Insights into the influences of carboxymethyl-β-cyclodextrin on DNA formulations characteristics and gene transfection efficiency

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Abstract

Gene therapy is an expanding field and it can treat genetic and acquired diseases.
It was found that formulations with DNA: CM-β–CD (Carboxymethyl-beta-cyclodextrin):
Pluronic-F127 1:3:3 and 1:3 DNA: CM-β–CD are the most stable formulations indicating
high incorporation of DNA within CM-β –CD. Gel electrophoresis revealed DNA with low
CM-β –CD concentration has formed a more stable complex. Samples 1:3 DNA: CM-β–CD
and 1:3:3 DNA: CM-β–CD: Pluronic-127 show no DNA fragment suggesting good
condensation of DNA inside cyclodextrin cavity. This was confirmed by fluorescence data
where fluorescence intensity was reduced for samples DNA: CM-β–CD 1:3. Overall the
findings showed that Carboxymethyl-beta-cyclodextrin (as a novel non-viral gene vector)
was able to provide condensation and protection to the DNA, with and without Pluronic-
F127, at low concentration. pDNA/CM-β–CD complex has not just shown to be able to
transfect COS 7 and SH-SY5Y cell lines but it gave a higher transfection efficiency than that
produced by the TransIT-LT1 commercial transfection reagent.

Key words:

1. Introduction

Gene therapy is the insertion of functioning genes into cells to correct cellular dysfunction or to provide new cellular functions (Mulligan *et al,* 1993). The concept of gene therapy emerged in the early 1960s when genetically marked cell transformation by papovaviruses polyoma and SV40 were identified (Hill *et al,* 1999). Over the past years there has been a remarkable expansion in gene therapy fields, which capture the interest of many researchers and much progress has been made. Many clinical trials have been conducted with highly promising results (Silva *et al, 2011;* Akinori *et al, 2009),* making gene therapy the medicine of future that could significantly contribute to the development of new treatment to cure both genetic and acquired conditions. In clinic, gene therapy has not been globally successful because of the inefficient delivery of genetic material to their correct intracellular sites and the poor toxicological profile of some vectors. For gene therapy success, gene must inter the target cell without any biodegradation or immune response (Liu and Huang, 2002).

Currently several methods have been used to deliver a gene into the nucleus of a cell in a process known as transfection. The first method is direct injection of DNA into cell nucleus using mechanical, ultrasonic, electric, hydrodynamic or laser-based energy, creating temporary weak points in the membrane of the target cell and allows the DNA to inter the cell by diffusion (Liu *et al,* 2002). This direct approach is considered a safe and effective method but is limited to specific applications that involve easily accessible and discrete targets (Howden, 2008). The second method involves the use of gene carriers, which can be either viral or nor-viral vectors. The viral vector has been the most utilized and most successful agent in the gene delivery because of their high transfection efficiency *in vivo*, but their high immunogenicity, high cost and limited size carrier, has led scientists to find alternative method (Hoag *et al,* 2005). Consequently non-viral vectors have been designed; these vectors are relatively inexpensive, show no signs of oncogenicity and have lower immune response. However low transfection efficiency has been reported due to differences in barrier permeation between the target cell nucleus and extracellular space (Cryan *et al*, 2004). Thus, the main points that should be taken into account for developing non-viral vectors are the introduction of the gene into the cell and increasing transfection efficiency.

Despite their relatively low efficiency, non-viral approaches are considered safer and preferable methods in gene delivery. Several different types of non-viral vectors have been investigated for gene therapy applications this includes: polymers, lipids/liposomes, dendrimers and cell penetrating peptides. The main function of these vectors is to deliver the therapeutic gene to the target cell to correct the defective or mutated gene. Non-viral vectors are generally used to transfer different types of nucleic acid including RNA, small and large DNA molecules (Hoag *et al*, 2005). Many barriers prevent the DNA from reaching their target and performing their function. After loading the genetic material to the vector, the vector must be delivered to the blood vessels and must be stable to avoid clearance by albumin as a result of their high surface charge. Next, the vector must pass through the epithelial tissues of blood vessels and enter the target cell through endocytosis process.

Although choosing the most appropriate vectors is the most critical step in achieving gene therapy, none of the currently available non-viral vectors fulfills ideal vector properties (Liu *et al,* 2002). This has led to research focus on suitable ideal non-viral vector delivery system. Gene delivery systems have focused in chemical carriers (lipid based, polymer based and

peptide based) as main non-viral vectors which have been designed to efficiently protect, transport and deliver the gene to target specific cells with minimum toxicity and high transfection rates.

Cyclodextrin as a non-viral gene carrier, cyclodextrin and its unique chemical structure has become an area of interest especially in DNA delivery, where it shows a great ability in forming inclusion complexes with DNA. Cyclodextrin (CDs) are naturally occurring cyclic oligosaccharides consist of (α-1,4)-linked α-D-glucopyranose units and contain a lipophilic central cavity and a hydrophilic outer surface. The most common CDs are α-, β- and γ-CDs, consisting of six, seven and eight α-D-glucopyranose units respectively (Cryan *et al,* 2004). The ability of CDs to improve drug bioavailability has been suggested to benefit from two features: (i) their membrane absorption enhancing properties and (ii) their ability to stabilise biomolecules in physiological media by shielding them from nonspecific interactions (Hao *et al*, 2012*)*. The successful delivery of DNA to the livening cell requires a complex formation of DNA with its carrier (CD). The driving force behind the non-covalent formulation of DNA-CD is the ability of CD to release water from the lipophilic cavity in favour of a more lipophilic entry. This causes a relaxation in the ring and decreasing the amount of strain felt by the cyclic system. All of this culminates in the complex becoming more stable.

In gene delivery applications, cyclodextrins have favourable properties where they function as absorption enhancers in therapeutics transfer, and play a role in modulating the cytotoxicity of other polymers (Martin *et al,* 2007). Native CD has failed to form stable complex with DNA and hence has limited transfection efficiency. However derivative CD where they constructed by amendment of molecular parameter such as density and hydrophobic-hydrophilic balance has induced transfection rate (Ortiga –caballero et al, 2008). In *vivo* the interaction of cyclodextrin with specific host molecules can be improved by increasing its stability and solubility, using primary and secondary alcohol group reactions. These CDs derivatives have different property and hence will have different level of transfection and toxicity (Jianshu *et al,* 2010).

