Gene Delivery Using Non-Viral Vectors (Cyclodextrins) with Pluronic®-F127 and Folic acid

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Abstract

Over the years, gene therapy has gained much attention across the field of research. The ability to deliver genes into cells offers the opportunities to treat various human genetic disease which results from mutation or deletion of gene(s). Effective gene delivery is highly dependent on its stability and ability to transfect across cell membrane and interferes with the host DNA. However, DNA is easily susceptible to enzymatic degradation and its large size and highly negatively charged surface are barriers towards successful transfection. Therefore, DNA has to be protected from degradation, neutralised and condensed into appropriate size for effective gene delivery. Currently, non-viral vectors are the preferred carrier systems as they are safer, and easier to manufacture. In this research, the use of β and γ-cyclodextrin as non-viral vectors with the incorporation of two different excipients (Pluronic®-F127 and folic acid) at different concentrations to stabilise the formulation was investigated. These formulations were characterised in fresh and freeze dried forms. The freeze dried and fresh solutions of DNA were prepared with cyclodextrins (β or γ), folic acid and Pluronic®-F127 in different ratios as shown in Table 1.

Table 1: The ratios of excipients to cyclodextrin to DNA in the formulations

Excipients	Ratio (Excipient: Cyclodextrin: DNA)
None	0:3:1 and 0:10:1
Folic Acid	3:3:1 and 10:10:1
Pluronic-F127	20:10:1

The DNA stability in the formulations was tested by determining the stability of DNA against enzymatic degradation (DNase test) using ultraviolet-visible spectroscopy. The degree of DNA inclusion into cyclodextrins was investigated using fluorescence spectroscopy. Fourier Transform Infrared Spectroscopy (FTIR) was employed to study the interaction between DNA

and excipients. Scanning Electron Microscope (SEM) was used in observing the surface morphology and uniformity of formed freeze dried particles and thermal behaviour was studied using Differential Scanning Calorimetry (DSC).The formulations were also stored in high humidity (RH=76%) over 5 weeks to access storage stability. In addition, charge measurement was conducted to figure out the transfection efficiency in vivo. It was observed that incorporation of Pluronic®-F127 (Table 1) produced the most stable formulations regarding enzymatic degradation, particularly in the freeze dried formulations. These formulations also show high percentage inclusion (>40%). Shift of peaks in FTIR data, appearance of uniform particulate as detected by SEM and changing in the denaturation temperature as demonstrated by DSC data for Pluronic®-F127 containing formulations confirms clear interaction between Pluronic®-F127 and the cyclodextrin/DNA complex which exhibits positive overall charge. DNA/cyclodextrin formulations containing Pluronic®-F127 also showed high stability and protection for the DNA after storage at 76%RH. Overall, it was noted γ-cyclodextrin provide better protection and inclusion compared to β-cyclodextrin. In summary, Pluronic®-F127 with β or γ -cyclodextrins is a promising combination to improve stability and delivery of DNA.

Keywords: Deoxyribonucleic acid (DNA), Gene delivery, Cyclodextrins, Pluronic®-F127, Folic acid, Non-viral vectors, DNA degradation

1. Introduction

The ability of gene(s) to treat various human genetic diseases has expanded the field of research over the past few years. The concept of gene therapy arose since the late 1960s and early 1970s and this has led to many new developments in the field of genetics. This concept offers the opportunities and potential to cure both genetic and acquired diseases for example haemophilia, cystic fibrosis and cancers (Anchordoquy et al, 2001). Gene therapy is described as the new age in medicine where application of genetic testing and pharmacogenomics are believed to direct treatment based on each person's own genetic makeup (Niidome, 2002). It is a suitable substitute for conventional protein therapy because problems such as bioavailability, systemic toxicity, manufacturing cost and in vivo clearance rate can be overcame (Wong et al 2007). Although, the concept of gene therapy looks promising, but current and ongoing clinical trials are unable to prove its efficacy. This is mainly due to challenges associated with safety of delivery systems, specificity of cell targeting, regulation of gene expression and efficiency of gene transfer and the stability of the gene itself (Park et al 2006).

DNA has a short half life highly susceptible to both intracellular and extracellular enzymatic degradation. In addition, the phosphate backbone of DNA is negatively charged at physiological pH leading to repulsion from anionic cell surface (Abdelhady et al, 2003, Lechardeur et al 1999). The large, bulky structure of DNA is also a barrier for effective transfection. Therefore, a good carrier system is needed for successful gene therapy. The ideal carrier system must be able to (i) neutralise the negative charge of DNA, (ii) condense DNA to an appropriate size (i.e. nanometers for receptor-meditated endocytosis or micrometers for pinocytosis or phagocytosis) (iii) protect DNA from enzymatic degradation in the blood. Moving

on from that, researches nowadays are developing gene carriers focusing on three main strategies: encapsulation, adsorption and electrostatic interaction (Wong et al, 2007, Park et al, 2006).

Basically, gene therapy is the insertion of a "normal" gene into an individual's cell to replace an "abnormal" or mutated gene. Effective gene therapy highly dependent on two important aspects: (i) ability of therapeutic gene to express itself at a target site and (ii) a delivery system which is able to deliver the gene safely to a specific target site (low immunogenicity)(Brown et al, 2001, Schaffer, 1998). Currently, there are a few methods to deliver gene into the cell nucleus, a process known as transfection. Firstly, it is through physical method where the naked deoxyribonucleic acid (DNA) is directly injected into the nucleus of the cell without a carrier. Direct injection without any carrier molecule is the safest and convenient route for gene administration. However, the application of this approach is limited due to the rapid degradation of DNA by DNase, high clearance by phagocyte in the blood and low DNA expression level at target site. This led to the establishment of various physical modifications techniques such as electroporation, gene gun (bioballistic), ultrasound and high pressure injection to improve the efficiency of direct injection (Herweijer, 2003, Williamson, 2008, Well, 2004).

Another method is via a vector where it can either be a viral or non-viral. Over the years, viral vectors have been the more popular choice among the two and have been used extensively because they are more superior in terms of transfection efficiency.

Non-viral vectors have low immunogenicity, easier to manufacture, no signs of oncogenicity and relatively inexpensive compared to viral vectors. However, due to differences

in barrier permeation between the target cell nucleus and the extracellular space, their transfection efficiency is significantly lower than viral carriers (Huang, 2000, Niidome, 2000, David, 2002)

In addition, issues such as limited expression duration and non-specific cell uptake were linked with non-viral systems. Therefore, various modifications methods have been employed to overcome these problems such as incorporating non-ionic hydrophilic polymer I.e. polyethylene glycol to prolong circulating half life in serum and reduce interactions with plasma proteins, lipoproteins or blood cells (Park et al, 2005). Examples of non-viral vectors are cationic lipid (lipoplexes), cationic polymer (polyplexes), chitosans, dendrimers and cyclodextrins (Lv et al, 2006).

The study of cyclodextrins as drug carriers has become an area of interest over the past few years especially in the delivery of DNA, proteins and peptides. Basically, cyclodextrins are a family of oligosaccharides which are made of glucopyranose units linked together by α-1,4 bonds. Three different types of cyclodextrins have been indentified, the α, β and γ forms (Del Valle, 2004, Loftsson, 2011, Rasheed et al, 2008).

Cyclodextrins contain a hydrophilic outer surface (polar) and a lipophilic inner cavity. The outer hydrophilic property due to large number of hydrogen bond donors and acceptors, making it freely soluble in water while the inner hydrophobic cavity, owing to the presence of $-CH_2-$ groups, enables poorly water soluble drug to be incorporated into this cavity (Rasheed et al, 2008, Szejtli, 1994). Recently, cyclodextrins have been used to incorporate gene into the inner cavity as well (Burckbuchler et al, 2008). β–cyclodextrin is the most commonly used cyclodextrin in pharmaceutical formulations and has been extensively studied in human.

The ability of cyclodextrins to form inclusion complex with many components is undoubtedly their most useful feature. Interestingly, the driving force of this complex formation is the release of water from the lipophilic cavity in favour of a more lipophilic entity and no covalent bonds are formed or broken during this process. The formation of inclusion complex is not fixed but rather is a dynamic equilibrium between the free water molecules and the hydrophobic molecule. This interaction is highly favourable as it reduces the ring strain leading to a more stable lower energy state (Rasheed et al, 2008, Stella, 1997). In addition, cyclodextrins has been proven to enhance solubility, bioavailability, safety and stability of drug molecules especially poorly water soluble drugs. In this study, β and γ-cyclodextrins will be used with different excipients (Pluronic®-F127 and folic acid).

Pluronic®-F127, a type of block co-polymer, has been proved to enhance gene expression via various delivery routes and different types of vectors including naked DNA itself (Strappe et al, 2005). In addition, Pluronic®-F127 showed significant ability to preserve the stability of polypeptide in both in vivo and in vitro. Folic acid has been studied to deliver genes through tissue specific targeting and a net positive charge was observed with folic acid formulations (Guo, 1999). The positive charge is highly desirable for effective cell transfection as cell membranes are negatively charged. In this study, the stability enhancement of Pluronic®-F127 and folic acid in addition to cyclodextrins will be evaluated.

One of the main factors which prevent a non-viral vector gene formulation to become a marketable product is its stability (Anchordoquy et al, 2001). Presently, there are a number of formulations have been investigated to prolong the storage of gene formulations which includes liquid, frozen and dehydrated formulations. Liquid formulations were not favourable

as they have the tendency to aggregate over time. Although attempts to reduce aggregation were successful, but decrease in cell transfection was observed (Anchordoquy et al, 2001). Moreover, the shelf life of liquid formulations is short, only lasts for hours or days where the minimum shelf life for a marketable pharmaceutical product is 18 months to 2 years (Anchordoquy et al, 2001).

