ENGINEERING FUNCTIONALIZED DNA NANOSPHERES AND THEIR APPLICATIONS

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ABSTRACT

DNA network structures are used as a material with versatile uses and applications. Spherical nanostructure made of DNA, termed the DNA nanosphere, is one type of such DNA network structure, and it has a potential for applications in various areas of biological engineering such as drug delivery and gene therapy. This work presents a study of DNA nanospheres for applications in two different areas of bioengineering that are yet to be explored with DNA nanospheres: cell-free protein expression and dye tracing in biological organisms. For cell-free protein expression, we believed that DNA nanospheres would induce a macromolecular crowding environment around the genes, leading to an increase in the efficiency of transcription and translation and thus the protein yield. To test this idea, we introduced DNA nanosphere as a crowding agent in a coupled cell-free protein expression system and analyzed the subsequent protein yields. Although further study needs to be done, the results show that DNA nanosphere may in fact enhance the protein yields from cell-free expression.

To functionalize DNA nanospheres as dye tracer, we attempted to fabricate dye-encapsulated DNA nanospheres with high structural stability and fluorescence signal. The effects of several engineering conditions such as photoreaction conditions and DNA building block structure were studied in order to find the optimized conditions for fabricating dye-encapsulated DNA nanospheres. It has been found that UV intensity, the presence of poly(ethylene glycol), photoinitiator concentration, and number of dyes on DNA building block monomer play a role in the structural stability of DNA and fluorescence intensity.

BIOGRAPHICAL SKETCH

Ja Eun Lee was born Seoul, South Korea in December, 1986. After graduating from Ehwa elementary school, she moved to the United States and attended Hamilton Middle School in Seattle, Washington. In 2006, she graduated from Portsmouth Abbey School in Rhode Island, and in the fall of the same year, entered Cornell University in Ithaca, New York as an undecided engineering major. During sophomore, she chose to major in biological engineering in the Department of Biological and Environmental Engineering, from which she got her Bachelor of Science degree in Spring, 2011. In January 2012, she is expected to obtain her Master of Engineering degree from the same department.

In Cornell University, she worked as a research assistant in the molecular bioengineering laboratory of Professor Dan Luo between June 2010 and December 2011. Her research focused on engineering and characterization of functionalized DNA nanospheres. Currently, she is expected to enter the Medical School of Catholic University of Korea in Seoul, South Korea in February 2012.

I dedicate this work to my family, who has always supported me with love and wisdom.

You gave me the courage and strength to endure anything that came in my way and to become who I am.

I love you and thank you endlessly.

ACKNOWLEDGMENTS

I would like to acknowledge Professor Dan Luo for his critical and insightful advices during my research experience in his laboratory. His advices fostered in me the direction and dedication for my research. Also, I would like to acknowledge my mentor Dr. Young Hoon Roh, who always gave me help and advices with patience to guide me through my research. His guidance and support are the reason I was able to become a better pursuer and learner of science. Thank you.

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LIST OF ABBREVIATIONS

1. Rluc = *Renilla* Luciferase
2. X-DNA = X-shaped DNA building block
3. RLU = Relative Luminescene Unit
4. CFPE = Cell-Free Protein Expression

1. Introduction

DNA has been extensively studied as the carrier of genetic information passed from generation to generation. However, specific base pair bonding, the unique structural feature of DNA, makes the molecules not only a key material in genetics, but also a material that has a huge potential and use in the world of nanotechnology[1]. Through a manipulation of its base pair sequence, arbitrary and artificial DNA polymeric structures can be formed, and structures of varying structural complexity such as branched DNA[2], dendrimer-like DNA[2], DNA buckyball[3], and DNA box with lid[4] have been constructed. Among these, DNA nanosphere is three-dimensional nanoscale DNA structure with a spherical morphology (Figure 1). This structure is composed of branched DNA building blocks that are polymerized via photoreaction to make a spherical network[5]. It has been reported that DNA nanospheres can be used as the drug-delivering vehicle for cells[5], but there may be many more potential areas for which these network structures can be used.

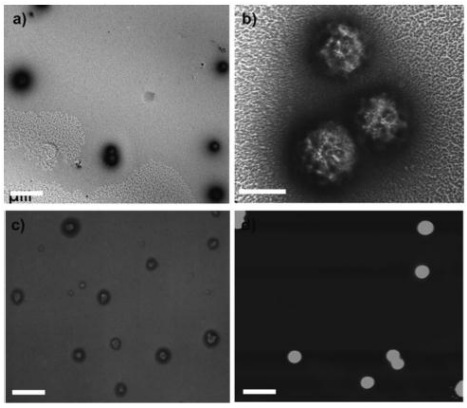


Figure 1. Microscope images of DNA nanosphere showing its spherical morphology [5]
(image reproduced from the work of Roh et al, 2010)
(a) & (b): TEM image of DNA nanospheres (c) SEM image of DNA nanospheres (d) AFM image of DNA nanospheres. Scale bar: (a), (c), (d): 1μm (b): 200nm

We postulated that one such area would be cell-free protein expression (CFPE). Previously, several attempts have been made to increase the efficiency of protein synthesis in

cell-free protein expression. One approach was using synthetic molecules to induce the macromolecular crowding in the expression environment[6]. In cells, the presence of crowding molecules reduces the diffusion of biomolecules and affects their kinetics and thermodynamic activity[7]; the introduction of macromolecular crowding in CFPE is thus an approach to increase the protein expression efficiency by imitating the cellular environment, the natural factory of protein production. Utilizing this concept, we decided to test the effect of DNA nanospheres as a novel, DNA-based crowding agent in CFPE. In addition, we decided to add another feature to our system—gene promixity—a unique characteristic of the protein-producing gel (P-gel) system; in this approach, the gel scaffold is composed of genes linked with DNA building blocks, achieving high expression yield through gene proximity and high gene local concentration[8]. In order to achieve such gene proximity, we decided to change the gene shape into a linkage of a mass number of genes, which we termed as the monster gene.

The second area studied for the application of DNA nanosphere was dye tracing in biological organisms. By functionalizing DNA nanospheres with fluorescent dyes, DNA nanosphere with dye encapsulation can be synthesized, which can be used for animal and cellular studies that require dye tracers. However, engineering such structure is not a simple task, as several qualities need to be achieved in concert—fluorescence intensity, photopolymerization efficiency, and stability of DNA material—in order to create dye-encapsulated DNA nanosphere applicable for animal or cellular studies. Therefore, to this end, optimization of the engineering process of dye-encapsulated DNA nanospheres was conducted. In particular, the photoreaction conditions, DNA building block structure, and the number of dyes on DNA building block were studied to see how they attribute to the aforementioned three qualities of dye-encapsulated DNA nanospheres.