Cyclodextrin has shown to be facile and effective approach in developing gene delivery when used as core system or alongside cationic lipid (Martin *et al*, 2013). Incorporation of cyclodextrin with excipient such as folic acid and pluronic- F127 significantly affect the stability of DNA formulations and provide safe and effective DNA formulations (Eng *et al,* 2014). The success of these formulations needs further investigations and transfection efficiency remains to be investigated.

In non-viral gene formulations, stability plays a major role in developing this therapy to become a marketable product (Anchordoquy et al, 2001). The storage of gene formulations has been investigated with various DNA formulations including liquid, frozen and dehydrated formulations. Aggregation and short shelf life are the main limitations of liquid formulations (Anchordoquy et al, 2001). The frozen formulations are more successful compared to liquid formulations, but damage to DNA structure was reported as result of cryolysis (the formation crack within the ice). Although the stability of frozen formulations was significantly improved by the addition of excipients, but difficult maintenance conditions were required. To overcome the limitation of liquid and frozen formulations, dehydrated formulations were developed. These formulations are stable at ambient temperature and can be ready to administer after a simple reconstitution step. The two main ways to remove water form liquid are spray drying and freeze-drying. Since spray drying could generate high shear forces and damage the gene vectors, freeze-drying become a preferable technique (Anchordoquy et al, 2001,).

The future of gene therapy requires the advancement of non-toxic and effective gene delivery systems. Therefore, this study will be focusing on improving gene stability and transfection efficiency using carboxymethyl-β-cyclodextrin (CM-β –CD) as a novel non-viral vector (without any structural modification; which is different to many publications such as Diaz-Moscoso et al 2009) with a type of block co-polymer known as Pluronic- F127. Pluronic – F127 as pharmaceutical excipient has been proved to enhance the transgene expression as well as the therapeutic effect of the transgene via several delivery routes and various types of vectors including naked DNA itself (Kabanov *et al*, 2002). Moreover, in both vivo and vitro Pluronic-F127 has also showed significant ability to preserve the stability of polypeptide. In this study, carboxymethyl-β-cyclodextrin will be used with and without Pluronic- F127 at different weight ratio to determine its effect on formulations stability. The stability of DNA formulations will be studied under fresh and freeze dried conditions. The most successful formulation will be used to transfect, two different cell lines and the results will be compared to that of TransIT-LT1 reagent (as a control).

2. Materials and Methods

2.1 Materials

Deoxyribonucleic acid (DNA) sodium salt from calf thymus was purchased from Sigma Aldrich (UK). TransIT-LT1 transfection reagent kit was obtained from Mirus (UK). Purity of DNA was checked by monitoring the absorption spectrum and the ratio of the absorbance at 260–280 nm. The ratio being 1.87 indicated that the DNA was fully free from proteins. Deoxyribonuclease (DNase from bovine pancreas), carboxymethyl-β-cyclodextrin and Pluronic-F127 were also purchased from Sigma Aldrich (UK). High glucose Dulbecco's modified Eagle's medium (DMEM), primocine, Fetal bovine serum (FBS), L- Glutamine and trypsin were also purchased from sigma Aldrich (UK).

2.2 Plasmid preparation

In this study, plasmid expressing green fluorescent protein (pc DNA3.1-GFP) was amplified by transformation of *E coli* to produce a large quantity of the plasmid. Cells were plated into the ampicillin (200 µg/ml) containing agar plates and stored at 37^0C overnight. A colony was picked from the plate and placed into 100mls of LB (Luria-Bertaini) medium and left for 48hours on the shaker at 37^oC. The DNA was extracted using a Maxiprep by following the manufacture protocol (Invitrogen, UK). Purity and quantity of the plasmid were checked using Nanodrop lite (thermo, UK). Purity was 1.9 (ratio of absorbance at 260nm/280nm) and quantity was 2µg/µl, this was also confirmed by taking UV measurement at 260nm and 280nm wavelengths.

2.3 Cell line used

African green monkey fibroblast cells COS 7 and SH-SY5Y neuroblastoma cell lines were obtained from the University of Sunderland labs. The cell line was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with o.5% primocine, 10% Fetal bovine serum (FBS) and 1% L- Glutamine. At 37^oc and 5% CO_2, cell were trypsinized and subcultured twice a week.

2.4 Preparation of fresh and freeze-dried DNA samples

The following preparations were made up in phosphate buffer saline (PBS) with pH 7.4 at various weight ratios of carboxymethyl-β-cyclodextrin(CM-β –CD) and pluronic-F127 (see table 1 for all DNA and non-DNA solutions). DNA concentration was kept constant at 20 μg /ml, preparations were made up to 20mls using the PBS.

Table 1: DNA samples made up to 20mls using PBS buffer

Samples. DNA/EXCEPIANTS	DNA (μg 20ml)	CM-β –CD (μg/ 20ml)	Pluronic – F127(μg/ 20ml)	Final DNA Concentratio (μg /ml)
1 (1:0)	400	0	0	20
2 (1:3)	400	1200	0	20
3 (1:3:3)	400	1200	1200	20
4 (0:3:3)	0	1200	1200	0
5 (1:10)	400	4000	0	20
6 (1:10:10)	400	4000	4000	20
7 (0: 10:10)	0	4000	4000	0

As drug delivery systems become more widespread, the challenge of their long-term stabilization will become of increasing importance (sameti *et al,* 2003). Freeze-drying is a well-established method used in the preservation of unstable pharmaceutical products over long periods of time, and has been shown to improve long term stability of DNA samples (Eng et al, 2014 and Martins *et al*, 2007). Non-viral vectors face two stages of stress during freeze drying process (freezing and drying), which has been reported to damage DNA especially during freezing process. However, these damages have not resulted in reduction of cell transfection (Allison and Anchordoquy 2000) and hence freeze drying method has been accepted for preparing dried formulations of proteins and non-viral gene vectors (Seville *et al,* 2002).

The samples (see Table 1), were frozen at -80 $^{\circ}$c for four hours, and then were taken to the freeze drier using Vir Tis benshtop k Freeze Drier (biopharma, UK). The condenser temperature was -105$^{\text{O}}$C, the shelf temperature was 21 $^{\text{O}}$C and the vacuum was set to 20mT. Solutions were left to dry for 48hrs and then characterised using Fluorescence, Fourier Transform Infra-red Spectroscopy (FT-IR), gel elechtrophoresis, and DNase activity. For Fluorescence measurement, UV absorbance, gel electrophoresis and DNase activity, freeze dried samples were reconstituted by hydrating the powder back to solution using distilled water (dH$_2$O).

