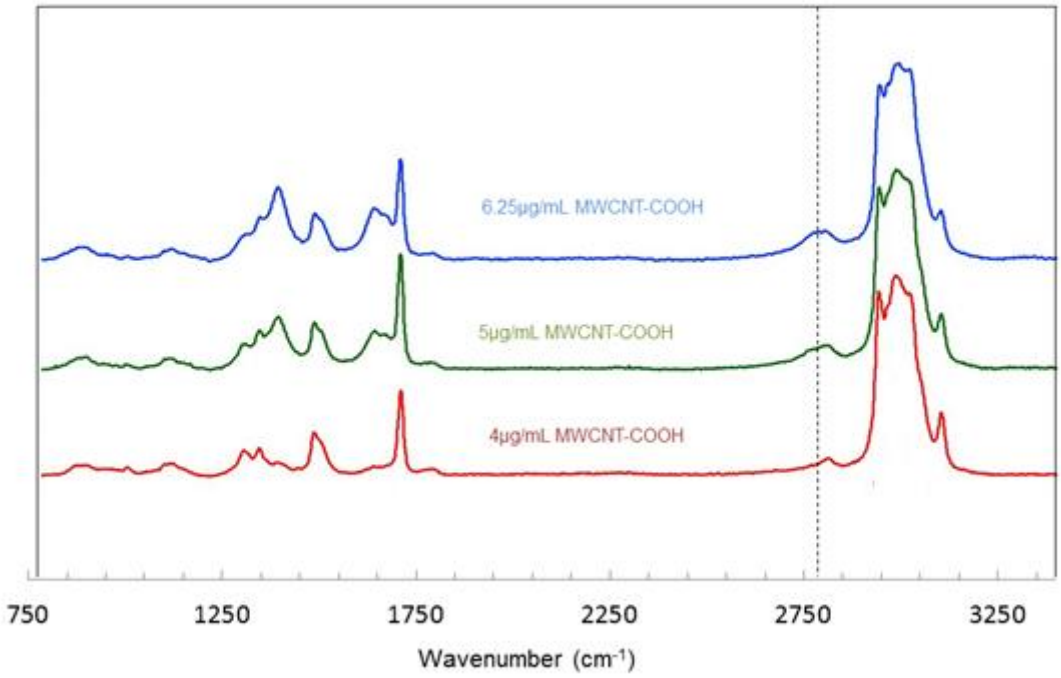
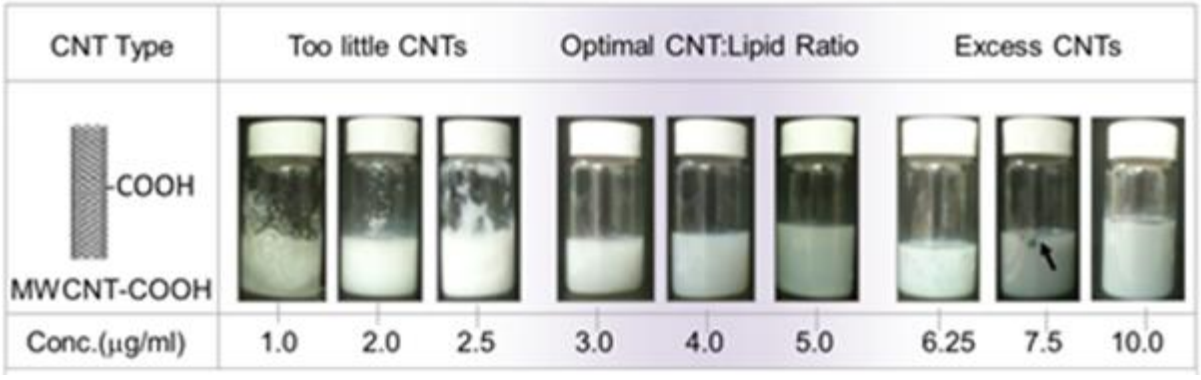
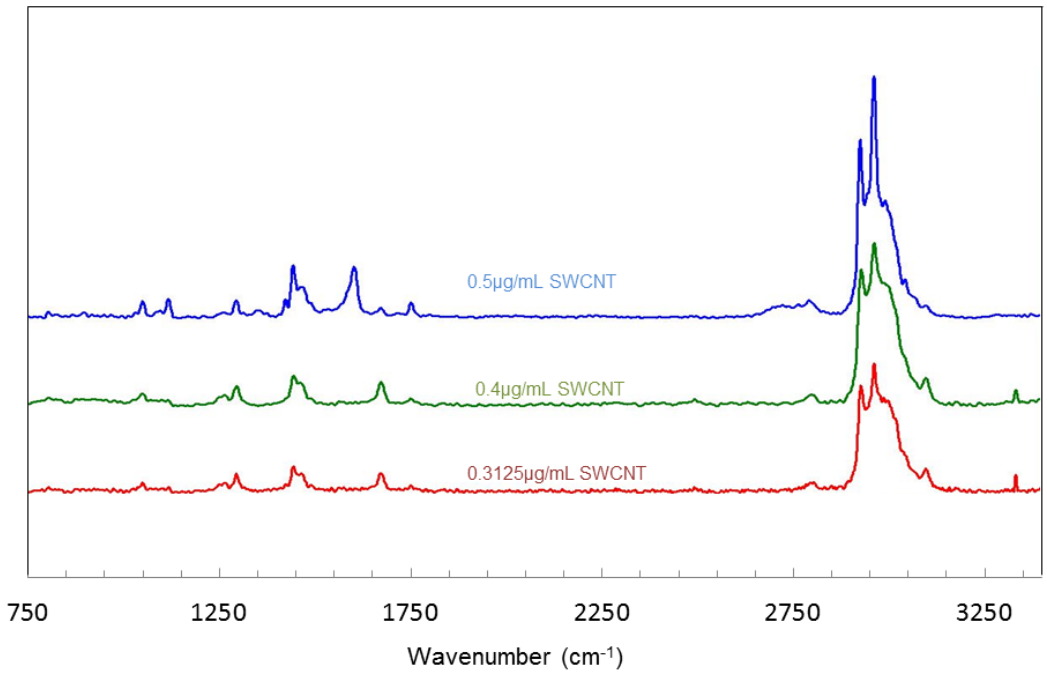
B
SWCNT
C
MWCNT-COOH