Similarly, frozen formulations could not preserve the stability of gene formulations but they are slightly better and more successful compared to liquid formulations. It was reported that freezing could damage the structure of DNA, which results in the formation of crack within the ice. This process in known as cryolysis and is dependent on the rate of cooling of the frozen samples (Lyscov and Moshkovsky, 1969). Addition of cryoprotectant excipients into frozen formulations has significantly improved the stability of frozen formulations. However, strict maintenance storage temperature is required to preserve the stability and prevent thawing or crystallisation of the excipients (Maitani et al, 2008, Anchordoquy et al, 1998). Maintaining these conditions are difficult, not practical and requires extra cost.

This then led to the development of dehydrated formulations which have the ability to overcome the limitations of liquid and frozen formulations (Shikama, 1965, Talsma et al, 1997). Dried formulations are stable at room temperature and can be ready to administer after a simple reconstitution step. Generally, there are two main ways to remove water from liquid formulations: (i) spray drying and (ii) freeze drying. However, spray drying is not appropriate because it can generate high shear forces which could damage the non-viral gene vectors thus freeze drying (lyophilisation) is preferable (Anchordoquy et al, 2001, Densmore et al, 2000).

2. Materials and Methods

2.1 Materials

The materials used are Deoxyribonucleic acid sodium salt from cell thymus, Deoxyribonuclease (DNase) from bovine pancrease, Folic Acid, Cyclodextrins and Pluronic®-F127, those materials were obtained from Sigma Aldrich Company (UK).

2.2 Preparation of samples

2.2.1 Fresh DNA aqueous samples

Samples containing DNA and equivalent samples without DNA samples with and without excipients (Pluronic®-F127 and folic acid) were prepared. The fresh samples were characterised using fluorescence and DNase I activity. The formulations prepared are listed in Table 2.

Table 2: Fresh DNA formulations and non-DNA formulations with either β- or γ- cyclodextrin, CD (with or without excipient)

Excipient	**Ratio**	
	DNA Sample **Excipient: cyclodextrin: DNA**	**Non-DNA Sample** **Excipient: cyclodextrin**
None	0 :3 :1	Only CD present at equivalent concentration as in DNA sample
None	0 :10 :1	Only CD present at equivalent concentration as in DNA sample
Folic Acid	3 :3 :1	3 :3
Folic Acid	10 :10 :1	10 :10
Pluronic®-F127	20 :10 :1	20 :10

2.2.2 Freeze dried DNA samples

Freeze drying is a good method to provide long term stability on heat sensitive products. All fresh DNA and non-DNA samples (see section 2.2.1) were frozen to -80℃. Once frozen, the samples were freeze dried using the VitTis Benchtop Freeze Drier (Gardiner, New York, USA).

The vacuum was set to 20mT and the condenser temperature was set to 105℃ while the shelf temperature at 21.1 ℃. The freeze dried samples were characterised using fluorescence, DNase I activity, FTIR and charge measurements. The freeze dried samples were reconstituted accordingly to obtain DNA concentration of approximately 20μg/mL. Reconstitution is needed to conduct fluorescence, DNase I activity and charge measurements. These characterisations are performed in order to compare the stability between freeze dried DNA samples and fresh DNA aqueous samples.

2.3 Calibration curve for Deoxyribonucleic acid (DNA) by UV-absorbance

Figure 1 shows the calibration of DNA, DNA solutions were prepared in PBS and the absorbance was measured at 260nm using M501 Single Beam ultraviolet visible spectrophotometer (Biochrom, UK).

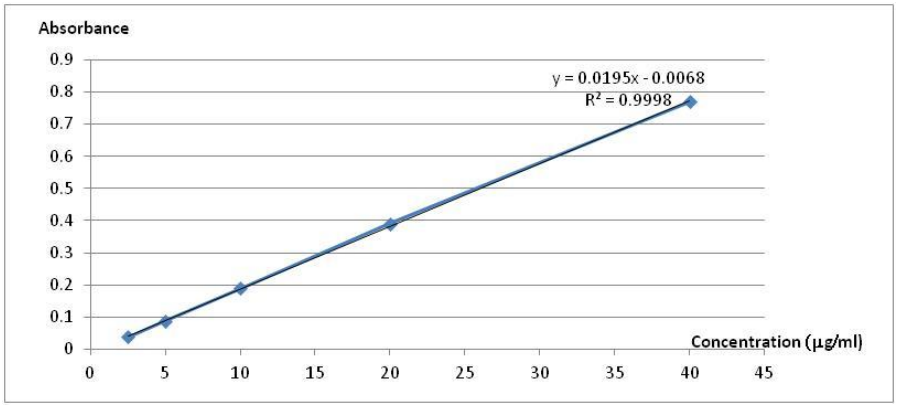


Figure 1: Absorbance calibration curve for DNA

2.4 Calibration curve for DNA by Fluorescence spectroscopy

Ethidium bromide (EtBr) exclusion assay was used to measure the fluorescence of DNA solutions. Fluorescence was determined using the Perkin-Elmer Luminescence

spectrophotometer (LS5OB, Perkin-Elmer, UK). The $\lambda_{excitation}$ and $\lambda_{emission}$ was set at 526nm and 592nm (Cryan et al, 2004), respectively as they are characteristic to DNA. Figure 2 shows the fluorescence calibration plot of DNA in PBS.

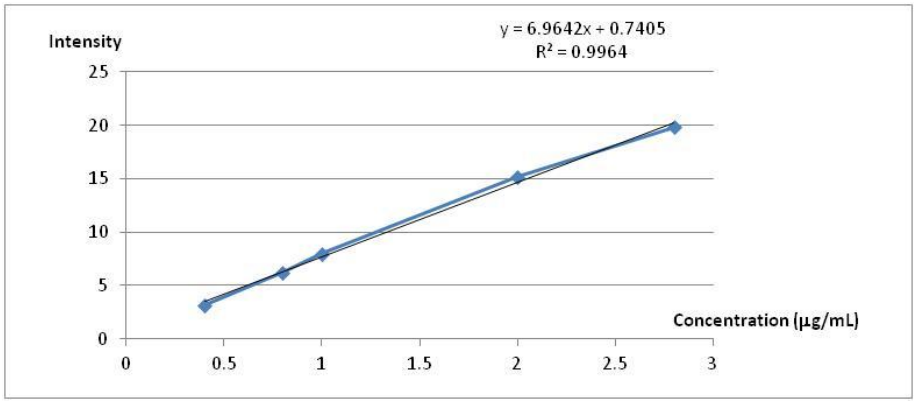


Figure 2: Fluorescence calibration curve for DNA

2.5 Fluorescence measurements for DNA samples

This test was done to evaluate the amount of DNA incorporated in the cyclodextrin. Samples were prepared to give final concentration of DNA in the samples of 1 µg/mL. Fluorescence measurements for both fresh and reconstituted freeze dried DNA aqueous samples were determined at $\lambda_{excitation}$ (526nm) and $\lambda_{emission}$ (592nm). The non-DNA samples were used as the blank instead of the pure PBS solution as the excipients may fluoresce at the wavelength used. These measurements in combination with the fluorescence calibration curve (Figure 2) can determine the concentration and % inclusion of DNA in each sample.

2.6 Deoxyribonuclease I (DNase I) activity measurements

The test was done to confirm the fluorescence data. It was conducted to evaluate whether DNA is present outside the cyclodextrin and to study the efficiency of the formulation to protect the DNA from DNase I enzymatic degradation. DNase I will hydrolyse the free DNA (not incorporated inside the cyclodextrins) and leading to increase in absorbance. Absorbance measurements were taken for fresh and freeze dried DNA aqueous solutions at three time points: 0, 5 and 15 minutes using the M501 Single Beam ultraviolet visible spectrophotometer (Biochrom, UK) at 260nm. The non-DNA samples were used as the blank.

2.7 Storage stability assessment of freeze dried DNA samples

All solid freeze dried DNA and non-DNA samples were subjected to stability testing. The samples were stored at high humidity (relative humidity: 76%) over 5 weeks. After 5 weeks, the samples were reconstituted with PBS accordingly to produce solution with DNA concentration of 20μg/mL. DNase I test was conducted to all DNA samples where absorbance measurements were taken at three time points: 0, 5 and 15 minutes using the M501 Single Beam ultraviolet visible spectrophotometer (Biochrom, UK) at 260nm. The non-DNA samples were used as the blank.

2.8 Fourier Transform Infrared (FTIR) Spectroscopy of DNA samples

This analysis was done to observe any shifts in the characteristic DNA peaks if there are interactions between DNA and the excipients. FTIR was conducted on the freeze dried DNA samples using the Perkin Elmer FT-IR Spectrum BX (Beakonsfield, UK) and software. The

analysis was conducted on the spectral region between 4000 cm^{-1} to 600cm^{-1} at 4cm^{-1} resolution.

2.9 Particles visualisation using Scanning Electron Microscope (SEM)

SEM analysis was done on solid samples of freeze dried DNA and non-DNA samples in ratios of [(10:10 and 10:20) cyclodextrin: excipient)]. Small amount of samples were attached to 15mm diameter aluminium stubs using double sided carbon adhesive tabs, Agar Silver Paint (Agar Scientific, Essex, UK). All samples were coated with a mixture of gold/palladium in a high vacuum coating unit using a Quarum Technology (Polaron Range) SC760 by exposing samples to an Argon atmosphere at about 10 Pascals. Samples were coated for 2x105 seconds (turning through 180 degrees in between) with a process current of 18-20mA. After coating, samples were examined using Hitachi S3000N Scanning Electron Microscope (Hitachi High Technologies UK-Electron Microscopes, Berkshire, UK). Particles shape, distribution, and morphology were analysed.