2.5 Characterization of fresh and reconstituted freeze dried DNA samples
2.5.1   *Fluorescence calibration curve for deoxyribonucleic acid (DNA)*

In order to measure the DNA fluorescence, Gel red was used to replace the highly toxic ethidium bromide. Gel red has been shown to be very safe and environmentally friendly. For fluorescence calibration curve, series of dilution were mad to contain, 0.5, 1, 2 and 2.5μg/ml from DNA stock solution of 20 μg/ml and 0.02% of Gel red. Samples were left for 30min to intercalate gel red with DNA. Fluorescence was then measured using the Perkin-Elmer Liminescence spectrophotometer (LS5OB, perkin-Elmer Ltd., UK) at λ$_{\text{excitation}}$ (592nm) and λ$_{\text{emssion}}$ (592) characteristic to DNA (Cryan *et al*, 2004).

2.5.2 Fluorescence measurements of DNA samples

Fluorescence is type of luminescence created by electromagnetic excitation, which generated when a substance absorbed light energy at a short wavelength, and then emits light energy at a longer wavelength. Fluorescence test was done to measure the % of DNA incorporate inside cyclodextrin. It is generally agreed that strong fluorescence enhancement accompanied intercalation of the dye into the double helix is a conformation of DNA presence (Olmestedll *et al,* 1977). Adding the gel red to DNA samples, it intercalates between the base pairs of DNA double helix and fluorescence the available DNA that has not been incubated inside the cyclodextrin. This measurement, via the calibration curve, can determine the concentration of DNA in each sample. The fluorescence measurements of DNA samples were taken from fresh and freeze dried samples using the Perkin Elmer luminescence spectrophotometer, (LS5OB, Perkin-Elmer Ltd., UK). 10mls of 0.02% gel red was mixed with 10mls of each sample to give a final concentration of 1μg/ml. The non-DNA solutions were used as blank to eliminate any excipients interference

2.5.3 DNase Activity measurements

DNase is an enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkage in the DNA backbone (Hedddi *et al*, 2010). Adding DNase to DNA solutions will determine whether DNA incorporated inside cyclodextrin cavity, any DNA that is not incorporated inside the cyclodextrin core will be available for DNase fragmentations and is an indication of unstable formulation. Fragmentation of DNA will lead to an increase of UV absorbance over time. One vial of DNase contain 0.93mg was diluted to 0.5ml with 0.15M NaCL. A volume of 0.1mls was further diluted to 1mls; 0.1ml was added to 3mls of each DNA containing samples and then absorbance measurement was taken at three different points 0, 5 and 15 minutes, using the M501 Single beam Scanning UV/Visible Spectrophotometer (Campsec Biochrom).

2.5.4 Gel electrophoresis

Gel electrophoresis is a method used to separate DNA, RNA and protein according to their sizes and charge (Hui-Qiao et al 2015). Interactions of DNA with excipients play a major role in gene therapy in order to protect DNA from any degradation. Agarose gel electrophoresis method was used to quantify the binding of DNA to cyclodextrin, any interaction between DNA and cyclodextrin will prevent DNA from traveling toward the positive charge. Agarose gels were prepared by dissolving 1g of agarose into 100ml of TAE buffer giving a gel concentration of 1%. 10μl of gel red was added to visualise the DNA when placed under UV light, then pour into electrophoresis apparatus. Two combs were used to make wells for fresh and freeze dried samples and left for 30min, then moved into gel box and cover with buffers, 8 μl of each sample was mixed with 5μl of loading dye and placed in each wall including DNA ladder. Solutions were left to run for one hour at 100V and imaged using the Gel Doc system (BioRad, UK).

2.6 Characterization of freeze dried DNA powders USING Fourier Transform Infra-red Spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM)

FT-IR spectroscopy is often used to characterize the nature of drug–DNA interactions and to monitor the effects of various drugs and excipients on DNA structure as well the binding sites of DNA (Ahmadi *et al*, 2010 and Hsiue, *et al* 1998). On this experiment FT-IR will determine any interaction between DNA and cyclodextrin. FT-IR was conducted on freeze dried DNA

powders; using Perkin-Elmer FT-IR Series (Beakonsfield, Buckinghamshire, UK) FT-IR was conducted at a resolution of $4cm^{-1}$.

SEM was used to study DNA–CD inclusion by monitoring any external morphology and chemical composition change. Freeze dried samples were added to 15mm diameter aluminium stub using double-sided carbone adhesive tabs. All samples were coated with mixture of gold/palladium in high vacuum coating unit using Quarum Technology (polaron range SC760) by exposing samples to an argon atmosphere at about 10 Pascals. Samples were coated for 2x105 second with process current of 18-20 mA. After coating, samples were examined using Hitachi S3000N Scanning Electron Microscopy (Hitachi High Technologies UK-Electron Microscopes, Berkshire, UK).

2.7 Preparation of transfection reagents
The most stable DNA formulation was used for transfection. 24hrs before transfection $0.6x10^5$ Cos 7 cells and SH-SY5Y neuroblastoma cell line were plated in 6 well plates with 2.5mls of DMEM, cells were ≥ 80 % confluence. TransIT-LT1 reagent were used as reference. 2µg DNA added to each formulation, CM-β –CD 1:3 and DNA: CM-β –CD: Pluronic-F127 1:3:3 and TransIT-LT1 and then mixed with 250µl of reduced –serum medium (Opti-MEM I). All formulations were mixed by pipetting gently and incubated for 30min at $37^{\circ}C$. Each formulation added to 6 well plate and measurement was taken at 24, 48 and 72 hours using the flow cytometry technique (BD Accuri, BD Biosciences, USA).

2.8 Statistical analysis
Univariate analysis of variance was performed for statistical analysis. Levene test was used to test the sample has equal variances. Equal variances cross samples is called homogeneity of variance. Tukey test was used as appropriate. The data are considered significant if the P value is less than 0.05.

3. Results and Discussion

The DNA condensation is prerequisite for successful gene delivery (Aviles *et al*, 2010). DNA undergoes a conformational transition from a semi-flexible coil to a more compacted state upon mixing with cyclodextrin, which has the ability to form polymers of nano-sized with DNA (Zerkoune *et al*, 2014). This formulation is attracted by electrostatic binding resulted from the phosphate group of the DNA backbone and the lipophilic inner cavity of the cyclodextrin (wang *et al*, 2002). In this study, the ability of CM- β-CD polymer to condense DNA was evaluated by Agarose gel electrophoresis, fluorescence, FT-IR, DNase activity and transfection

3.1 Fluorescence measurements for fresh and freeze dried DNA samples
For fluorescence intensity measurements for fresh DNA samples, the results were applied to the equation of line of regression from the DNA fluorescence calibration curve (see Equation 1) to determine the DNA concentration.

y= 33.5-7.1   Equation 1

The % of DNA inclusion into cyclodextrin cavity was calculated using Equation 2. Decrease in fluorescence intensity would results in higher % inclusion due to the unavailability of DNA to gel red dye.