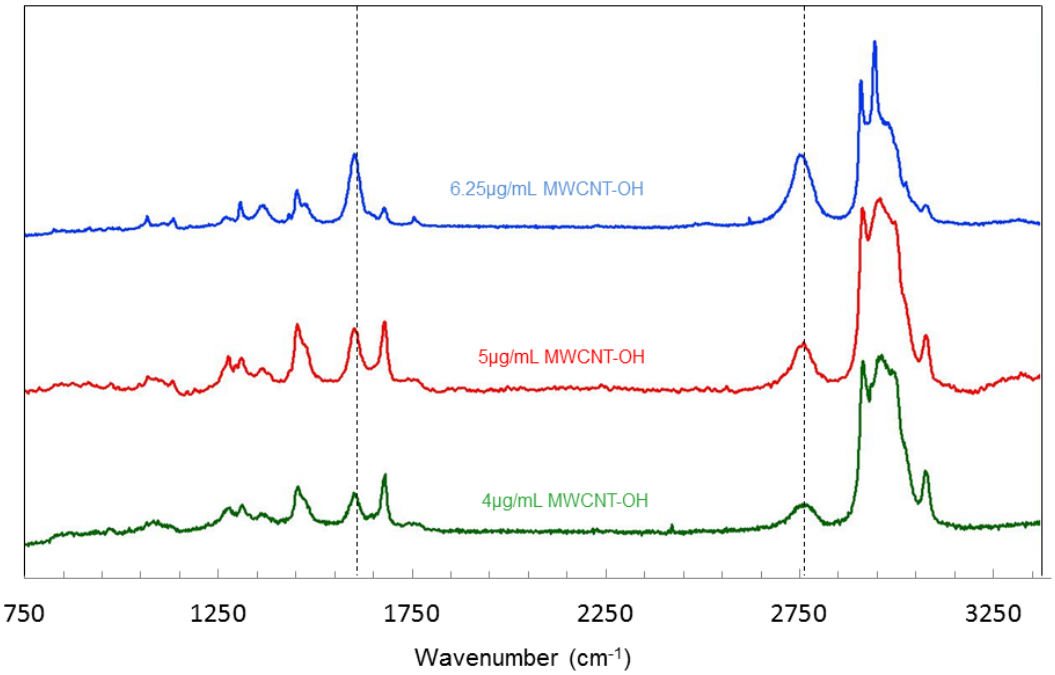

1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
Intensity (a.u.)
DryLipid
FreezeDriedLipidNanoparticlesWithCNT-COOH
FreezeDriedLipidNanoparticlesWithSurfactant
PristineCNT-COOH
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
2θ (°)