2. Methods

2.1 Cell-Free Protein Expression with Monster Gene and Crowding Agent

Crowding Agent Synthesis

Two types of crowding agents made of DNA were used. The first was DNA building block with four branched arms: X-DNA. These building blocks were synthesized from four single stranded DNA oligonucleotides whose base sequences were designed so that the oligonucleotides assemble into X-DNA upon hybridization (see Appendix for base sequence). All oligonucleotides used in this and other experiments discussed in this report were commercially synthesized and desalted (Integrated DNA Technologies, Coralville, Iowa).

The other type of crowding agent, DNA nanosphere, was synthesized from polymerizing photocrosslinkable X-DNA (PEGA X-DNA) with UV light. First, single stranded oligonucleotides with amine groups were conjugated with PEGA (poly(ethylene glycol) acrylate) (Nektar, Huntsville, Alabama) by mixing the two and incubating the mixture at room temperature overnight. The sequences for the oligonucleotides can be found in Appendix. Unreacted products were removed from the PEGA-oligonucleotide reaction batch via high performance liquid chromatography (HPLC). Xbridge C18 column with photodiode array detector to detect UV at 260nm wavelength was used for this process (Waters Corp., Milford, Massachusetts)[5]. After purification, the four PEGA-conjugated oligonucleotides were mixed in equimolar ratio and hybridized to make PEGA X-DNA: X-DNA with four photoreactive groups. To make DNA nanosphere, PEGA X-DNA were mixed with 1-[4-(2-hydroxyethoxy)phenyl]-2-hydroxy-2-phenylpropan-1-one (Ciba Specialty Chemicals, Tarrytown, New York)—molecules that initiate the photopolymerization reaction—to the final concentration of 0.05% (w/v). The PEGA X-DNA and initiator mixture was put in 0.5mL microcentrifuge tube, and photoreaction

was conducted for 10 minutes under the UV intensity of 6.5mV/cm^2 in XL-1000 UV crosslinker (Spectronic Corp., Westbury, New York). The phoreacted sample was tested for the presence of DNA nanospheres by agarose gel electrophoresis at 90V for 60 minutes with 3% agarose gel. After the presence was confirmed, the sample was used for protein expression without further processing.

Monster Gene Synthesis

Monster gene was synthesized from linking a mass number of individual linear *Renilla* luciferase (Rluc) plasmids. pIVEX1.3 Rluc plasmids were cut with ApaI restriction enzyme, producing linear plasmids with palindromic sticky ends on each side (provided by Edward Rice in Prof. Dan Luo's lab). Differential amounts (100ng, 200ng, 400ng, 600ng, 800ng) of linearized genes were mixed with T4 DNA ligase (Promega, Madison, Wisconsin) in order to promote ligation among the genes, and the ligased product was used as the monster gene.

Procedures for Cell-Free Protein Expression

For CFPE, RTS 100 continuous-exchange cell-free protein expression platform was used, which was based on wheat germ lysate (5 Prime Inc., Gaithersburg, Maryland). For monster gene experiment, each of the five amounts of monster gene (100ng, 200ng, 400ng, 600ng, 800ng) was mixed with nuclease-free water to total volume of 15µl. As a control, linear gene of 400ng was prepared in the same way.

For crowding agent experiments, one type of gene shape (linear or monster gene) and one type of crowding agent (X-DNA or DNA nanosphere) were mixed together in nuclease-free water to total volume of 15µl. Four different types of gene and crowding agent mixture were thus

made: 1. linear gene + X-DNA, 2. linear gene + DNA nanosphere, 3. monster gene + X-DNA, 4. monster gene + DNA nanosphere. Differential amounts of crowding agents were added to genes; for X-DNA, 3, 10, 30 50, 70, 90 μg of X-DNA (corresponding to 0.20, 0.67, 2.0, 3.33, 4.67, 6.00 μg/μl) were added to 400ng of either linear or monster gene. For DNA nanosphere, 0.20, 0.48, 1.2, 3, 7.5 μg (corresponding to 0.013, 0.032, 0.08, 0.20, 0.50 μg/μl) of DNA nanosphere were added to 400ng of either linear or monster gene.

For all expressions, the gene and crowding agent mixture batches (all 15μl) were mixed with protein expression reagents (lysate, reaction mix, feeding mix, amino acids, methionine, reconstitution buffer) according to the RTS 100 CFPE kit manual and then incubated for 24 hours at temperature of 24℃ and shaking speed of 900 rpm in Proteomaster (Roche, Basel, Switzerland). The completed expression products were stored at -20℃ unless used for assays.

Quantification of Functional Rluc Yield

Functional Rluc was defined as luciferases that catalyze the oxidative reaction of luciferin substrate to produce blue light[8]. In order to measure the concentration of functional Rluc produced from CFPE, luciferase assay kit from Promega (Catalog #: E1500) was used. Each expression sample was diluted 10^6 times, and 5 μl of the diluted sample was taken for luminescence reading by luminometer (Promega, Catalog #: E5331). The measurements from the luminometer were reported in relative luminescence unit (RLU). Calibration curve using standard concentrations of commercial Rluc (Prolume Ltd., Pinetop, Arizona) had been previously made, and all luminescence readings from the expression samples were converted to weight concentrations using the conversion factor of $7.5*10^{13}$ RLU/mg Rluc produced from the calibration curve previously made[8].

Comparison of Total R. Luciferase Yield

For analysis of total Rluc yield, western blot was used. For primary antibody, monoclonal anti-Rluc antibody from mouse was used to detect the Rluc (Millipore, Billerica, Massachusetts, Catalog #: MAB 4400). For secondary antibody, anti-mouse antibody from goat was used (Millipore, Catalog #: AP124A) that is conjugated with alkaline phosphatase. Proteins in the expression samples were first separated using SDS PAGE and then transferred to PVDF membranes. Membranes with transferred proteins were blocked with 5% skim milk (w/v) TBS blocking solution for one hour. Then, membrane was immersed in 5% skim milk blocking solution mixed with primary antibody and was incubated at 4℃ overnight. Secondary antibody was added and incubated for 1.5 hours at room temperature. Afterwards, Western Blue stabilized substrate for alkaline phosphatase (Promega, Catalog #S3841) was used to develop the color for Rluc protein bands. The band intensities of different samples were compared using ImageJ (U.S. National Institute of Health, Bethesda, Maryland).