2.10 Charge measurements of DNA samples

Cell transfection is highly dependent on the overall charge of the formulation. An overall positive charge is highly desirable for effective cell transfection. This test was performed for the most promising formulations regarding inclusion and stability against DNase I degradation. The freeze dried DNA samples chosen were β-CD: DNA 3:1, β-CD:DNA 10:1, β-CD:Pluronic-F127:DNA 10:20:1 and γ-CD:Pluronic-F127:DNA 10:20:1. The charge measurements were conducted using Zeta PALS1 zeta potential analyser (Brookhaven Instruments Corporation, USA.

2.11 Differential Scanning Calorimetry (DSC) of DNA samples

DSC was conducted to determine changes in phase transitions between DNA and non-DNA freeze dried formulations where any changes would suggest interactions between DNA and excipients (Cooper et al, 2000). Each freeze dried samples were weighed (2mg) and sealed into aluminium pans. The DSC analysis was run using the DSC Refrigerated Cooling System (Model Q100, TA instruments, UK). The reference and sample pans were kept at 20℃ for 5 minutes to ensure isothermal starting conditions. The samples were heated to 230℃ at a rate of 10℃/min.

2.12 Statistical Analysis

Statistical analysis was performed using the SPSS version 16. Test performed including one way ANOVA, Levene test (to check homogeneity of variances), and Scheffe Test for normally distributed data. Kruskal Wallis non-parametric test was conducted for not normally distributed data. The data are considered significant if the p value is less than 0.05

3. Results and Discussions

3.1 UV absorbance measurements for DNA samples for fresh and freeze dried DNA samples

The actual DNA concentrations in all formulations were calculated, to investigate if there is any change in the theoretical DNA concentration, based on the regression line equation from the DNA absorbance calibration curve. The equation is as below (Equation 1):

Equation 1: Concentration (μg/mL) $=(Absorbance + 0.0068)/0.0195$

The theoretical DNA concentration was 20μg/ml, β-CD:Folic acid:DNA 3:3:1 and γ-CD:Folic acid:DNA 3:3:1 samples deviate from the theoretical value by 19% and 10.4% respectively. For freeze dried samples, all of the samples deviate from the theoretical values except β-cyclodextrin with DNA (10:1).

Both fresh and freeze dried samples were made up to contain 20μg/mL of DNA. However, not all of the samples contain exactly this concentration. Pluronic®-F127 containing formulations both fresh and freeze dried were significantly different from the theoretical concentration of 20μg/mL. This may be explained by the interaction affecting DNA due to freeze drying or due to excipients.

3.2 Fluorescence measurements for DNA samples

3.2.1 Fresh DNA aqueous samples

Tables 3 and 4 below contain the fluorescence determining concentrations and %DNA inclusion, respectively for fresh DNA aqueous samples. The corresponding concentrations were calculated based on the regression line equation (see equation 2) from the fluorescence calibration curve.

Equation 2: Concentration (μg/mL) =$(Fluorescence\ Intesity - 0.7405)/6.9642$

Table 3: Concentrations of DNA in each fresh DNA samples from the fluorescence calibration curve

Sample	Concentration (μg/ml)				Standard Deviation
	1	2	3	Average	
β-CD + DNA 3:1	0.931	0.953	0.96	0.948	0.015
β-CD + DNA 10:1	0.917	0.893	0.931	0.914	0.019
β-CD + Folic acid + DNA 3:3:1	0.811	0.798	0.832	0.814	0.017
β-CD + Folic acid + DNA 10:10:1	1.028	1.023	1.008	1.020	0.010
β-CD + Pluronic®-F127 + DNA 10:20:1	0.807	0.789	0.788	0.795	0.011
γ-CD + DNA 3:1	0.855	0.867	0.835	0.852	0.016
γ-CD + DNA 10:1	0.696	0.721	0.747	0.721	0.026
γ-CD +Folic acid + DNA 3:3:1	1.016	1.01	0.990	1.005	0.014
γ-CD + Folic acid + DNA 10:10:1	0.930	0.954	0.959	0.948	0.016
γ-CD +Pluronic®-F127 + DNA 10:20:1	0.789	0.792	0.745	0.775	0.026

The % inclusion of DNA into the cyclodextrins was calculated using the initial/ practical concentration of DNA (see equation 3). Decrease in fluorescence intensity would results in higher % inclusion due to the unavailability of DNA to ethidium bromide.

Equation 3: $\%\ \text{Inclusion} = \left[(1) - \left(\frac{Concentration\ \ of\ DNA\ measured\ \ by\ fluorescence}{Practical\ \ concentration\ \ of\ DNA}\right)\right] x\ 100\%$

Table 4: Percentage inclusion of DNA into cyclodextrin complex and initial concentration of DNA in each fresh DNA aqueous samples

Sample	Initial/ Practical Concentration (μg/mL)	% inclusion				Standard Deviation
		1	2	3	Average	
β-CD + DNA 3:1	0.989	5.9	3.6	2.9	4.1	1.56
β-CD + DNA 10:1	0.995	7.8	10.3	6.4	8.2	1.98
β-CD + Folic acid + DNA 3:3:1	0.807	**-0.5**	**1.1**	**-3.1**	**-0.8**	2.12
β-CD + Folic acid + DNA 10:10:1*	-	-	-	-	-	-
β-CD + Pluronic®-F127 + DNA 10:20:1	1.023	21.1	22.9	23.0	22.3	1.07
γ-CD + DNA 3:1	0.997	14.2	13.0	16.2	14.5	1.62
γ-CD + DNA 10:1	0.995	**30.1**	**27.5**	**24.9**	**27.5**	2.60
γ-CD +Folic acid + DNA 3:3:1	0.895	**-13.5**	**-12.8**	**-10.6**	**-12.3**	1.51
γ-CD + Folic acid + DNA 10:10:1*	-	-	-	-	-	-
γ-CD +Pluronic®-F127 + DNA 10:20:1	1.035	**23.8**	**23.5**	**28.0**	**25.1**	2.52

* The theoretical DNA concentration and % inclusion data could not be calculated since absorbance readings are not available

Based on the results, γ-CD:DNA 10:1, γ-CD:Pluronic®-F127:DNA 10:20:1 and β-CD:Pluronic®-F127:DNA 10:20:1 formulations were the most stable fresh formulations followed by γ-CD: DNA 3:1, β-CD: DNA 10:1 and β-CD: DNA 3:1 as moderately stable. The samples which show no inclusion of DNA were β-CD: Folic acid: DNA 3:3:1 and γ-CD: Folic acid: DNA 3:3:1. One way ANOVA analysis was done to determine whether the samples differed significantly since the variance is homogenous. It was found out that the most stable freshly prepared aqueous formulations (γ-CD:DNA 10:1, γ-CD:Pluronic®-F127:DNA 10:20:1 and β-CD:Pluronic®-F127:DNA 10:20:1 differed significantly ($p<0.05$) from all the fresh samples.

3.2.2 Freeze dried DNA samples

Tables 5 and 6 below display the fluorescence determining DNA concentrations and % DNA inclusion, respectively for reconstituted freeze dried DNA samples.

Table 5: Concentrations of DNA in freeze dried DNA samples from the fluorescence calibration curve

Sample	Concentration (μg/ml)				Standard Deviation
	1	2	3	Average	
β-CD + DNA 3:1	0.870	0.917	0.915	0.901	0.027
β-CD + DNA 10:1	0.793	0.778	0.802	0.791	0.012
β-CD + Folic acid + DNA 3:3:1	0.910	0.942	0.897	0.916	0.023
β-CD + Folic acid + DNA 10:10:1	1.022	1.026	1.007	1.018	0.010
β-CD + Pluronic-F127 + DNA 10:20:1	0.468	0.508	0.484	0.487	0.020
γ-CD + DNA 3:1	0.893	0.900	0.946	0.913	0.029
γ-CD + DNA 10:1	0.842	0.826	0.834	0.834	0.008
γ-CD +Folic acid + DNA 3:3:1	0.970	0.944	0.999	0.971	0.028
γ-CD + Folic acid + DNA 10:10:1	0.693	0.655	0.658	0.667	0.021
γ-CD +Pluronic-F127 + DNA 10:20:1	0.478	0.469	0.485	0.477	0.008

Table 6: Percentage inclusion of DNA into cyclodextrin complex and initial concentration of DNA in each freeze dried DNA samples

Sample	Initial/ Theoretical Concentration (μg/mL)	% inclusion				Standard Deviation
		1	2	3	Average	
β-CD + DNA 3:1	1.165	**25.3**	**21.3**	**21.5**	**22.7**	2.25
β-CD + DNA 10:1	1.010	**21.4**	**23.0**	**20.6**	**21.7**	1.22
β-CD + Folic acid + DNA 3:3:1	1.070	15.0	12.0	16.2	14.4	2.16
β-CD + Folic acid + DNA 10:10:1*	-	-	-	-	-	-
β-CD + Pluronic-F127 + DNA 10:20:1	0.933	**49.8**	**45.6**	**48.1**	**47.8**	2.11
γ-CD + DNA 3:1	0.875	-2.1	-2.9	-8.11	-4.37	3.26
γ-CD + DNA 10:1	0.885	4.9	6.7	5.8	5.8	0.90
γ-CD +Folic acid + DNA 3:3:1	1.061	8.6	11.0	5.8	8.5	2.60
γ-CD + Folic acid + DNA 10:10:1*	-	-	-	-	-	-
γ-CD +Pluronic-F127 + DNA 10:20:1	0.920	**48.0**	**49.0**	**47.3**	**48.1**	0.85

*The theoretical DNA concentration and % inclusion data could not be calculated since absorbance readings are not available

The most stable freeze dried DNA formulations regarding inclusion were γ-CD:Pluronic®-F127:DNA 10:20:1 and β-CD:Pluronic®-F127:DNA 10:20:1. They showed highest % inclusion (>45%) compared to other freeze dried DNA formulations. Based on the statistical analysis (one way ANOVA), both these formulations were significantly differed ($p<0.05$) from the least stable formulation (γ-CD:DNA 3:1) and the rest of the freeze dried formulations although β-CD:DNA 3:1, β–CD:DNA 10:1 and β-CD:Folic acid:DNA 3:3:1 formulations did show good inclusion compared to γ-CD:DNA 10:1 and γ-CD:DNA 10:1. Both fresh formulations and freeze dried formulations were made to contain 1μg/mL of DNA but none of the samples contain exactly this concentration. Most of the samples show a decrease in fluorescence intensity/concentration which might suggest some degree of inclusion of DNA into the cyclodextrin complex. Some formulations demonstrated negative % inclusion which might suggest instability of the cyclodextrin-excipient complex formed.