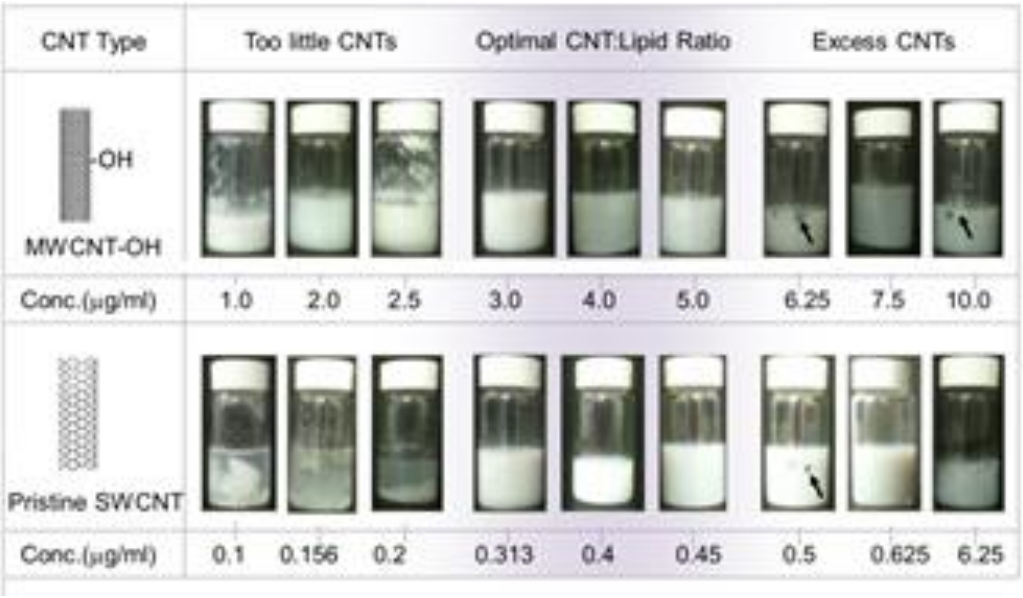

Emulsion stability
10 minutes
2 days
2 months
3 months
After shaking


6.25μg/mL MWCNT-COOH
5μg/mL MWCNT-COOH
4μg/mL MWCNT-COOH
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)

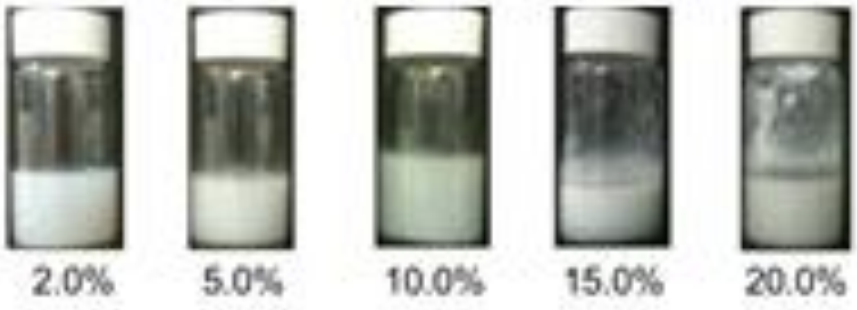

CNT Type
Too little CNTs
Optimal CNT:Lipid Ratio
Excess CNTs
-COOH
MWCNT-COOH
Conc.(μg/ml)
1.0
2.0
2.5
3.0
4.0
5.0
6.25
7.5
10.0

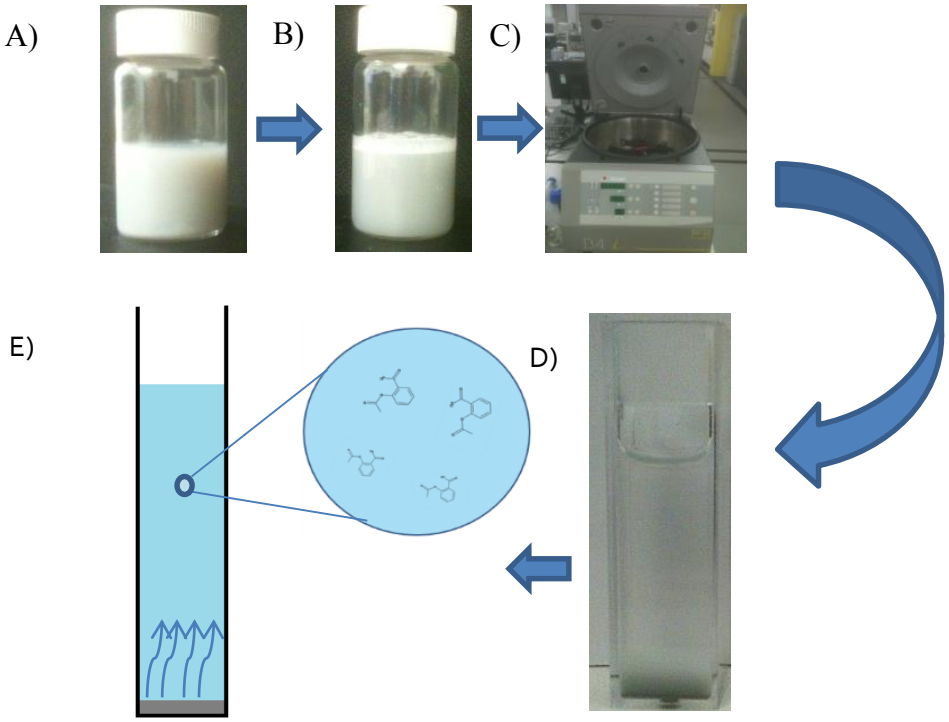

0.5µg/mL SWCNT
0.4µg/mL SWCNT
0.3125µg/mL SWCNT
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)