2.2 Dye-Encapsulated DNA Nanospheres

DNA Building Block Synthesis

Two types of X-DNA building block were synthesized for the purpose of fabricating dye-encapsulated DNA nanospheres. First, X-DNA with four photocrosslinkable arms (PEGA X-DNA) was synthesized using the method described in the previous section. Another type of X-DNA was synthesized from oligonucleotides conjugated with either photoreactive group or fluorescent dye (FITC (green dye) or Alexa CY5 (red dye)). In order to make X-DNA with one dye, three oligonucleotides with photoreactive group and one oligonucleotide with dye were

hybridized, and to make X-DNA with two dyes, two oligonucleotides with photoreactive group and two oligonucleotides with dye were hybridized. For convenience, X-DNA with no dye conjugation will be referred to as non-dyeX and X-DNA with dye will be referred to as dyeX in the discussion body.

DNA Nanosphere Synthesis

The two types of X-DNA (dyeX and non-dyeX) were mixed in varying ratio (10:0, 7.5:2.5, 5:5, 2.5:7.5, 0:10) and then mixed with photoinitiator, and the mixture batches were exposed to UV light for 10 minutes in XL-100 UV crosslinker. The intensity of UV light and final concentrations of photoinitiator were varied during the experiments to find the optimized conditions. PEGA X-DNA used in this procedure was made by the method described in previous section (section 2.1).

Detection of Fluorescence in DNA Nanosphere

For FITC-encapsulated DNA nanospheres, fluorescence microscopy was used to observe the presence and fluorescence of the DNA nanospheres. 5µl of sample was dropped onto the microscope glass and covered with cover glass, both of which were cleaned with nitrogen gas. The images were observed with Olympus IX70 fluorescence microscope (Olympus America Inc., Center Valley, Pennsylvania) under the 100X objective. Images were acquired with MetaMorph microscopy image analysis software (Molecular Devices, Inc., Sunnyvale, California). For Alexa CY5-encapsulated DNA nanospheres, spectrophotometer (Nanodrop 1000, Wilmington, Delaware) was used to measure the fluorescence present in 2µl of photoreacted samples. As controls, fluorescence from 2µl of the same mixture batch of X-DNA before

photoreaction was also measured. After the absorbance curve was obtained, the area under the curve with the peak at 650nm (wavelength) was calculated by dividing the area into several trapezoids and summing up the individual trapezoidal areas using Microsoft Excel software (Microsoft Corp., Redmond, Washington). The area under the curve from photoreacted samples were divided by the area under the curve from non-photoreacted samples, which was subtracted from 1 and multiplied by 100 to obtain the percentage loss of fluorescence for dye-encapsulated DNA nanospheres.

3. Results and Discussion

3.1 Cell-Free Protein Expression with Monster Gene and DNA Crowding Agent

Because the linear Rluc genes had palindromic sticky ends on each side, ligating the genes produced monster genes that could be of various shapes. Figure 2 shows possible shapes of monster genes resulting from ligation of linear genes.

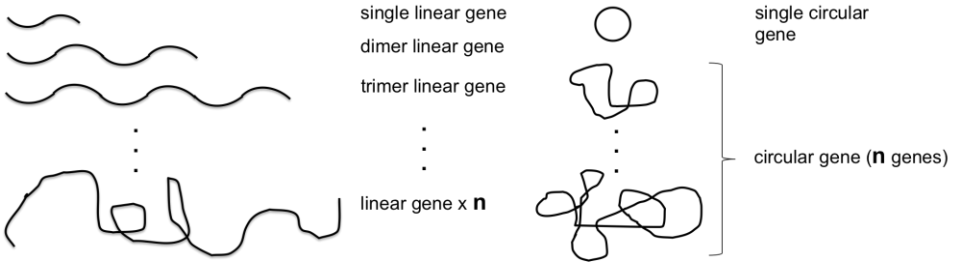


Figure 2. Possible Shapes of Monster Gene

Although Figure 2 shows various possible shapes of monster gene, a detailed characterization of monster genes must be done in order to reveal the structural nature of these genes, which may be accomplished through gel electrophoresis and/or microscopy.

When luciferase assay was conducted to measure the luminescence from proteins expressed from monster gene, the functional Rluc yields from these monster genes increased with increasing amounts of monster genes from 100ng to 800ng (Figure 3). In Figure 3, Each Rluc concentration is the average of five measurements taken from the same expression sample. For 100, 200, 400, 600, 800ng of monster genes expressed, functional Rluc of 0.012, 0.025, 0.30, 0.73, 1.08 mg/ml was produced, respectively. On the other hand, expression of 400 ng linear gene produced 0.021 mg/ml of Rluc, which was closest to the yield from using 200 ng of monster gene. From the expression of the same amount of genes (400ng), linear genes produced only 0.021 mg/ml of functional protein, whereas monster genes produced up to 0.30 mg/ml of

functional protein. These results suggest that monster gene may be a gene shape leading to more efficient protein expression; replications of this experiment should reveal of the effects seen in this experiment are true.