% Of inclusion = (theoretical DNA concentration- concentration of DNA measured by Fluorescence) x 100
Theoretical DNA concentration (1µg/ml)

Equation 2

Results from Table 2 demonstrated that DNA : CM-β –CD 1:3 and DNA 1:10 were the most stable formulations with highest % of DNA inclusion 20.51% and 20.3% respectively. Followed by samples DNA: CM-β –CD:Pluronic F127 1:3:3 with 16.44% while sample DNA: CM-β –CD:Pluronic F127 had only 14.9% inclusion. Unianova, analysis of variance was done to determine whether the samples differed significantly ($p<0.05$). It was found that the most stable freshly prepared aqueous formulations CM-β-CD:DNA 1:3 and 1:10 differed significantly ($p<0.05$) from DNA : CM-β –CD:Pluronic F127 1:3:3 and 1:10:10.

Table 2 shows % of inclusion of DNA (as average and S.D.) inside cyclodextrin from fresh and freeze dried DNA samples

Samples	Average for fresh samples	SD	Average for freeze dried samples	SD
DNA: CM-β –CD 1:3	20.51%	0.08	44.20%	0.01
DNA: CM-β –CD:Pluronic F127 1:3:3	16.44%	0.09	33.70%	0.08
DNA: CM-β –CD 1:10	20.32%	0.07	45.87%	0.02
DNA: CM-β –CD:Pluronic F127 1:10:10	14.93%	0.04	25.87%	0.01

The results for DNA concentration and % DNA inclusion for freeze dried samples are illustrated in Table 2. Formulations DNA: CM-β –CD 1:3 and 1:10 had the highest % of inclusion and were the most stable formulations compared to other freeze dried samples. Based on the statistical analysis (Unianova,), both these formulations were significantly

differed (p<0.05) from the least stable formulations (DNA:CD:Pluronic F127 (1:10:10) and the rest of freeze dried formulations.

Fresh and freeze dried DNA formulations were made to contain 1μg/mL of DNA. The decrease in fluorescence intensity in most samples indicates some degree of DNA inclusion into the cyclodextrin complex. In comparison, freeze dried formulations to fresh formulations, freeze dried samples provided higher % of DNA inclusion inside cyclodextrin cavity. Where nearly double the % of DNA inclusion was reported for freeze dried samples (Table 2) compared to fresh DNA aqueous solutions (Table 2).

The stability of DNA- CM-β –CD complex was also investigated in the presence of Pluronic F-127 for both fresh and freeze-dried formulations. Although the addition of Pluronic F-127 as stabilising excipients to the samples was expected to enhance the stability of formulations, results show no significant (P > 0.05) effect of Pluronic F-127 on the samples. Moreover, a slight decrease in the % of DNA inclusion was noticed in some DNA-CD formulations. The decrease in DNA inclusion into cyclodextrin in the presence of Pluronic F-127 might be related to the formation of micelles. Pluronic F127 is an amphiphilic molecule, which forms spherical micelles with hydrophobic cores and hydrophilic coronas, above critical micelles concentration (CMC) in aqueous solution forms (Rajib et al 2013).

Fluorometry is a very sensitive method used to measure the concentration of DNA and it is known to be more accurate than absorbance at 260nm (Rengajaran et al, 2002). This method is highly specific in terms of differentiating double stranded DNA from single stranded or RNA, and able to detect a small amount of double stranded DNA. Ethidium bromide fluorophore is a preferable stain that usually used in this method. However due to health and safety issues it was replaced with Gel-red which is widely used stain in molecular biology that also known to be sensitive, safe and an easy stain for DNA.

3.2 DNase I activity measurements

The stability and DNA incorporation for fresh and freeze dried DNA formulations were investigated using DNase enzymes activity measurements. The degree of DNA formulation stability is obtained from the difference between the absorbance readings from time 0 to 15 minutes, the smaller the difference in absorbance the higher the stability.

For DNase I activity measurements for fresh DNA samples, the results of DNase I activity measurements at time 0, 5 and 15 minutes for fresh DNA aqueous samples are illustrated in Table 3.

Table 3: DNase I activity measurements against fresh and freeze dried DNA samples

Fresh Samples	Absorbance At t^a=0	Absorbance At t^a=5	Absorbance At t^a=15	Change in absorbance At t^a=15
DNA	0.412	0.419	0.421	0.009
DNA- CM-β –CD 1:3	0.440	0.442	0.443	0.003
DNA- CM-β –CD - pluronic 1:3:3	0.356	0.359	0.361	0.005
DNA- CM-β –CD 1:10	0.347	0.352	0.359	0.011
DNA- CM-β –CD -Pluronic 1:10:10	0.309	0.309	0.319	0.010
Freeze Dried Samples	**Absorbance At ta=0**	**Absorbance At ta=5**	**Absorbance At ta=15**	**Change in absorbance At ta=15**
DNA alone	0.327	0.331	0.344	0.017
DNA-CD 1:3	0.125	0.126	0.126	0.001
DNA-CD-pluronic 1:3:3	0.171	0.172	0.175	0.004
DNA-CD 1:10	0.279	0.290	0.293	0.014
DNA-CD-Pluronic 1:10:10	0.268	0.280	0.281	0.013

Results of DNase I activity measurements for fresh DNA samples revealed that formulations with DNA: CM-β –CD:Pluronic-F127 1:3:3 and 1:3 DNA: CM-β –CD had the smallest change in absorbance. Thus, these formulations are the most stable DNA formulations indicating high incorporation of DNA within the CM-β –CD. Samples DNA: CM-β –CD 1:10 and DNA : CM-β –CD:Pluronic F 127 1:10:10 showed less DNA protection against DNase I degradation.

For DNase I activity measurements for freeze dried DNA samples, Table 3 shows the results of DNase I activity measurements at time 0, 5 and 15 minutes for freeze dried DNA samples.

Based on the results, the most stable freeze dried formulations that provide most protection from DNase I degradation were DNA: CM-β –CD 1:3 and DNA: CM-β –CD:Pluronic F127 1:3:3. While samples DNA-CD 1:10 and DNA:CM-β –CD:Pluronic F127 1:10:10 showed

higher change and less stability ($p < 0.05$).