Generally, freeze dried formulations provide higher inclusion compared to fresh formulations. In comparison, formulations containing excipient with those without excipient, the addition of Pluronic®-F127 as stabilising excipient into DNA cyclodextrin formulations (fresh and freeze dried) clearly enhanced the stability of the DNA formulation with both β and γ–cyclodextrins. In contrast, addition of folic acid decreased the stability of the formulations (fresh and freeze dried) as lower % inclusion was observed. Fluorometry is considered as one of the most sensitive method to measure DNA concentrations and is more accurate than absorbance at 260nm (Rengajaran et al, 2002). Fluorometry can detect small quantities of double stranded DNA and more specific in terms of differentiating double stranded DNA from single stranded or RNA. Ethidium bromide fluorophore was chosen because it is a sensitive and an easy stain for DNA and binds preferably to double stranded DNA.

Some formulations showed low or negative % inclusion and this might be due to the repulsion between cyclodextrins and the DNA as both of them are negatively charged at pH 7.4.

3.3 Deoxyribonuclease I(DNase I) activity measurements

This test was performed to study DNA availability and stability. The smaller the difference between the absorbance readings from time 0 to 15 minutes, the higher the stability of the DNA formulation.

3.3.1 Fresh DNA aqueous samples

Table 7 exhibits the results of DNase I activity measurements at time 0, 5 and 15 minutes for fresh DNA aqueous samples.

Table 7: Absorbance measurements of fresh DNA aqueous samples at specific time upon addition of DNase I

Samples	Absorbance at time (min)			Δ Absorbance
	0	5	15	
β-CD + DNA 3:1	0.370	0.370	0.373	0.003
β-CD + DNA 10:1	0.220	0.220	0.222	0.002
β-CD + Folic acid + DNA 3:3:1	**1.253**	**1.263**	**1.270**	**0.017**
β-CD + Folic acid + DNA 10:10:1*	-	-	-	-
β-CD + Pluronic®-F127 + DNA 10:20:1	**0.412**	**0.410**	**0.415**	**0.003**
γ-CD + DNA 3:1	0.329	0.329	0.329	0.000
γ-CD + DNA 10:1	0.387	0.388	0.388	0.001
γ-CD +Folic acid + DNA 3:3:1	**0.111**	**0.109**	**0.119**	**0.008**
γ-CD + Folic acid + DNA 10:10:1*	-	-	-	-
γ-CD +Pluronic®-F127 + DNA 10:20:1	**0.310**	**0.312**	**0.312**	**0.002**
Pure DNA	0.780	0.790	0.801	0.021

*The UV spectrophotometer was unable to provide any absorbance readings for these samples

Based on the results, formulations which provide most protection from DNase I degradation were β-CD:Pluronic®-F127:DNA 10:20:1 and γ-CD:Pluronic®-F127:DNA 10:20:1. Similarly γ-CD:DNA 3:1, γ-CD:DNA 10:1, β-CD:DNA 3:1 and β-CD:DNA 10:1 also showed good stability. The least stable formulations were β-CD:Folic acid:DNA 3:3:1 and γ-CD:Folic acid:DNA 3:3:1 compared to pure DNA. These findings were consistent with the % inclusion of fresh DNA aqueous samples (see Table 4) where both Pluronic®-F127 containing formulations showed highest % inclusion while both β-CD:Folic acid:DNA 3:3:1 and γ-CD:Folic acid:DNA 3:3:1 demonstrated no inclusion of DNA (negative % inclusion).

3.3.2 Freeze dried DNA samples

Table 8 shows the results of DNase I activity measurements at time 0, 5 and 15 minutes for freeze dried DNA samples after reconstitution.

Table 8: Absorbance measurements of freeze dried DNA samples at specific time upon addition of DNase

Sample	Absorbance at time (min)			Δ Absorbance
	0	5	15	
β-CD + DNA 3:1	0.288	0.286	0.293	0.005
β-CD + DNA 10:1	0.028	0.029	0.028	0.000
β-CD + Folic acid + DNA 3:3:1	**0.918**	**0.930**	**0.925**	**0.007**
β-CD + Folic acid + DNA 10:10:1*	-	-	-	-
β-CD + Pluronic®-F127 + DNA 10:20:1	**0.295**	**0.294**	**0.296**	**0.001**
γ-CD + DNA 3:1	0.261	0.263	0.265	0.004
γ-CD + DNA 10:1	**0.297**	**0.293**	**0.298**	**0.001**
γ-CD +Folic acid + DNA 3:3:1	**0.128**	**0.140**	**0.175**	**0.047**
γ-CD + Folic acid + DNA 10:10:1*	-	-	-	-
γ-CD +Pluronic®-F127 + DNA 10:20:1	**0.278**	**0.279**	**0.278**	**0.000**

*The UV spectrophotometer was unable to provide any absorbance readings for these samples

The most stable freeze dried samples were β-CD:Pluronic®-F127:DNA 10:20:1, γ-CD:Pluronic®-F127:DNA 10:20:1 and β-CD:DNA 10:1. The least stable formulation were β-CD:Folic acid:DNA 3:3:1 and γ-CD:Folic acid: DNA 3:3:1. The promising stability against DNase I provided by both freeze dried Pluronic®-F127 containing formulations was consistent with the % inclusion observed for freeze dried DNA formulations (Table 6). The addition of Pluronic®-F127 into the formulations increases the amount of DNA incorporated into the cyclodextrins, therefore more DNA are protected from enzymatic degradation.

Generally, freeze drying has shown to improve the stability of the formulations over fresh DNA aqueous samples in particular with Pluronic®-F127 containing formulations. The increase in stability for these samples may be due to the formation of favourable bonds or

interaction between the excipients and cyclodextrin-DNA complex during the lyophilisation process. This was confirmed by FTIR data and SEM images (see below). Interestingly, samples containing folic acid as an excipient shows contraindicating results when incorporate either with β-CD and γ-CD. The stability of sample (β-CD:Folic acid:DNA 3:3:1) increased after freeze drying but the stability of sample (γ-CD:Folic acid:DNA 3:3:1) significantly deteriorate after freeze drying. This might briefly suggest that folic acid and β-cyclodextrin interaction can provide better protection against enzymatic degradation than folic acid γ–cyclodextrin after freeze drying. This interaction should be further evaluated. On the other hand, the stress imparted during freeze drying might have completely destabilised the integrity of the folic acid with γ–cyclodextrin+DNA complex (Li et al, 2000, Pozo-Rodriquez et al, 2009).

It was also noted that fresh β and γ–cyclodextrin+DNA formulations were stable on their own without excipients. This might be due to the protection offered by cyclodextrins themselves. Cyclodextrins are type of sugar and basically sugars have been proven to exhibit stabilising properties (Anchordoquy et al, 2001, Matani et al, 2008). However, after undergone the freeze drying process, only formulations with the higher concentration of cyclodextrin remain stable. Again, this phenomenon might be due to the stress imposed during freezing and drying destroying a finite number of cyclodextrin molecules. The 10:1 ratio may provide sufficient amount of cyclodextrin molecules to overcome the loss, therefore still able to protect the DNA from DNase I degradation whereas the 3:1 ratio formulations did not have the capability.

As observed, γ-cyclodextrin provides better protection than β-cyclodextrin for both fresh and freeze dried DNA aqueous samples except for folic acid containing formulations. Folic

acid might not be compatible with γ-cyclodextrin, thus leading to unfavourable outcome. The additional unit of glucopyranose structure in γ-cyclodextrin might have increase the stability of the complex formed. This additional unit could have provided the optimum spatial space required for DNA and Pluronic®-F127 to interact more favourably with the cyclodextrin. Moreover, γ-cyclodextrin has a wider inner cavity compared to β-cyclodextrin, thus more DNA molecules can be incorporated into γ-cyclodextrin. This reduces the amount of free DNA outside the cyclodextrin, therefore making it less susceptible to enzymatic degradation.

3.3.3 Storage stability for freeze dried DNA samples

Table 9 contains the results of DNase I activity measurements at time 0, 5 and 15 minutes for freeze dried DNA samples after 5 weeks storage to access the stability of the formulations upon storage at RH=76%.