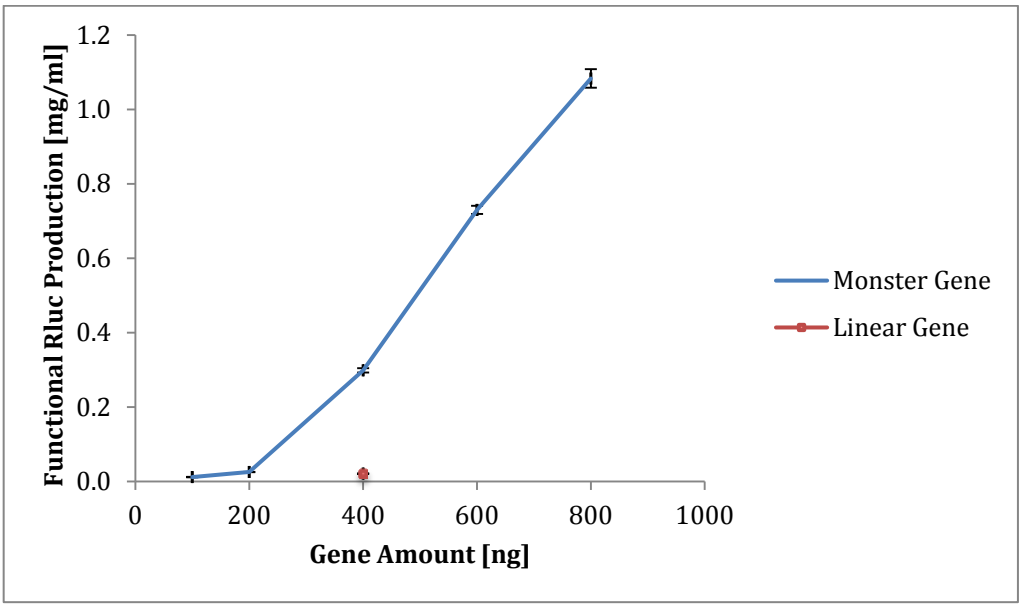


Figure 3. Functional Rluc Yield from Two Types of Gene Shape

When free-floating X-DNA was used as crowding agent for linear genes, the functional protein yield ranged from 0.021 mg/ml to 0.63 mg/ml, while for monster genes, the yield ranged from 0.30 mg/ml to 1.08 mg/ml (Figure 4). Each data point in Figure 4 is an average of five measurements from the same sample and error bars indicate standard deviation based on the five measurements. For both types of gene shape, the yield initially increased with X-DNA concentration but fell steeply after reaching its peak. For both linear and monster gene, the peak yields were reached at 0.67 μg/μl of X-DNA.

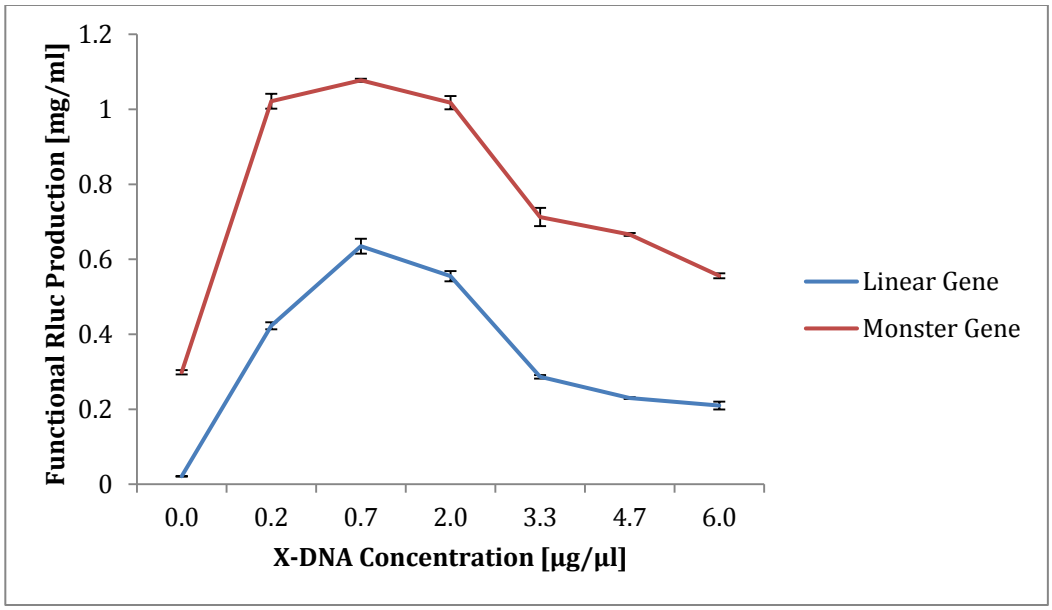
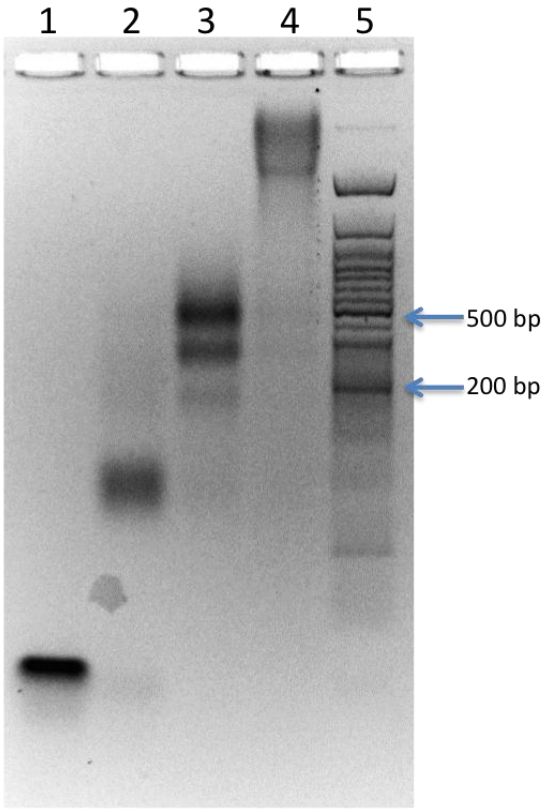


Figure 4. Functional Rluc Yield Produced with X-DNA as Crowding Agent

In order to use DNA nanosphere as another crowding agent, the presence of DNA nanosphere from photoreaction of PEGA X-DNA was first confirmed via agarose gel electrophoresis (Figure 5). In Figure 5, lane 4 is a photoreacted product of PEGA X-DNA (lane 3). The change in the band position shows that the electrophoretic mobility of the sample has significantly shifted from that of PEGA X-DNA, confirming the synthesis of DNA nanospheres.

Using DNA nanospheres as crowding agent produced a yield trend similar to that from using free-floating X-DNA; the yield initially increased then decreased as the concentration of DNA nanosphere increased (Figure 6). The peak yields were 0.33 mg/ml and 0.81 mg/ml for linear and monster genes, respectively. Unlike X-DNA, the concentrations of crowding agent at which the peak yields were reached were different for linear gene (0.20 µg/µl) and monster genes (0.013 µg/µl).