The results of DNase I activity from both freeze dried and fresh formulations have revealed that low concentration of CM-β–CD provided better protection for DNA than higher concentration. The effectiveness of CD at low concentration was also noticed in the fluorescence measurements (see Table 2) where samples with low CM-β–CD concentration showed high % of DNA inclusion with no benefit from the increase of CM-β–CD concentration. Results suggested that CM-β–CD provide protection for DNA at low concentration with and without the presence of Pluronic F127. The increase in formulations stability at low CM-β–CD concentration was also confirmed by gel electrophoresis (see below).

The decrease in formulations stability at high level of CM-β–CD may be due to the formation of favourable bonds or interaction between the CM-β–CD molecules at the hydrophilic site leaving DNA with little or no site to bind to (Allison et al, 2000).

3.3 Gel electrophoresis

The phosphate molecules that make up the backbone of DNA molecules have a high negative charge. When DNA is placed on a field with an electric current, these negatively charged DNA molecules migrate toward the positive end of the field creating DNA bands. Formulations that have high stability and good DNA inclusion inside cyclodextrin will show little or no bands of DNA.

Gel elcectrophoresis for fresh DNA samples

Figures 1 and 2 show the results of gel electrophoresis of fresh DNA aqueous samples and freeze dried DNA samples, respectively.
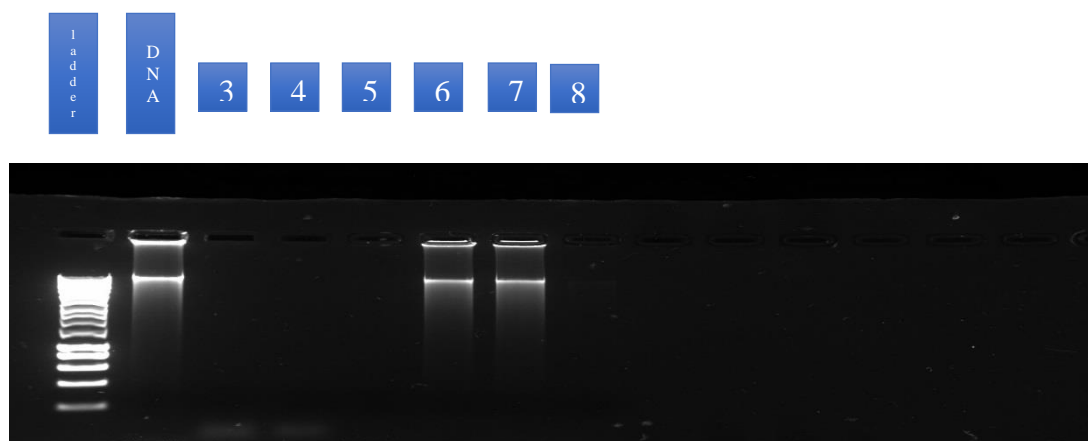

Figure 1: Agarose gel electrophoresis for β-CD-cm:DNA:Pluronic-F127 samples from fresh samples. Lane 1: DNA ladder; lane 2: pure DNA; lane 3: DNA:CD 1:3 lane 4: DNA:CD:Pluronic 1:3:3; Lane 5: CD:PLURONIC 3:3; lane 6: DNA:CD 1:10; lane 7: DNA:CD:Pluronic 1:10:10; lane 8: CD:Pluronic 10:10.
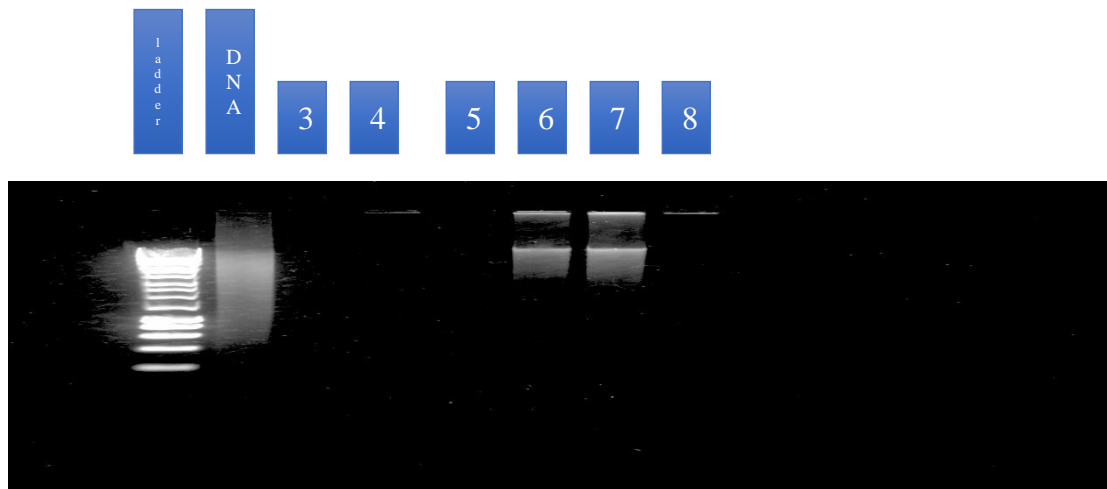
Figure 2: Agarose gel electrophoresis for β-CD:DNA:PluronicCF127 samples from freeze dry samples. Lane 1: DNA ladder; lane 2: pure DNA; lane 3: DNA:CD 1:3 lane 4: DNA:CD:Pluronic 1:3:3; Lane 5: CD:PLURONIC 3:3; lane 6: DNA:CD 1:10; lane 7: DNA:CD:Pluronic 1:10:10; lane 8: CD:Pluronic 10:1

Results from gel electrophoresis for fresh DNA samples showed no movement of DNA for lane 3 (DNA: CM-β –CD 1:3) and line 4 (DNA: CM-β –CD:Pluronic F127 1:3:3) (see Figure 1). Similar results were also obtained from freeze-dried samples. Samples 3 and 4 (1:3 DNA: CM-β –CD and 1:3:3 DNA: CM-β –CD:Pluronic F127) show no DNA fragment (see Figure 2) suggesting good condensation of DNA inside cyclodextrin cavity.

These results have confirmed that DNA with low CM-β –CD concentration has formed more stable complex than DNA with higher CM-β –CD concentration, which support the results from Fluorescence and DNase activity measurements as explained above.