Table 9: Absorbance measurements of freeze DNA samples at specific time upon addition of DNase I after 5 weeks storage

Sample	Absorbance at time (min)			Δ Absorbance
	0	5	15	
β-CD + DNA 3:1	0.345	0.340	0.340	-0.005
β-CD + DNA 10:1	-0.161	-0.173	-0.178	-0.017
β-CD + Folic acid + DNA 3:3:1	0.473	0.466	0.466	-0.007
β-CD + Folic acid + DNA 10:10:1*	-	-	-	-
β-CD + Pluronic-F127 + DNA 10:20:1	**0.270**	**0.269**	**0.273**	**0.003**
γ-CD + DNA 3:1	0.303	0.300	0.301	-0.002
γ-CD + DNA 10:1	0.450	0.440	0.439	-0.011
γ-CD +Folic acid + DNA 3:3:1	-0.065	-0.073	-0.074	-0.009
γ-CD + Folic acid + DNA 10:10:1*	-	-	-	-
γ-CD +Pluronic-F127 + DNA 10:20:1	**0.310**	**0.312**	**0.311**	**0.001**

*The UV spectrophotometer was unable to provide any absorbance readings for these samples

After storing at high relative humidity for 5 weeks, clearly only Pluronic®-F127 containing formulations remain stable against DNase I degradation with γ cyclodextrin providing better stability than β cyclodextrin. Only an increase of 0.001 in absorbance reading was observed over 15 minutes for γ-CD:Pluronic®-F127:DNA 10:20:1 sample compared to 0.003 for β–CD:Pluronic-F127:DNA 10:20:1. This proves that γ-cyclodextrin provide better protection against enzymatic degradation than β-cyclodextrin. All other formulations displayed decrease in absorbance, therefore the samples were assumed not stable since the readings were anomalous or may be due to changes in DNA original conformation. The high humidity employed could have destroyed the integrity of the formulations leading to instability of the cyclodextrin:DNA complex. The addition of Pluronic®-F127 into the formulations has genuinely maintained the stability of the cyclodextrin:DNA complex for at least five weeks at high relative humidity (RH=76%). Looking at the chemical structure of Pluronic®-F127 and DNA, interaction could occur between the phosphate group of DNA with the OH group of Pluronic®-F127.

3.4 Fourier Transform Infrared (FT-IR) Spectroscopy of DNA samples

Five main peaks were identified in the pure freeze dried DNA spectra: 1648.35, 1221.76, 10862.96, 1054.92 and 962.2 cm^{-1}. These peaks were consistent with the results from other published papers (Mao et al, 1993, Ruiz-Chica et al, 2001, Ouameur, 1977). β–Cyclodextrin molecule has distinct peaks at 1152.12, 1077.24, 1022.78, 937.87 and 855.15cm^{-1} whereas γ– cyclodextrin has peaks at 1080.43, 1020.61 and 939cm^{-1}.The spectra of pure DNA can be seen in Figure 3a.

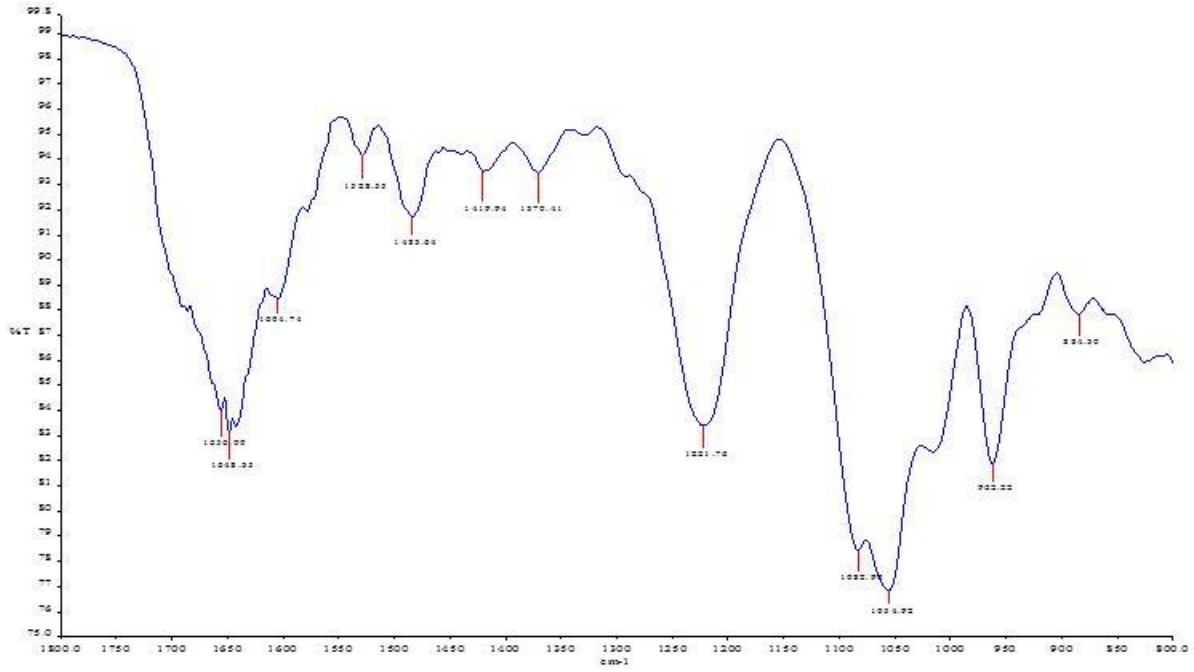


Figure 3a: Pure DNA spectrum (blue)

3.4.1 Freeze Dried DNA solid samples

Figure 3 b and c contains some of the FTIR spectra for freeze dried DNA solid samples. Generally, the peak at 1648.35cm^{-1} and 1086cm^{-1}(present in pure DNA spectrum) were absent in all formulations. This stretch at 1648.35cm^{-1} is characteristic to the carbonyl bond in the planar DNA bases. The change in this band might indicate perhaps cyclodextrins reacts with the phosphate sugar backbone of DNA and disrupt the base stacking to some extent (Mao et al, 1993).

Comparing between spectra of β-CD:DNA 3:1, β-CD:Folic acid:DNA 3:3:1 and β-CD:Pluronic®-F127:DNA 10:20:1 with pure DNA (Figure 3b), generally all the three spectra demonstrated similar stretch with Pluronic-®F127 containing formulation showing the highest intensity in terms of band stretching. It was noted that the β–Cyclodextrin peak at around

1022cm^{-1} was absent. This might be due to broadening of peaks i.e. two peaks merge together as one which may be caused by strong hydrogen bonding. In contrast, the spectrum of γ-CD:Pluronic®-F127:DNA 10:20:1 demonstrated the presence of many peaks around the region 1528 to 1270cm^{-1} (Figure 3c). The peaks from 1270 to 800cm^{-1} were much broader and intense compared to γ-CD:DNA 3:1, γ-CD:Folic acid:DNA 3:3:1 (Figure 3c) and also β-CD:Pluronic®-F127:DNA 10:20:1 spectrum. This might explain why γ–cyclodextrin offer better protection than β–cyclodextrin as more favourable interaction was observed. In addition, an extra peak was observed in both Pluronic®-F127 containing formulations at around 719cm^{-1}.

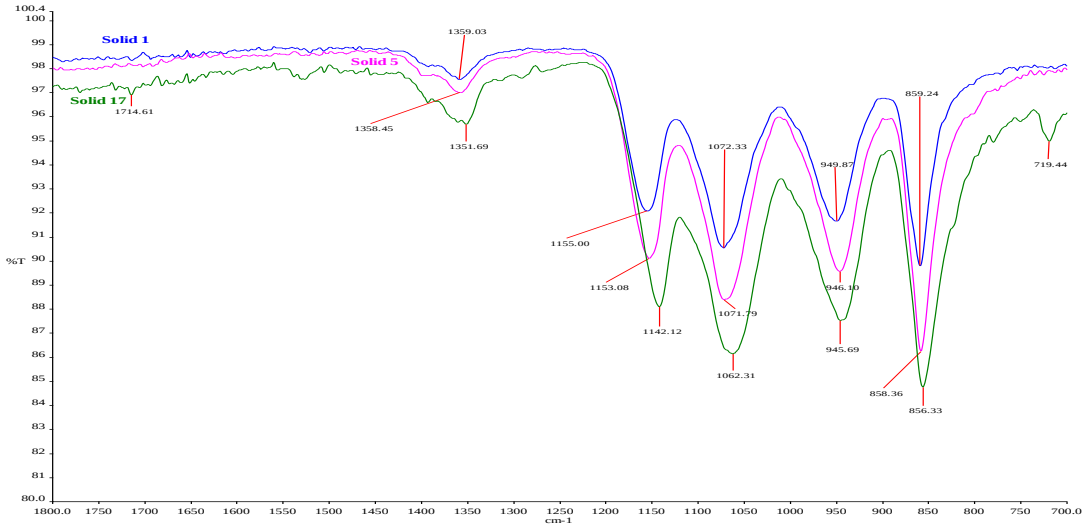


Figure 3b: Spectra of Solid 1 (β-CD:DNA 3:1)blue; Solid 5(β-CD:Folic Acid:DNA 3:3:1) pink; Solid 17 (β-CD:Pluronic®-F127:DNA 10:20:1) green