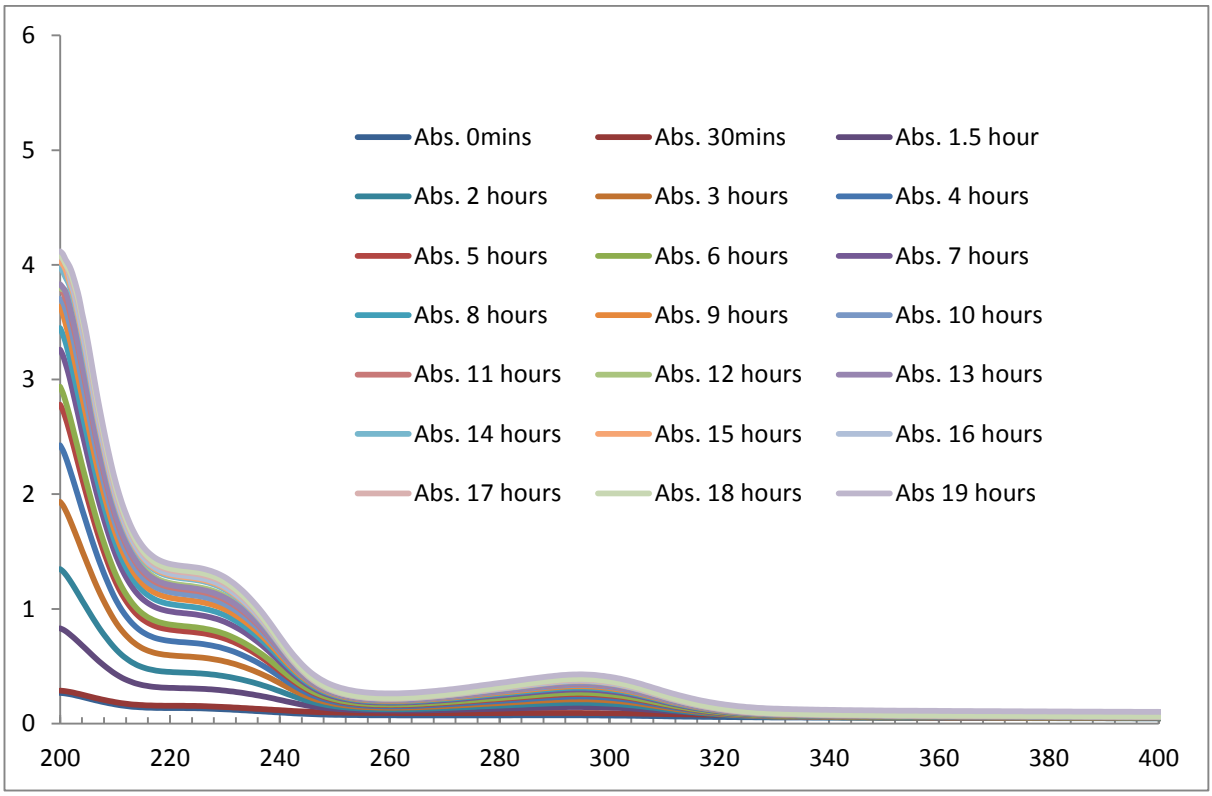

6.25µg/mL MWCNT-OH
5µg/mL MWCNT-OH
4µg/mL MWCNT-OH
750
1250
1750
2250
2750
3250
Wavenumber (cm-1)