Lane 1: single stranded oligonucleotide
Lane 2: single stranded oligonucleotide with PEGA
Lane 3: PEGA-X-DNA
Lane 4: Photoreated PEGA-X-DNA (DNA nanosphere)
Lane 5: 50 bp DNA ladder

Figure 5. Agarose Gel Electrophoresis Image of PEGA X-DNA and DNA nanosphere
3% agarose, 90V, 60min
Image Provided by Jae Hyon Park

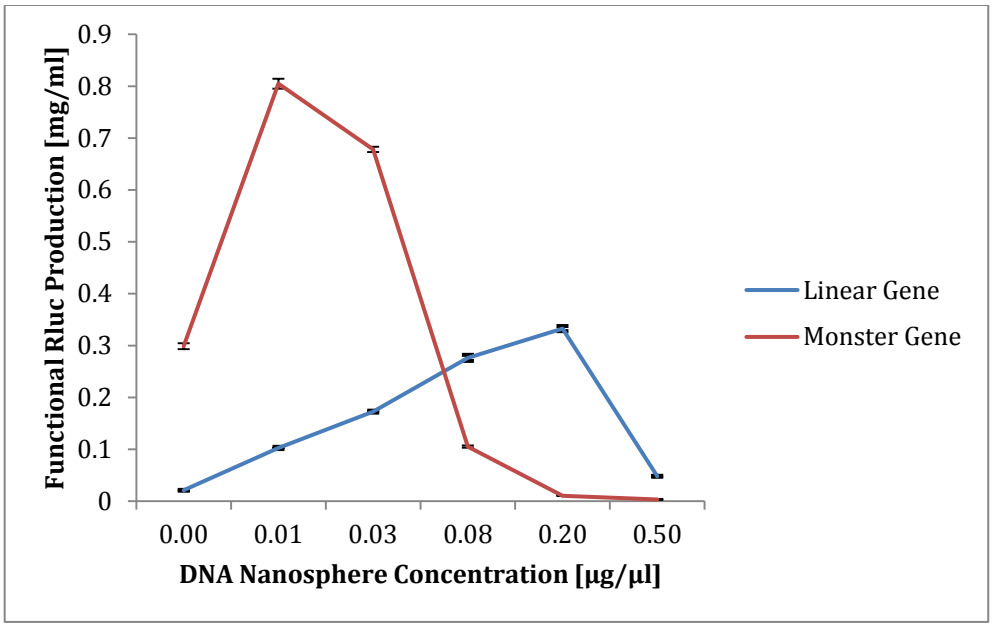


Figure 6. Functional Rluc Yield Produced with DNA Nanosphere as Crowding Agent

The results of functional assay suggest that having DNA crowding agents may increase the protein yield produced from CFCE. More importantly, it seems possible that DNA nanospheres produce a Rluc yield comparable to that from X-DNA by using a far less DNA material; whereas X-DNA achieved 1.08 mg/ml of functional Rluc expression at 0.67 µg/µl, DNA nanospheres produced 0.81 mg/ml of functional Rluc at 0.013 µg/µl DNA nanosphere. Although the yield difference is only 0.27 mg/ml, the amount of DNA required for DNA nanosphere was 52 times less than that for X-DNA. This suggests that DNA nanosphere is potentially a more efficient and economical crowding agent than X-DNA. However, it should be noted that like the monster gene experiment, replication of these functional assays must be carried out in order to confirm the effect of DNA nanosphere and/or X-DNA as truly yield-increasing factors. Further, protein yields at more detailed range of X-DNA and DNA nanosphere may to be done in order to find if higher protein yields are possible.

The total Rluc yield was also analyzed using western blot. On western blot, expressions from linear gene, monster gene, linear gene & DNA nanosphere, and monster gene & DNA

nanosphere were compared to see both the individual and combined effect of gene shape and DNA nanosphere-induced crowding. (Figure 7).

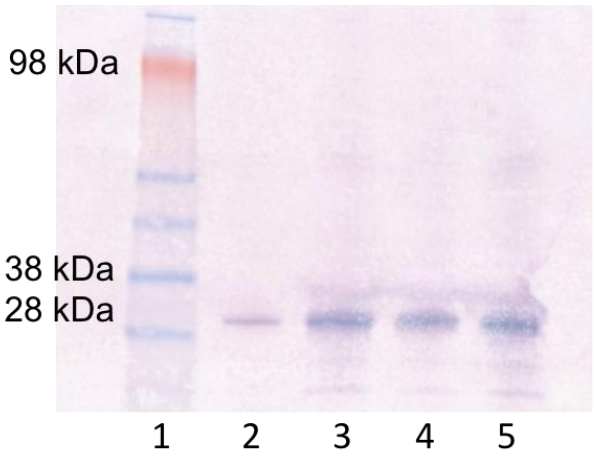


Figure 7. Western Blot Image of Rluc Expressions
Lane 1: SeeBlue® Plus2 Pre-Stained Protein Standard (Invitrogen, Carlsbad, CA)
Lane 2: Rluc produced from using 400ng of linear gene
Lane 3: Rluc produced from using 400ng of monster gene
Lane 4: Rluc produced from using 400ng of linear gene & 0.032 µg/µl DNA nanosphere
Lane 5: Rluc produced from using 400ng of monster gene & 0.032 µg/µl DNA nanosphere

Analysis of band intensities from ImageJ was converted to relative total Rluc yields in comparison to the yield from expressing 400ng of linear gene (Table 1). 400ng of linear gene was chosen as the comparison point because it did not have any manipulations in either gene shape or crowding environment, serving as the "purest" form of protein expression template. Here, the initial intention was to include Rluc standard with known concentration, which would have made quantification of absolute total Rluc yield possible; however, it could not be accomplished due to a problematic commercial Rluc purchased.

Table 1. Relative Total Rluc Yields from Various Protein Expression Template

Type & Amount of Gene	Type & Amount of Crowding Agent	Relative Total Rluc Yield
Linear gene 400ng	-	1
Monster gene 400ng	-	8.9
Linear gene 400ng	DNA nanosphere 0.032 µg/µl	5.7
Monster gene 400ng	DNA nanosphere 0.032 µg/µl	10.3

In comparison to linear gene, monster gene of the same amount produced 8.9 times more total Rluc. Having 0.032 μg/μl of DNA nanosphere as crowding agent produced about 5.7 times more Rluc than just linear gene. However, when the presence of monster gene and DNA nanosphere were combined, the yield was 10.3 times more than that from linear gene, which was highest among the four samples. It should be noted that there is still a need for the quantification of total Rluc yields produced from the various expression templates, and this should and can be accomplished by including a standard Rluc in the western blot procedure.