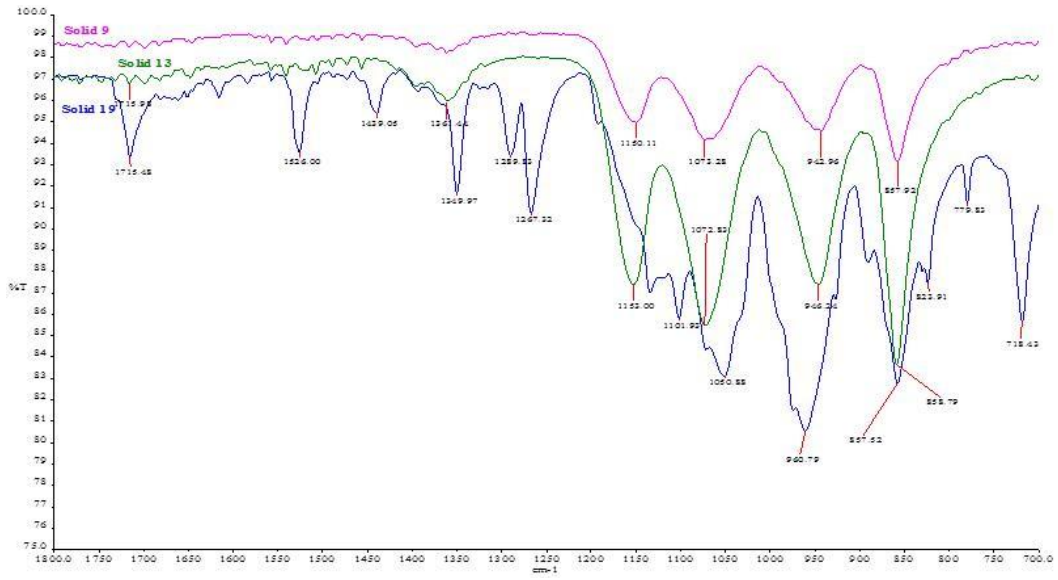
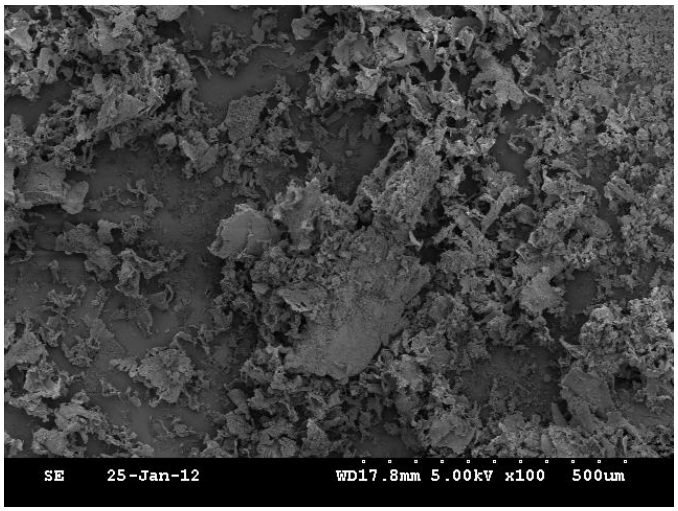


Figure 3c: Spectra of Solid 9 (γ-CD:DNA 3:1)pink; Solid 13 (γ-CD:Folic acid:DNA 3:3:1) green; Solid 19 (γ-CD:Pluronic®-F127:DNA 10:20:1) blue

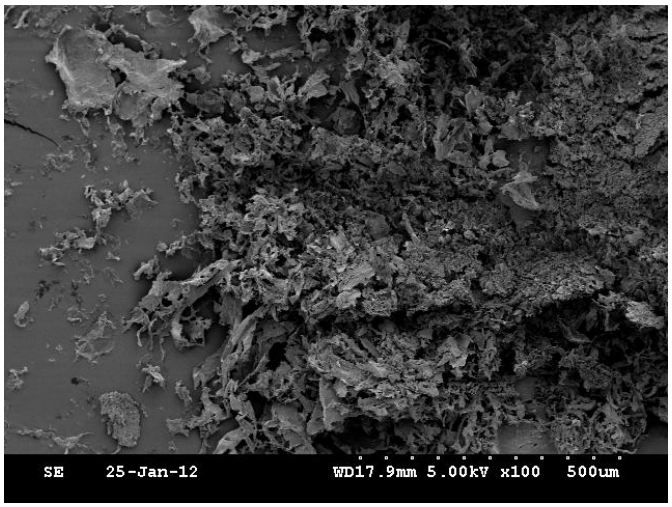
3.5 Particles visualisation using Scanning Electron Microscope (SEM)

Figures 4, 5 and 6 shows some of the freeze dried DNA solids SEM images at different magnification (100x, 1000x and 1500x). Generally, β-cyclodextrin and γ-cyclodextrin DNA formulations with no excipient showed similarity in terms of particles size distribution. Interestingly, the morphology, shape and distribution of Pluronic®-F127 containing formulations particles dramatically changes compared to the structure of pure DNA, β-CD and γ-CD. The presence of cubic or crystalline-like structure were observed in both β-CD:Pluronic®-F127:DNA 10:20:1 and γ-CD:Pluronic®-F127:DNA 10:20:1. The porous, circular like structure in pure DNA and pure cyclodextrin molecule were not seen in Pluronic®-F127 containing formulations. The changes in the morphology clearly demonstrated that interaction occurred between the cyclodextrin:DNA complex with Pluronic®-F127. This interaction was also showed in the FTIR spectra and the DSC thermograms. These interactions led to desirable outcomes in

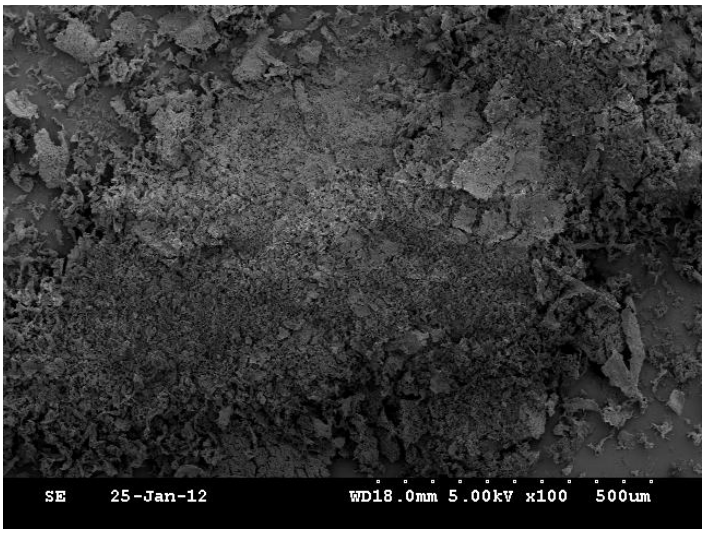
terms of % inclusion, DNase I activity and charge measurements. Similarly, folic acid containing formulations also displayed changes in the particles shape, morphology and distribution. Cubic or crystalline-like morphology was also observed in γ-CD:Folic Acid:DNA 10:10:1 but not in β-CD:Folic Acid:DNA 10:10:1. This suggests that the surface of folic acid interacts differently with β-Cyclodextrin and γ-cylcodextrin compared to Pluronic®-F127. This finding was also observed in the FTIR and DNase I activity.