3.4 Fourier Transform Infra-red Spectroscopy (FT-IR)

FT-IR was used to indicate any interaction between DNA and cyclodextrin for dried samples. Interaction between CM-β –CD: DNA will result in shifting DNA peaks. FT-IR will give structure information of the entire molecule and any conformational change to DNA biomolecules. Since FT-IR is a fast method and only required small amount of sample, it becomes ideal for the study of DNA (Deepak *et al* 2010). Results of FT-IR showed that cyclodextrin binds to DNA through direct interaction with DNA bases (guanine, thymine, adenine and cytosine), with a small perturbation of phosphate group of DNA backbone. It could be inferred that this interactive model of CM-β –CD with DNA is an electrostatic binding (Wang *et al* 2002, Zhao *et al* 1999). The measurements were taken at 1800-650 cm^{-1} spectral range with a resolution of 4 cm^{-1}. Background spectra were collected and subtracted before each measurement. Four main peaks have been identified in pure freeze dried of DNA as shown in Figure 3: 1155.36 cm^{-1} (the sugar-phosphate band of the

C—O of the ribose-phosphate bond), 1074.35 cm^{-1} (arise from the antisymmetric and symmetric vibrations of the PO_2^- groups), 954.76 cm^{-1} (arise from C–C and C–O vibrations in deoxyribose) and 858.32cm^{-1} (reflect the phosphate-sugar backbone). The present results were expected and have been reported by other studies (Eng *et al,* 2014 and Mao *et al,* 1993). Cyclodextrin molecule has peaks at 858.43 cm^{-1}, 956.69 cm^{-1}, 1066.64 cm^{-1}, 1153.43 cm^{-1}. It is clear that, 954.76cm^{-1} peak from DNA and 1066 cm^{-1} and 956.69 cm^{-1} from cyclodextrin no longer present on samples of 1:3 DNA:CM-β-CD and 1:3:3 DNA: CM-β-CD:Pluronic F-127 (data not shown). However, there is new peak appeared at 947.05cm^{-1} that could be as results of two peaks overlapped and created broader peak, indicating an interaction of DNA with CM-β –CD.

The inclusion of DNA with CM-β-CD and pluronic F-127 at low concentration 1:3 and 1:3:3 showed similar results to higher concentration 1:10 and 1:10:10. This inclusion has supported our result from DNase activity and Florescence. The peak at 954.76cm^{-1} was shifted to 945.12cm^{-1} (see Figure 3); this could be explained by that the sugar molecules of cyclodextrin binding together at higher concentration leaving no site for DNA binding.
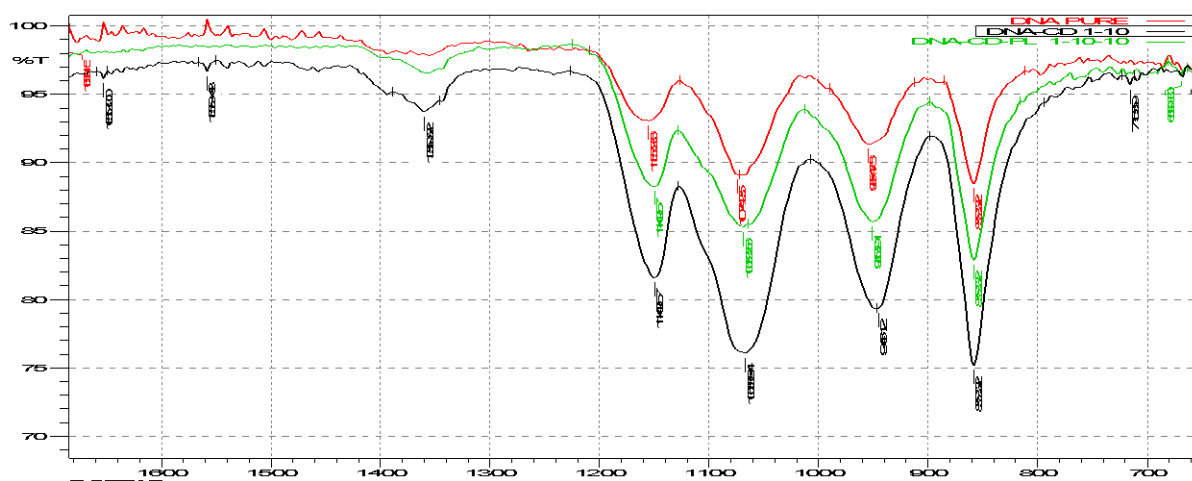

Figure 3: FT-IR graphs for DNA alone (red line), DNA :CD 1:10(green line) and DNA :CD:PL (1:10:10) (black line)

3.5 Scanning Electron Microscopy (SEM)

SEM was used to give information about the sample including external morphology (texture), crystalline structure and orientation of materials making up the sample. Figure 4 shows the SEM image at the same magnification of 10000x. There are changes in the external morphology and shape that can be seen in all formulations with CM-β –CD compared to the morphology of DNA alone. This is an indication that DNA has interacted within CM-β –CD formulations.
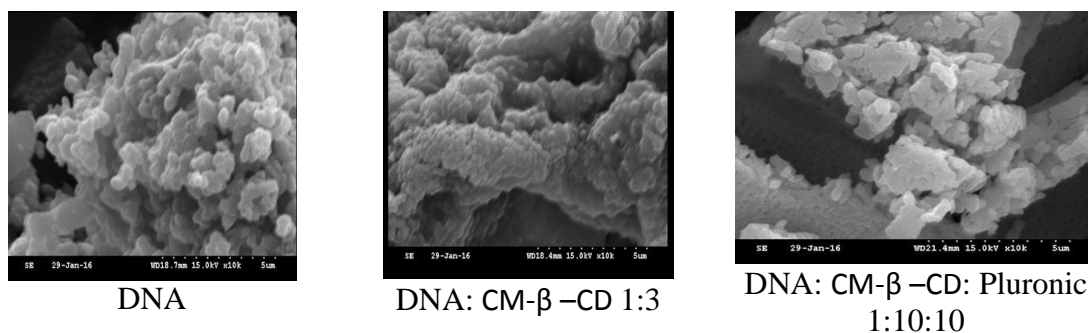
| DNA | DNA: CM-β –CD 1:3 | DNA: CM-β –CD: Pluronic 1:10:10 |

Figure 4: SEM photomicrographs for DNA and DNA: CM-β –CD formulations

3.6 Transfection

The physiochemical property of CM- β –CD and its ability to form complexes of nano-sized with DNA has shown to be the first step to transfection. Before transfection the ability of CM-β-CD to condense DNA was confirmed by gel electrophoresis where CM- β –CD has the ability to condense DNA at weight ratio of 1:3. The enhancement of gene transfection, when liposomes were incorporated with less polar cyclodextrin, could be as result of non-specific interaction with cell membrane ingestion by endocytosis rather than an electrostatic interaction (Kannan et al 2004). Figures 5 and 6 reveal the data for gene transfection containing CM-β-CD.