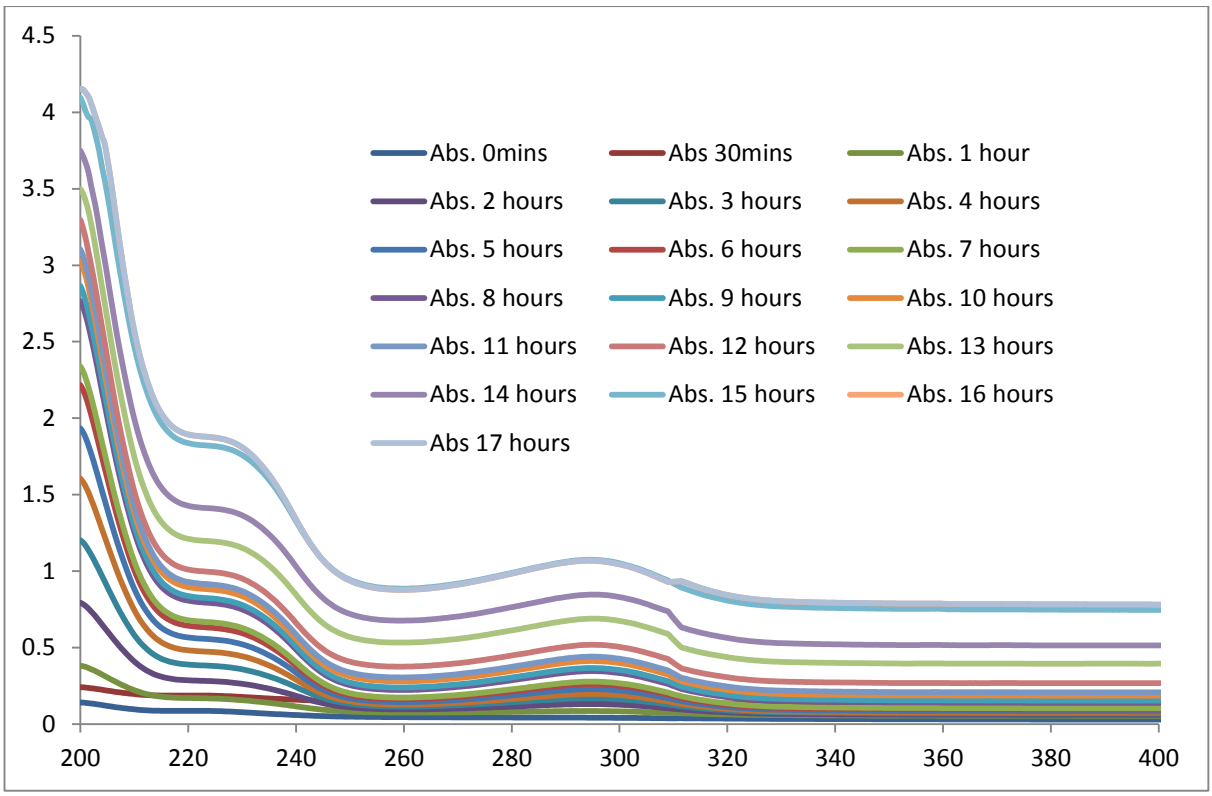

CNT Type
Too little CNTs
Optimal CNT:Lipid Ratio
Excess CNTs
-OH
MWCNT-OH
Conc.(μg/ml)
1.0 2.0 2.5 3.0 4.0 5.0 6.25 7.5 10.0
Pristine SWCNT
Conc.(μg/ml)
0.1 0.156 0.2 0.313 0.4 0.45 0.5 0.625 6.25