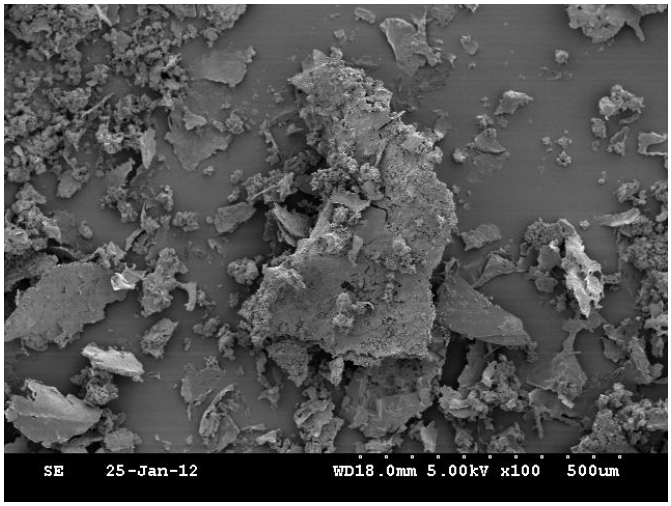
a) β-CD:DNA 10:1



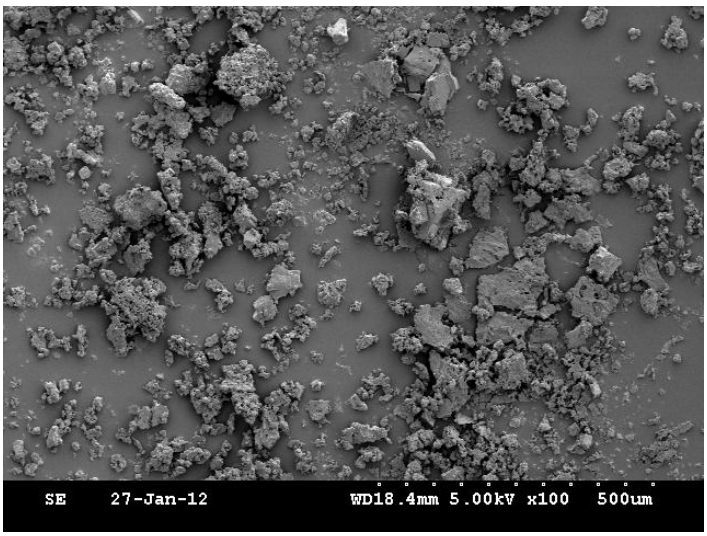
b) β-CD:Folic Acid:DNA 10:10:1



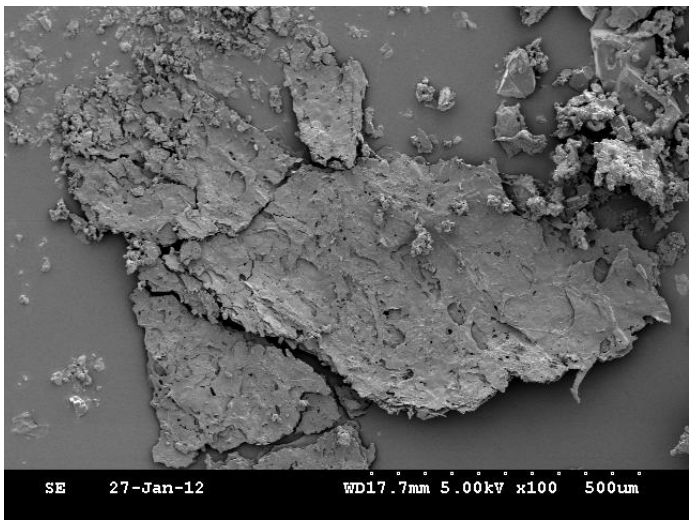
c) γ-CD:DNA 10:1



d) γ-CD:Folic Acid:DNA 10:10:1

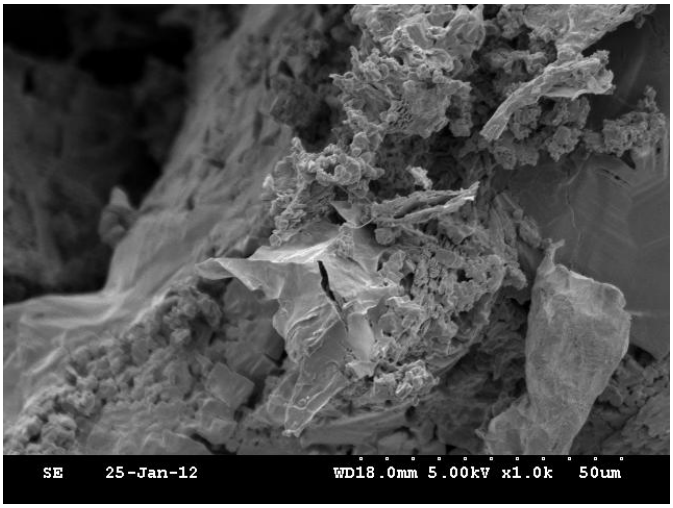


e) β-CD:Pluronic®-F127:DNA 10:20:1

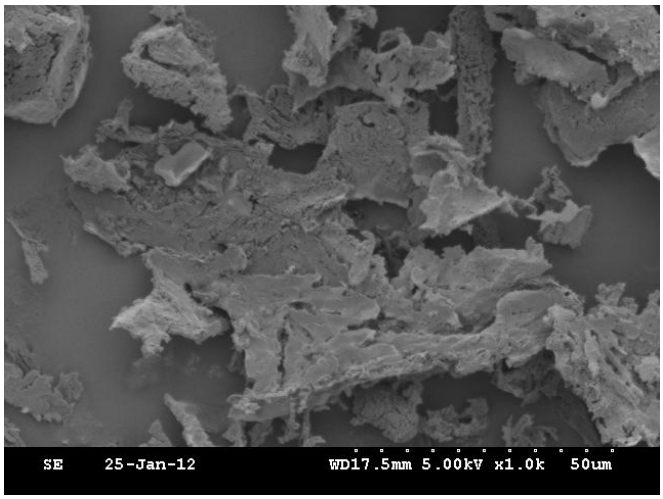


f) γ-CD:Pluronic®-F127:DNA 10:20:1

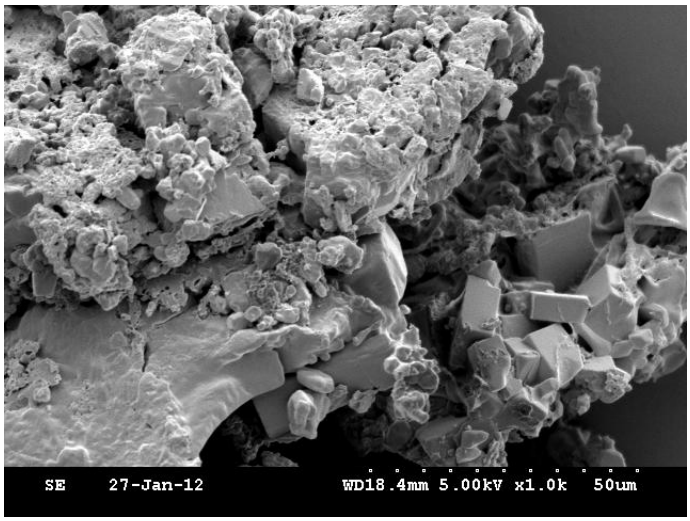
Figure 4: SEM images of freeze dried DNA samples at 100x