Following the transfection of COS 7 cell line with both LT1 and CM-β –CD for 48hrs the expression of GFP is showing in Figure 5, the highest level of expression was detected after 48hrs of transfection with CM-β –CD this has shown that transfection with CM-β –CD containing pDNA gave similar or even better results compared to LT1 containing pDNA.

SH-SY5Y neuroblastoma cell is showing to be harder to transfect than COS 7. However, pDNA:CM-β –CD still showing 20.4% transfection (Panel 2) which is higher than LT1 (as a reference) with transfection of 18.2% (Panel 4). CM-β –CD and LT1 both were used to transfect different type of cells COS 7 and SH-SY5Y. CM-β –CD has the ability to form complex with pDNA and it shows higher transfection rate than LT1. Different transfection rate was recorded for different cell lines and higher transfection rate for pDNA:CM-β –CD was persistently confirmed.
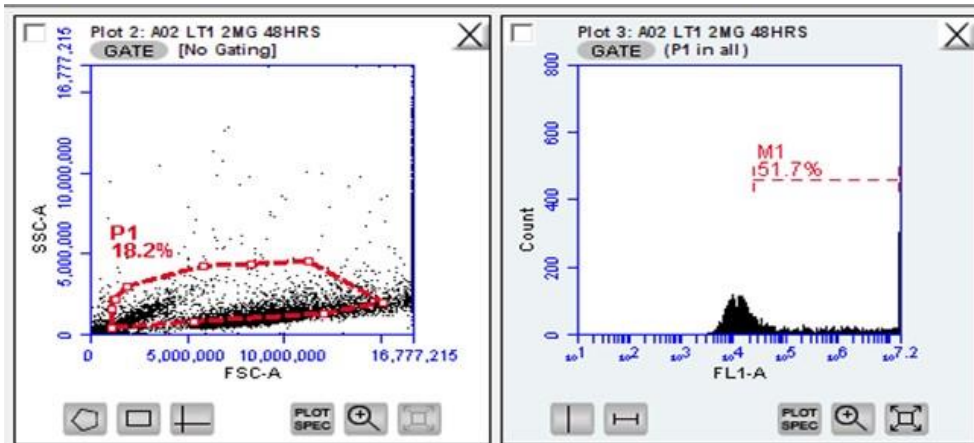
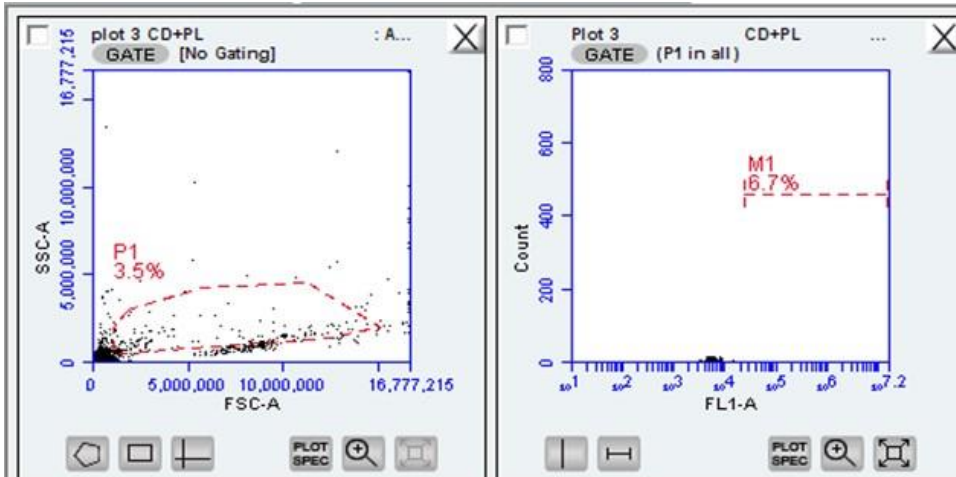Panel 1   Panel 2
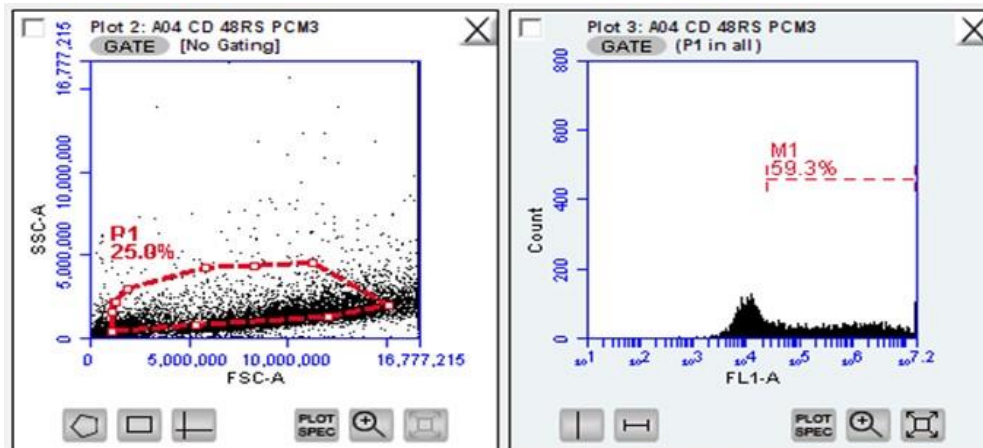
Panel 3   Panel 4
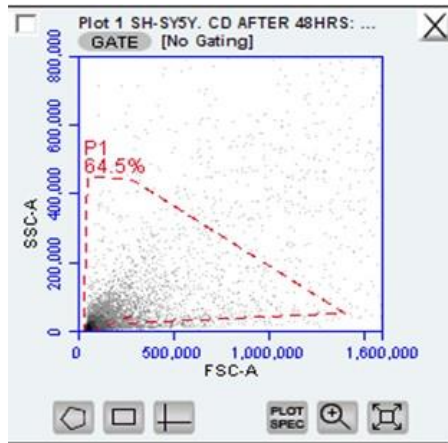