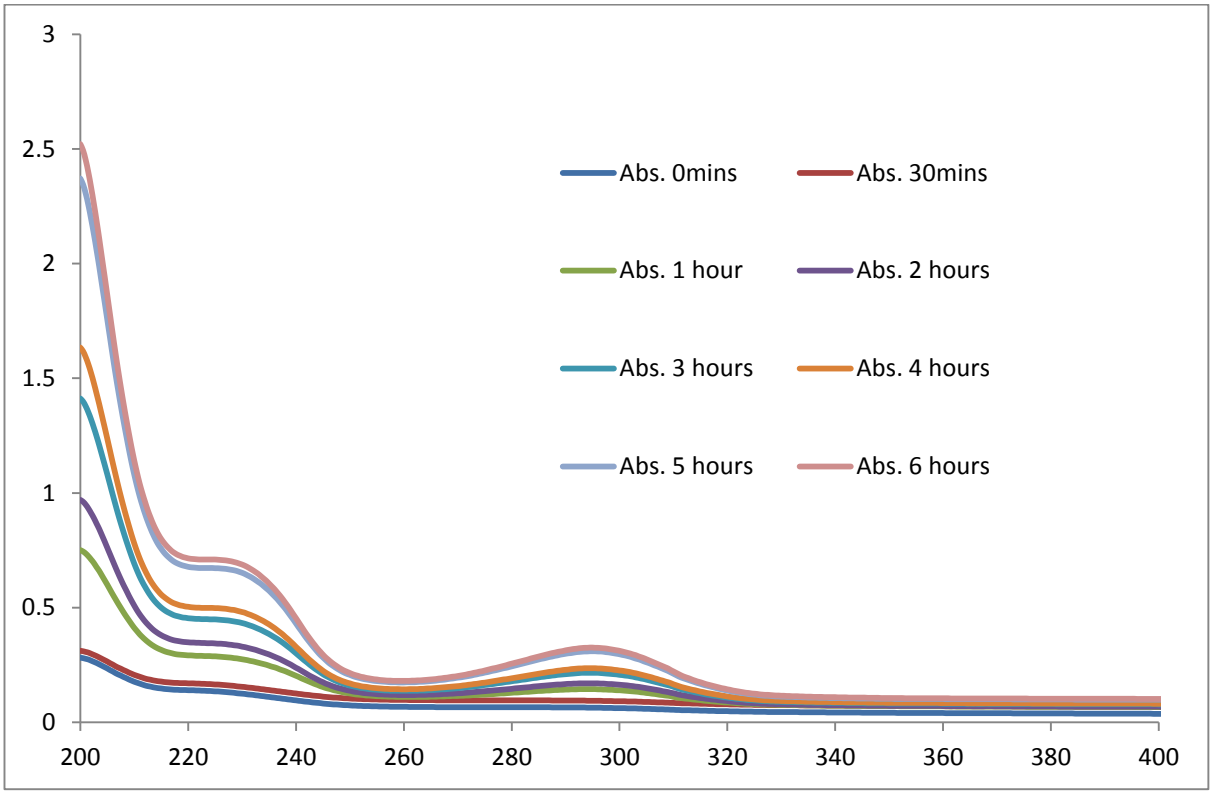

Original
Freeze-dried
Re-dispersed
After 2 days