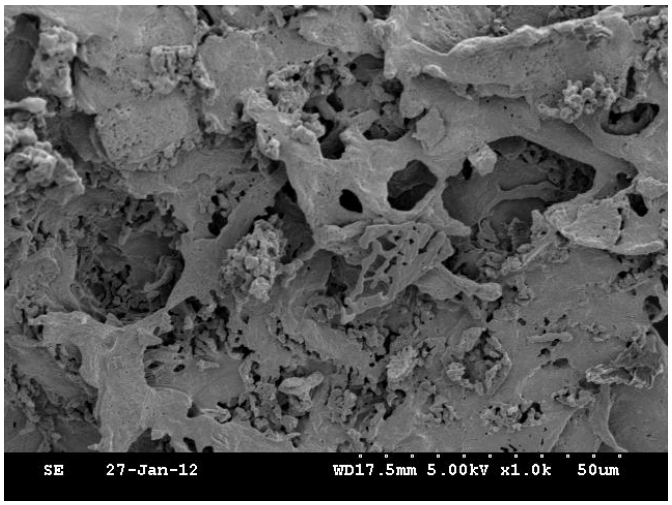
a) γ-CD:Folic Acid:DNA 10:10:1



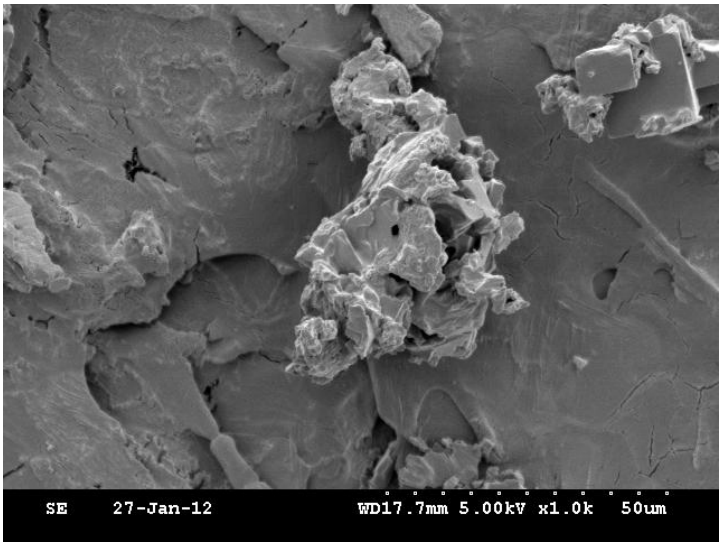
b) γ-CD:Folic Acid 10:10



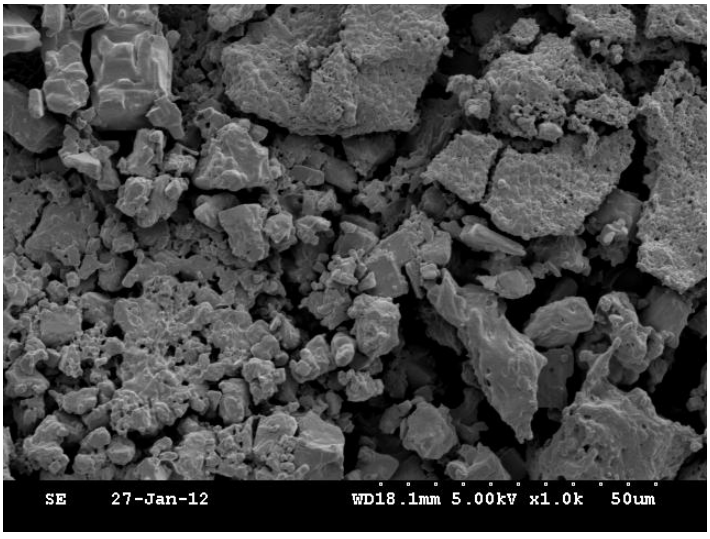
c) β-CD:Pluronic®-F127:DNA 10:20:1



d) β-CD:Pluronic®-F127 10:20

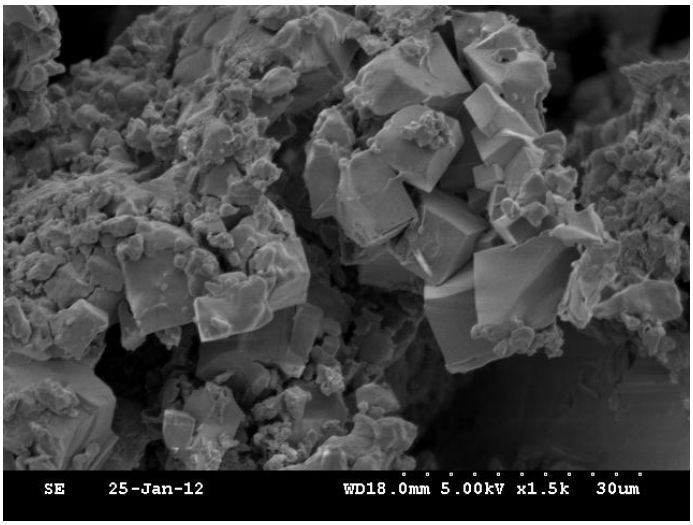


e) γ-CD:Pluronic®-F127:DNA 10:20:1

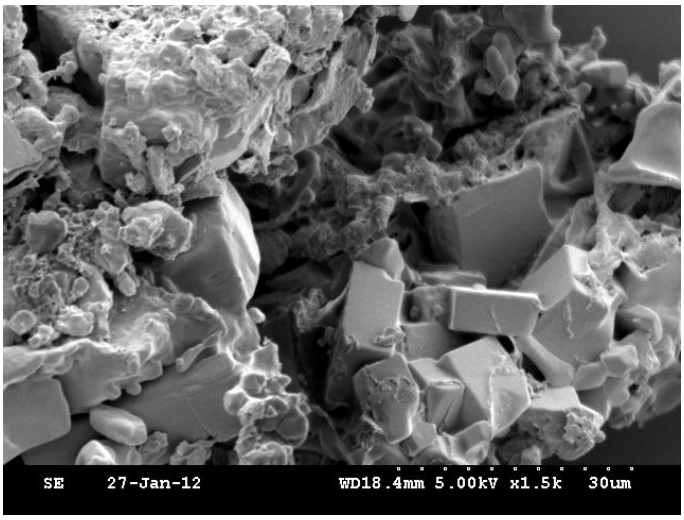


f) γ-CD:Pluronic®-F127 10:20

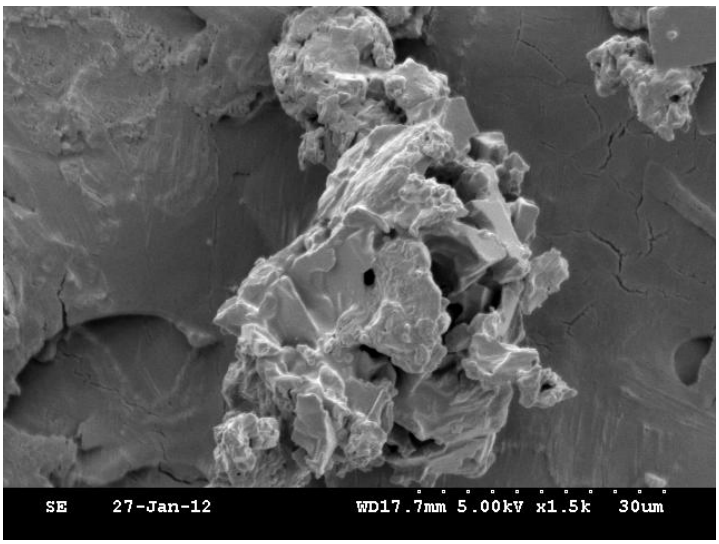
Figure 5: SEM images of freeze dried DNA and non-DNA samples at 1000x



a) γ-CD:Folic Acid:DNA 10:10:1



b) β-CD:Pluronic®-F127:DNA 10:20:1



c) γ-CD:Pluronic®-F127:DNA 10:20:1

Figure 6: SEM images of freeze dried DNA samples at 1500x

3.6 Charge measurements of DNA samples

The zeta potential for freeze dried DNA sample of β-CD: DNA 3:1, β-CD:DNA 10:1, β-CD:Pluronic®-F127:DNA 10:20:1 and γ-CD:Pluronic®-F127: DNA 10:20:1 were-2.6, 11.18, 0.18 and -5.06mV, respectively. Looking at the charge measurements, only freeze dried DNA formulations of β-CD:DNA 10:1 and β-CD:Pluronic®-F127:DNA 10:20:1 were positively charged. Therefore, they would be suitable for cell transfection.

Previously, Pluronic®-F127 containing formulations were investigated at lower concentrations [(3:3:1 and 10:10:1) (β-CD:Pluronic-F127:DNA)], it was thought that by increasing the concentration of Pluronic®-F127, the negative charge of DNA can be completely neutralised (Beba and Elkordy 2011). It was observed in this present study, by doubling the concentration of Pluronic®-F127, an overall positive was obtained. Since Pluronic®-F127 is a type of non ionic surfactant, thus increasing the concentration of Pluronic®-F127 can lead to the formation of micelles. This ability has been demonstrated in various studies (Zhang and Lam, 2006, Linse, 1993, Bohoquezza et al 1991). Therefore by increasing the concentration of Pluronic®-F127, more micelles will be formed and this might protect or shield the negative charge phosphate backbone of DNA from the outside. However, the exact concentration of Pluronic®-F127 in γ-cyclodextrin with DNA still showed an overall negative charge. This might be due to the larger size of γ-cyclodextrin and since cyclodextrins are generally negatively charged, perhaps higher concentration of Pluronic®-F127 is needed. More research is needed to further evaluate these findings.

Interestingly, β-cyclodextrin with DNA alone (no excipient) 10:10 ratio showed overall positive charge. As observed, by increasing the amount of β-cyclodextrin in the formulation, the

overall charge changes from negative to positive. The only plausible explanation for this is that the increased amount of β-cyclodextrin incorporated more DNA molecules into its inner cavity, thus shielding the negative charge phosphate backbone of DNA. The addition of excipients i.e. Pluronic®-F127 might cause direct competition with DNA for the cyclodextrin molecule, reducing the amount of DNA shielded, therefore lower positive current was observed in β-CD:Pluronic-F127:DNA 10:20:1 compared to β-CD:DNA 10:1. The ratio of excipient: cyclodextrins: DNA has to be evaluated for optimum transfection.

3.7 Differential Scanning Calorimetry (DSC) of DNA samples

DSC is used to determine the thermal stability of the DNA samples. It measures the difference in temperature between the sample and a reference (the amount of energy required to maintain the sample at constant temperature as the reference). Changes in transition temperature, alteration in shape or area of the peak or the appearance of the peak on the DSC thermograms indicate an interaction occur between the components of the samples. This interaction could be favourable or unfavourable. Table 10 demonstrates the apparent denaturation temperature for some of the freeze dried samples.

Table 10: T_m values for freeze dried DNA samples

Sample	T_m [a] (°C)
β-CD + Folic acid + DNA 3:3:1	230.06
β-CD + Pluronic-F127 + DNA 10:20:1	224.98
DNA alone	209.52

Based on the thermograms, all formulations showed temperature difference when compared with DNA alone. This can be indicative of interactions between cyclodextrin and

DNA and the excipients. All formulations demonstrated increased in denaturation temperature upon addition of cyclodextrin, Pluronic®-F127 or folic acid. Thus, the solid state of those samples would have extended shelf-life.

4. Conclusion

The use of cyclodextrins as non-viral gene carriers with the incorporation of Pluronic®-F127 or folic acid as excipients has dramatically affected the stability of the gene formulations. The addition of Pluronic®-F127 into the DNA formulations improved the overall stability while conflicting results were observed with folic acid containing formulations. The stability of the DNA formulations was significantly increased through freeze drying.

FTIR, SEM and DSC analysis confirmed interactions occurred between the cyclodextrins, DNA and the excipients for most formulations. Significant changes in FTIR spectra characterised by changes in peak intensity, broadening and shifting of peaks were observed in β-CD:Pluronic®-F127:DNA 10:20:1, γ-CD:Pluronic®-F127:DNA 10:20:1, β-CD:Folic acid:DNA 10:10:1, γ-CD:Folic acid:DNA 10:10:1 and β-CD:DNA 10:1 spectra. In addition, formation of cubic or crystalline-like structure as seen in SEM were demonstrated in β-CD:Pluronic®-F127:DNA 10:20:1, γ-CD:Pluronic-F127:DNA 10:20:1 and γ-CD:Folic acid:DNA 10:10:1. Moreover, DSC thermographs indicated changes in denaturation temperature for β-CD:Pluronic-F127:DNA 10:20:1 and γ-CD:Folic acid:DNA 3:3:1 formulations when compared to DNA alone and the presence of an extra peak in the DSC thermograph of β-CD:Pluronic®-F127:DNA 10:20:1 suggests significant interactions are occurring between the excipients.

These significant interactions led to favourable results for Pluronic®-F127 containing formulations in contrast to folic acid containing samples in terms of % DNA inclusion, protection from DNase I and stability at high humidity (RH=76%). Both fresh and freeze dried DNA formulations of β-CD:Pluronic®-F127:DNA 10:20:1 and γ-CD:Pluronic®-F127:DNA 10:20:1 showed highest % DNA inclusion and provide enough protection against enzymatic degradation. Their stability against DNase I remained the same even after storage at RH=76% for 5 weeks.

On the other hand, fresh samples of γ-CD:Folic acid:DNA 3:3:1 and β-CD:Folic acid:DNA 3:3:1 showed no inclusion of DNA and offered little protection against DNase I before and after storage at RH=76% for 5 weeks . Although, lyophilisation generally improved the % of DNA inclusion into the cyclodextrin, but folic acid containing samples were still considered the least stable when compared to other DNA samples.

Freeze dried β-CD: DNA 10:1 sample also showed favourable results in relation to % inclusion and DNase I and is considered suitable for cell transfection as it was the only formulation with an overall positive charge besides β-CD:Pluronic-F127:DNA 10:20:1.

Overall, it was noted that γ-cyclodextrin offered better stability than β-cylodextrin.

5. Acknowledgements

We would like to thank Mrs. Rita Haj-Ahmad and Mr. Malcolm Haswell for their assistance in conducting charge measurements and operation of Scanning Electron Microscope.

6. References

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