The analyses of Rluc yields suggest that monster gene and DNA crowding agents may in fact increase both the functional and total protein produced from CFPE. However, in order to confirm that the effects are real, replication of these experiments and reproduction of similar results are imperative. Then, if it is found that the effects of monster gene and DNA crowding agent are true, their mechanisms will need to be determined by further experiments. Here, it is hypothesized that gene proximity provided by monster gene creates a system where RNA polymerases work with "higher turnover rate" by being able to move from one gene to another faster. Also, it can be hypothesized that if DNA crowding agents do in fact induce more efficient protein expression, it is because they work as excellent mimicry of biomolecular crowding present in the *in vivo* cell environment. Specifically, the presence of X-DNA and DNA nanosphere may be providing a hindrance against the free, diffusive movement of genes and enzymes in the system, forcing more frequent contacts between these molecules and a higher rate of transcription and/or translation. Also, if DNA nanosphere is revealed as more efficient DNA crowding agent than free-floating X-DNA, it may be that the spherical, aggregated morphology of DNA nanosphere induce the contacts between genes and polymerases more efficiently than X-DNA. The fact that the effect of DNA nanosphere was more prominent with monster gene than

with linear gene in these preliminary experiments bolsters this hypothesis. Since the length and size of monster gene are probably far greater than those of linear gene, the diffusion of monster gene through DNA nanospheres may be more arrested because monster genes can be "tangled" around the DNA nanospheres due to their length.

3.2 Dye-Encapsulated DNA Nanosphere

Initially, dye-encapsulated DNA nanospheres were fabricated from 10 μM of X-DNA without the conjugation to PEG. The photoreactions conditions were 10 mV/cm^2 UV intensity, 0.05% photoinitiator, and 10 minutes of reaction. Here, the dye used was FITC. Two types of X-DNA—non-dyeX and dyeX—were mixed in varied molar ratio for two reasons; first, to see its effect on polymerization efficiency and second, to find a condition that produces the best fluorescence intensity. Here, green dye was used as part of the preliminary optimization process of dye-encapsulated DNA nanosphere fabrication.

DIC microscope images of the DNA nanospheres (Figure 8) show that DNA nanospheres formed as a result of photoreaction of dyeX and non-dyeX. However, in corresponding fluorescence images (Figure 8), it is seen that not all of the DNA nanospheres possess the green fluorescence. It is seen from the fluorescent images that non-dyeX:dyeX ratio of 2.5:7:5 yields the highest number of dye-encapsulated DNA nanospheres. However, it was later found that even DNA nanosphere produced from this condition had a fluorescence signal that was not strong enough to be used in animal injection study.

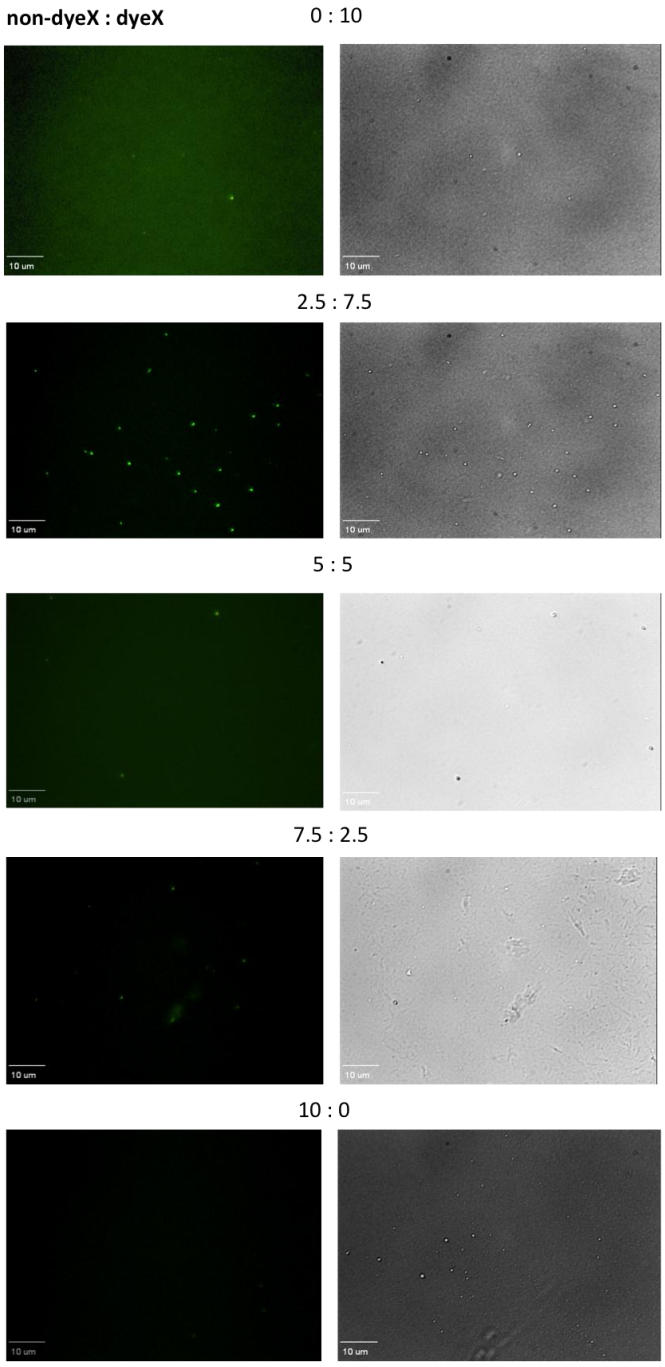


Figure 8. DIC and Fluorescent Microscope Images of FITC-Encapsulated DNA Nanospheres
(Magnification: 100X, Scale Bar: 10μm)
captions above each row of images indicate the molar ratio of non-dyeX to dyeX
Image Provided by Jae Hyon Park

During the fabrication process of DNA nanospheres, it was found that there is a significant amount of DNA damage resulting from photoreaction, as free radicals released from photoinitiator molecules upon UV exposure attack and break down the DNA backbone and base pair bonding[9,10]. Hence, experiments were carried out to optimize the parameters involved in photopolymerization of DNA building block to minimize the loss of DNA. As a result, it was found that the phopolymerization conditions can be manipulated to achieve reduced DNA loss with uncompromised polymerization efficiency; for example, the presence of PEG conjugated to X-DNA works as a protectant of DNA building block against the damage occurred by radicals during photoreaction. Initially, the damage was up to 90% in 10 μM of photoreacted X-DNA without PEG. With PEG, the damage was reduced to 40% for 5 μM of X-DNA. Also, it was found that higher UV intensity and initiator concentration increase the DNA loss. Finally, it was determined that when 5 μM of PEGA X-DNA, 0.05% initiator, 6.5-8 mV/cm^2 and 10 minutes of reaction time are used, DNA nanosphere were successfully formed without significant DNA loss[9].