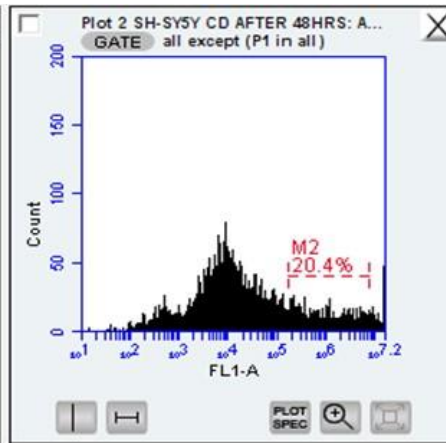Panel 5   Panel 6

Figure 5 shows FCS results of COS 7 when transfected with LT1 (a reference in the form of commercial liposomes) after 48hrs or pDNA:CM-β –CD. LT1 has resulted in 51.7% transfection efficiency (Panel 2) when comparing with pDNA:CM-β –CD (1:3) which resulted on 59.3% transfections (Panel 4). Panels 5 and 6 are for excipients (CM-β-CD and Pluronic F-127).
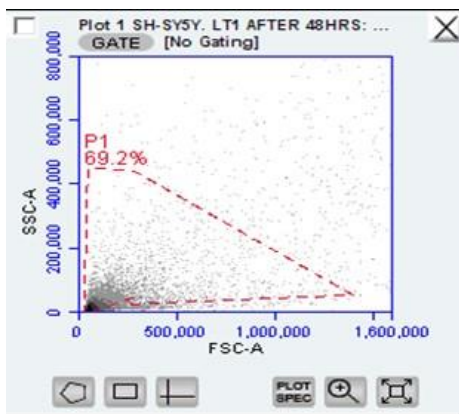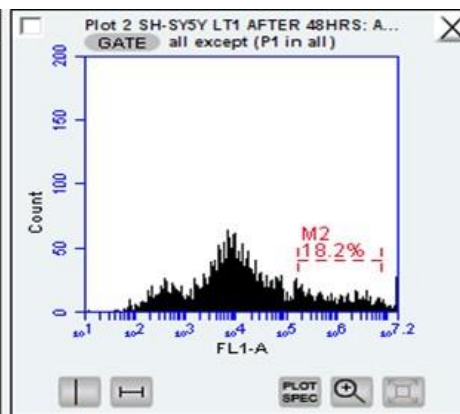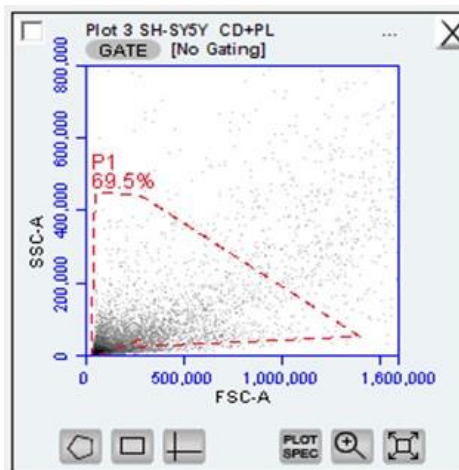
Panel 1

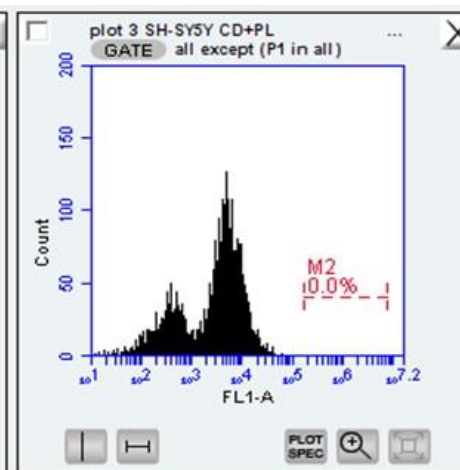Panel 2

Panel 3

Panel 4

Panel 5

Panel 6

Figure 6 shows FCS results of SH-SY5Y neuroblastoma cell when transfected with LT1 (a reference in the form of commercial liposomes) after 48hrs or pDNA:CM-β –CD. pDNA:CM-β –CD (1:3) resulted in 20.4% transfections (Panel 2) compared to 18.2% transfection efficiency (Panel 4) from pDNA/LT1. Panels 5 and 6 are for excipients (CM-β-CD and Pluronic F-127).

4. Conclusion

All formulations DNA: CM-β-CD (1:3), DNA- CM-β-CD (1-10), DNA: CM-β:CD: Pluronic–F127 (1:3:3) and DNA:CD:PL F-127 (1:10:10) have shown to provide protection against DNase degradation. Formulations with lower CM-β-CD and Pluronic F-127 provided more protection than the DNA: CM-β-CD: Pluronic–F127 at high ratio. After freeze-dried all formulations increased their protection to DNA from DNase degradation. DNase results were also supported by gel electrophoresis, where formulations with low DNA: CM-β-CD ratio has provided better inclusion between DNA and CM-β-CD. The Fluorescence measurement for fresh formulations showed the % of inclusion for both DNA:CM- β -CM CD (1:3) and (1:10) 20% and hence there was no benefit from increasing cyclodextrin concentration. The addition of Pluronic F-127 to both formulations resulted in small reduction in the % of inclusion. Freeze dried formulations significantly increase the % of inclusion for both DNA-CM-β –CD (1:3) and DNA- CM-β –CD (1:10) to 44% and 45% respectively. The FT-IR showed that all formulations have resulted in some level of interaction (broadening and shifting of the peaks). The change only occurs at peak 954 cm^{-1}. This peak is a result of the (PO_4^{3-}) group in the sugar-phosphate backbone of DNA. This is a confirmation that CM-β-CD may interact with the anionic group (PO_4^{3-}) in the sugar-phosphate backbone of DNA and hence this was observed in DNA: CM-β –CD (1:3), DNA: CM-β –CD (1:10) and DNA- CM-β –CD: Pluronic–F127 (1:3:3; 1:10:10), which also confirms that only small part of DNA was bind with the cyclodextrin. Freeze dried formulations have shown to be more stable than freshly prepared formulations, this could be as a result of the advantage of converting solutions into solid forms which will prevent the particles from aggregation and degradation. The increase of inclusion after freeze-drying could be a result of favourable bond been formed. These bonds are most likely to be non-covalent in nature such as electrostatic interaction, hydrophobic, hydrogen bonds and Van der Waals, which has also been confirmed by FT-IR data. In conclusion, it was found that cyclodextrin at ratio of DNA: CM-β –CD 1:3   provide a good protection for DNA, with and without Pluronic F-127 and no advantage in increasing cyclodextrin concentration. Transfecting COS 7 and SH-SY5Y neuroblastoma cell lines using CM-β-CD has not just shown to be able to transfect both cell line it also revealed higher transfection efficiency compare to TransIT-LT1 reagent. Results have also confirmed that the addition of Pluronic–F127 resulted in low or no transfection.

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