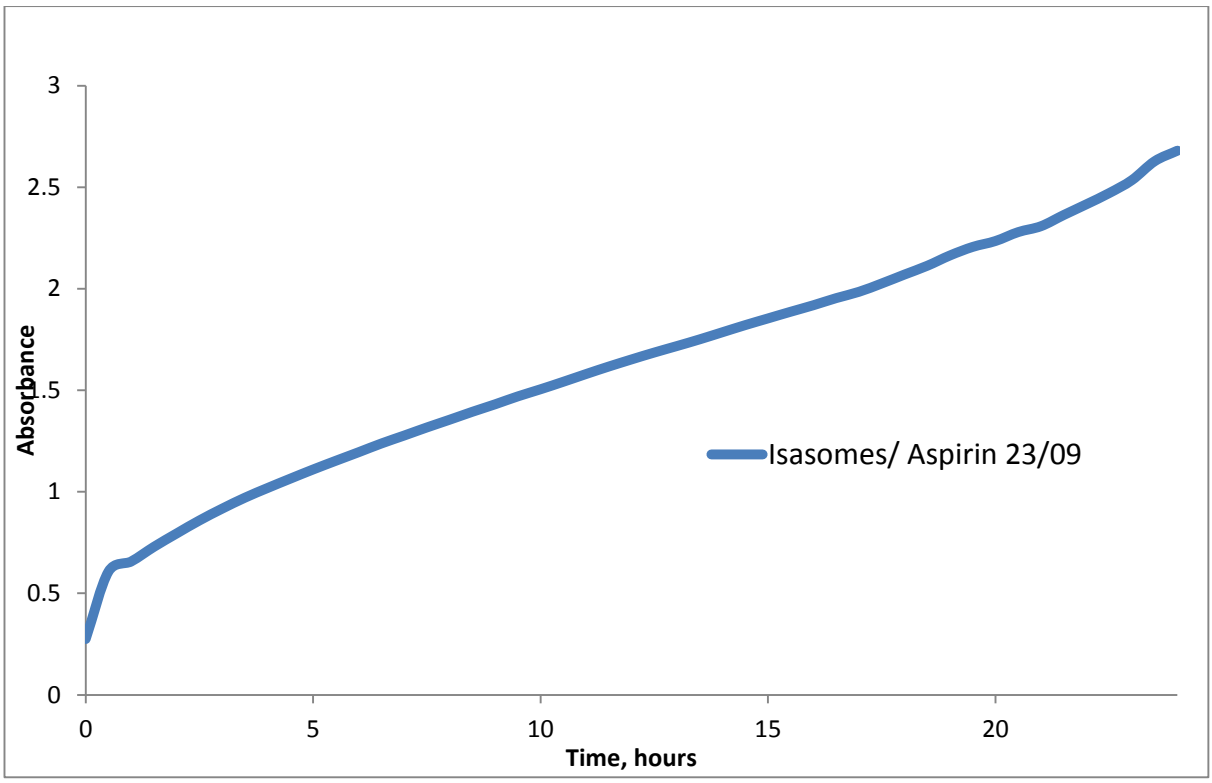

2.0%
5.0%
10.0%
15.0%
20.0%

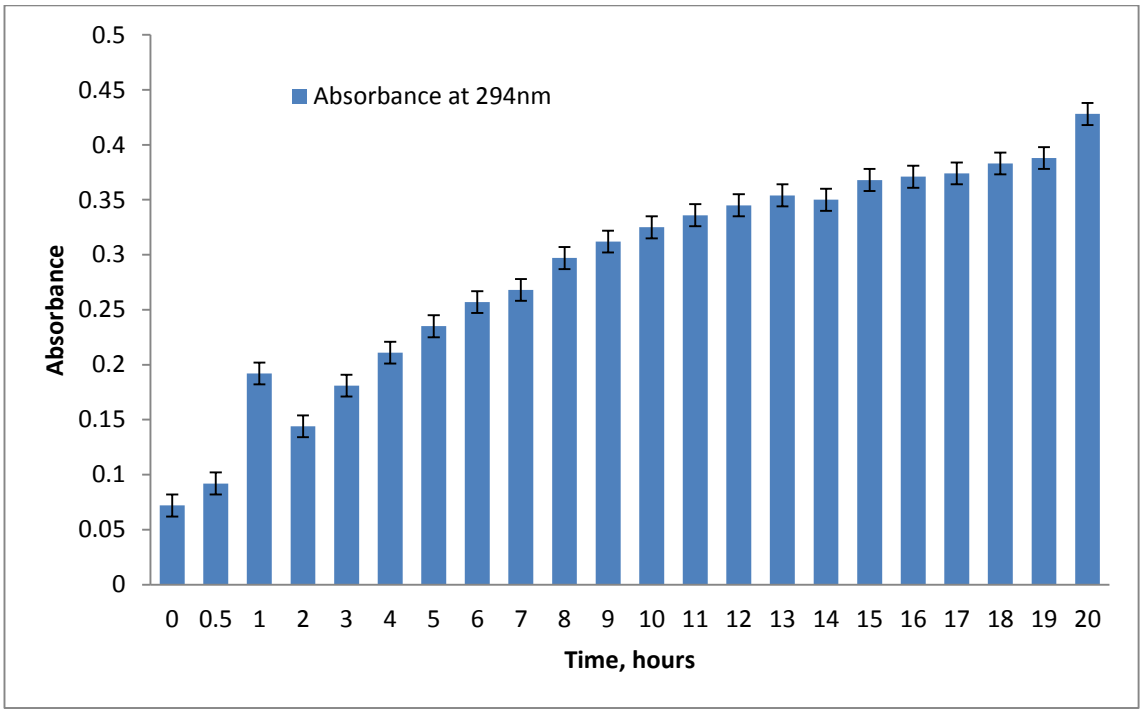

A)
B)
C)
D)
E)