After the conditions for photoreaction have been optimized to minimize the DNA loss from UV exposure, the newly set conditions were applied to the photopolymerization of dye-encapsulated DNA nanospheres; specifically, PEG was added as a protectant for X-DNA, and the UV light intensity was reduced from $10mV/cm^2$ to $6.5mV/cm^2$. Also, the DNA concentration was reduced to 5μM. Based on these conditions, dye-encapsulated DNA nanospheres were again attempted to be made. At this stage, the fluorescence intensity of DNA nanosphere was the issue at interest; therefore, in order to see the fluorescence intensity of photoreacted dyeX, dyeX

conjugated with Alexa CY5 were photoreacted based on the new conditions and its fluorescence intensity was measured with spectrophotometer (Figure 9).

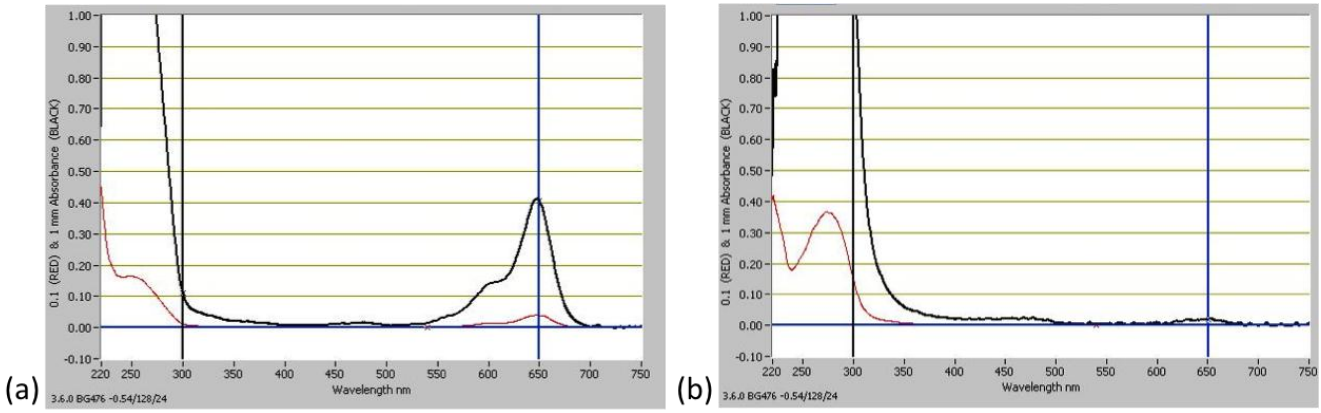


Figure 9. Spectrophotometer Results of Fluorescence Detection from Alexa CY5-conjugated X-DNA

Initiator = 0.05%, # Dye/X-DNA=1
(a) before photoreaction (10 minutes)
(b) after photoreaction (10 minutes)

The results showed that the percent loss of fluorescence from dyeX after photoreaction was 93.8%. The significant loss of fluorescence detected by spectrophotometer indicates that fluorescent molecules were destroyed during the UV exposure; otherwise, spectrophotometer would detect the signals from fluorescence molecules regardless of whether they are free or encapsulated into DNA nanosphere. This indicates the dyes became nonfunctional, probably because their structures have been disturbed. Hence, even if the problem of DNA being damaged from photoreaction was resolved, the protection of fluorescence intensity remained as a critical difficulty in the fabrication of dye-encapsulated DNA nanospheres.

In order to solve this issue, several other parameters were changed. First, the photoinitiator concentration was reduced to 0.03% (w/v). Next, the number of dye molecules was increased to two per dyeX. Figure 10 shows the spectrophotometer results of dye-encapsulated