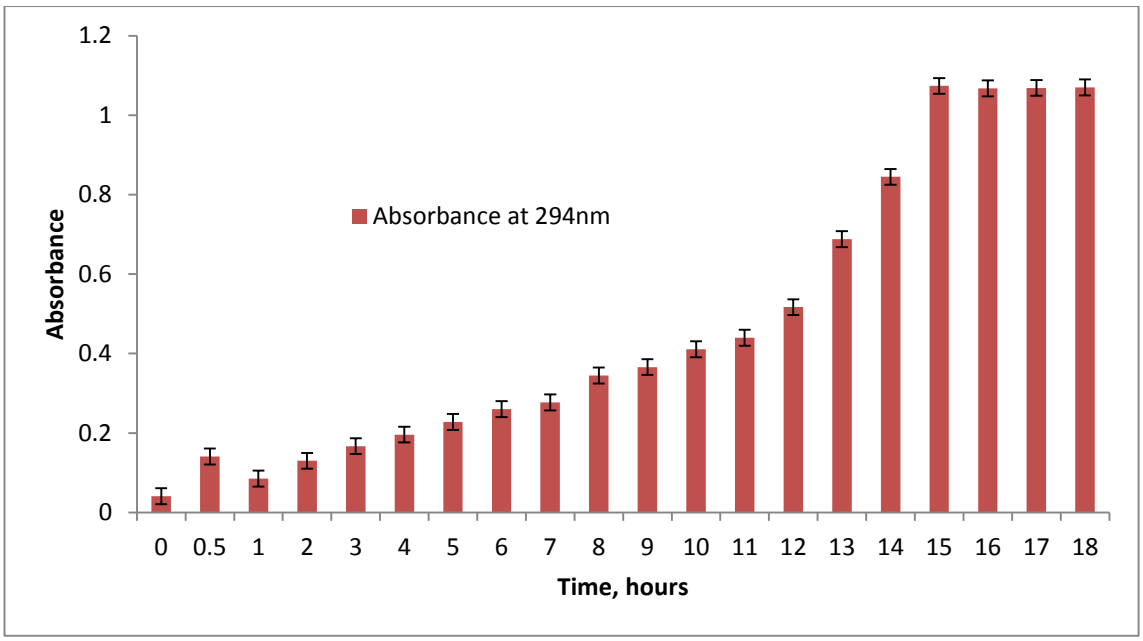

6
5
4
3
2
1
0
200
220
240
260
280
300
320
340
360
380
400
Abs. 0mins
Abs. 30mins
Abs. 1.5 hour
Abs. 2 hours
Abs. 3 hours
Abs. 4 hours
Abs. 5 hours
Abs. 6 hours
Abs. 7 hours
Abs. 8 hours
Abs. 9 hours
Abs. 10 hours
Abs. 11 hours
Abs. 12 hours
Abs. 13 hours
Abs. 14 hours
Abs. 15 hours
Abs. 16 hours
Abs. 17 hours
Abs. 18 hours
Abs 19 hours

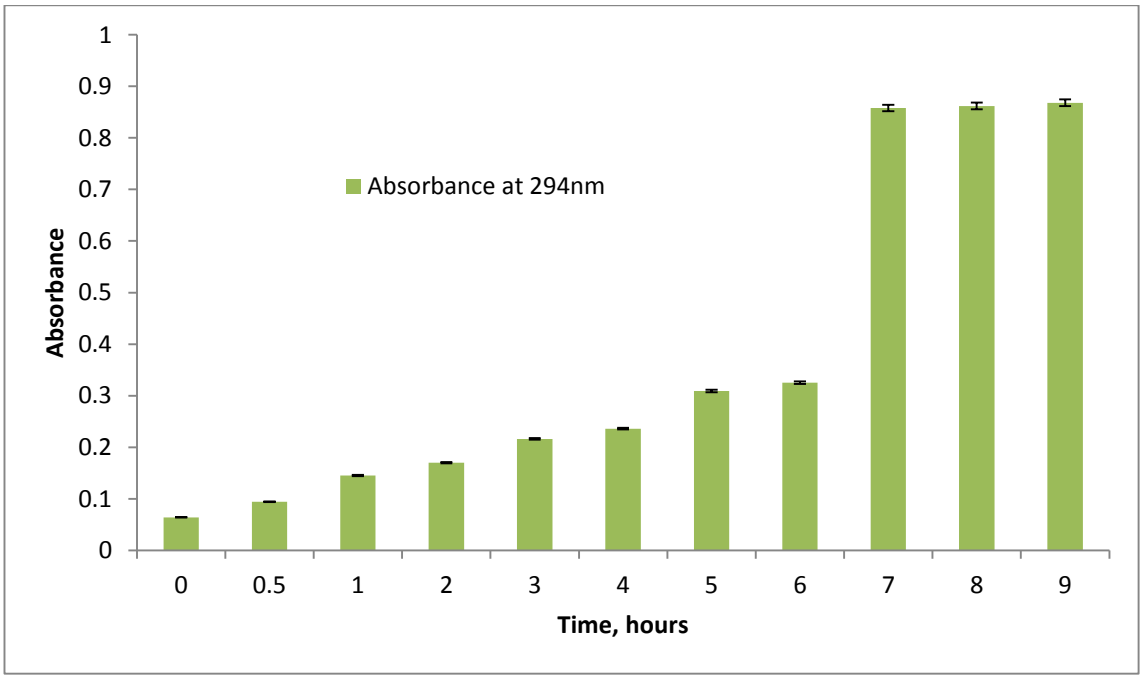

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
200
220
240
260
280
300
320
340
360
380
400
Abs. 0mins
Abs 30mins
Abs. 1 hour
Abs. 2 hours
Abs. 3 hours
Abs. 4 hours
Abs. 5 hours
Abs. 6 hours
Abs. 7 hours
Abs. 8 hours
Abs. 9 hours
Abs. 10 hours
Abs. 11 hours
Abs. 12 hours
Abs. 13 hours
Abs. 14 hours
Abs. 15 hours
Abs. 16 hours
Abs 17 hours


3
2.5
2
1.5
1
0.5
0
200
220
240
260
280
300
320
340
360
380
400
Abs. 0mins
Abs. 30mins
Abs. 1 hour
Abs. 2 hours
Abs. 3 hours
Abs. 4 hours
Abs. 5 hours
Abs. 6 hours


3
2.5
2
1.5
1
0.5
0
Absorbance
0
5
10
15
20
Time, hours
Isasomes/ Aspirin 23/09


0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance
Absorbance at 294nm
0 0.5 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time, hours


1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
Absorbance at 294nm
0 0.5 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time, hours


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
Absorbance at 294nm
0 0.5 1 2 3 4 5 6 7 8 9
Time, hours