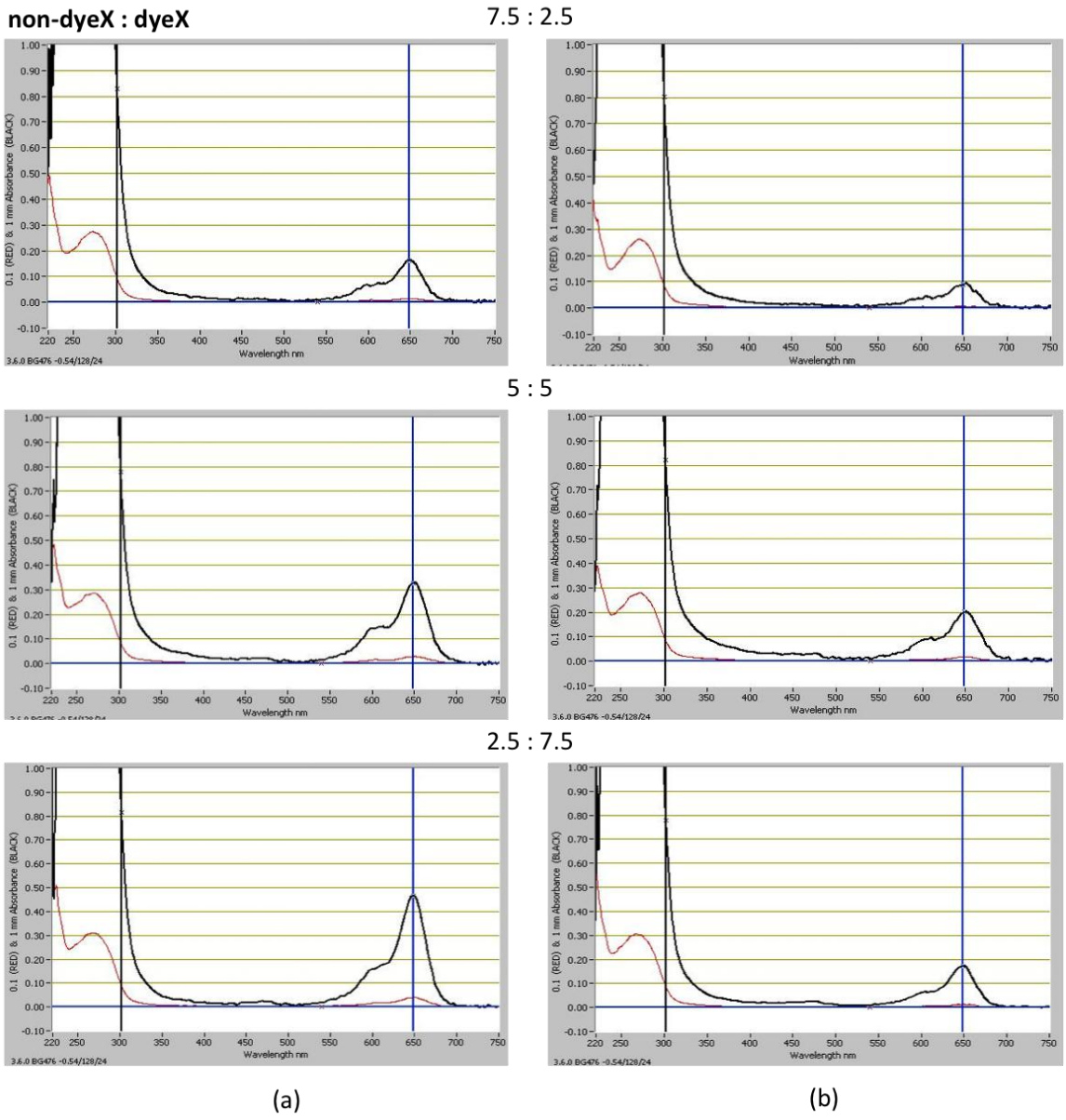


Figure 10. Spectrophotometer Results of Fluorescence Detection from Alexa CY5-conjugated X-DNA and non-dye X-DNA

I = 0.03%, # Dye/X-DNA=2
(a) before photoreaction (10 minutes)
(b) after photoreaction (10 minutes)

DNA nanosphere based on these changes for different non-dyeX and dyeX ratio. The florescence losses for each molar ratio as determined from the absorbance graphs are shown in Table 2.

Table 2. Percent Loss of Fluorescence after Photoreaction of non-dyeX and Alexa CY5-conjugated X-DNA

Ratio of DyeX : nonDyeX	% Loss of Fluorescence after Photoreaction
7.5:2.5	68.1
5:5	22.0
2.5:7.5	35.6

The loss of fluorescence was 22.0% when the molar ratio of dyeX to non-dye-X was 5:5, which was the smallest loss achieved in the dye-encaspulated DNA nanosphere experiments. On either side of the ratio range, the loss of fluorescence increased, but when the amount of dyeX was more than that of non-dyeX, the fluorescence loss increased more steeply (68.1%) compared to when the amount of dyeX was less than that of non-dyeX (35.6%), indicating that presence of non-dyeX (PEGA X-DNA) may be playing a role in protecting the fluorescent molecules during UV exposure. However, in order to have a better idea on the trend of fluorescence loss, more ratios (10:0 and 0:10) will have to be studied. Also, the effects of lower initiator concentration and higher number of dyes on dyeX need to be separately studied.

The spectrophotometer results show that optimization of photoreaction conditions (lower UV intensity, presence of PEG) to reduce the loss of DNA during DNA nanospheres synthesis is not enough when it comes to fabricating DNA nanospheres functionalized with dyes. In addition to those parameters, other factors need to be optimized in order to retain the necessary fluorescence intensity during photoreaction; lowering the initiator concentration and increasing the dye number per X-DNA are promising approaches to fabricating DNA nanosphere with sufficient dye encapsulation based on these experimental results.

Although the fluorescence detection results from spectrophotometer indicate that the fluorescence loss can be reduced, it remains to be studied how these conditions affect the efficiency of photopolymerization. Increasing the number of dye on dyeX leads to a smaller number of photoreactive groups present on dyeX, and it is expected that the efficiency of photopolymerization subsequently decrease. A further study on the characterization of DNA nanospheres formed from dyeX with only two crosslinkable arms will need to be performed in the future, and this can be done by observing the morphology of dye-encapsulated DNA nanospheres with various microscopes such as fluorescenc microscopy, transmission electron micropy, and atomic force microscopy.

Lastly, a study of the mechanism of fluorescence loss from photoreaction would enhance the understanding behind the synthesis of dye-encapsulated DNA nanospheres. It is not yet known how the fluorescent molecules lose their function during photoreaction, and it remains as an area to be further studied during the optimization process of dye-encapsulated DNA nanosphere fabrication.

4. Conclusion

In CFPE, DNA nanospheres, along with free-floating X-DNA, may work as effective crowding agents that increase the protein yields, and this may be because these DNA crowding agents create an environment that more closely represents the real intracellular condition, which is populated with bulky biomolecules. However, the effects of the two agents are different and DNA nanosphere may be a more powerful inducer of crowding effect than X-DNA. Also, changing the gene shape into mass linkage of genes—the monster gene—may also increase the efficiency of protein expression via gene proximity. A detailed study of the effects of monster gene and DNA nanosphere-induced crowding can potentially lead to establishment of a novel CFPE technique that is based purely on manipulation of DNA as material.

In addition to being a possible enhancer of protein yields in CFPE, DNA nanospheres can also be used as dye tracer for animal and cellular studies. Engineering dye-encapsulated DNA nanospheres requires considerations of three qualities: DNA stability, fluorescence intensity, and photopolymerization efficiency. While DNA stability can be achieved from optimized photoreaction conditions and conjugation of PEG to DNA building block, high fluorescence intensity can be achieved by reducing the phoinitiator concentration and/or incorporating more than one dye molecule per DNA building block. A thorough study of the effects of these two parameters as well as other possible factors will lead the way to finding the optimum condition for dye-encapsulated DNA nanospheres fabrication. Such dye-encapsulated DNA nanosphere will have structural stability and fluorescence intensity that are appropriate for animal or cellular studies.

APPENDIX

Oligonucleotide sequences to make X-DNA

Only strand X1 had a sequence for sticky end part (highlighted in red) and was phosphorylated at 5' end. However, the sticky end played no functional role in the protein expression experiments. The rest of the strands had no sticky end part. The base sequences are a slight modification from the base sequence of strands used to make X-DNA in protein-producing gel[8].

Table 3. Base sequence of singled stranded DNA oligonucleotides used to make X-DNA

Strand	Base Sequence
X1	5'-p-GGCCCGACCGATGAATAGCGGTCAGATCCGTACCTACTCG-3'
X2	5'-CGAGTAGGTACGGATCTGCGTATTGCGAACGACTCG-3'
X3	5'-CGAGTCGTTCGCAATACGGCTGTACGTATGGTCTCG-3'
X4	5'-CGAGACCATACGTACAGCACCGCTATTCATCGGTCG-3'

Oligonucleotide sequences for PEGA X-DNA

All strands had an amine group attached at 5' end for conjugation of PEGA[5].

Table 4. Base sequence of singled stranded DNA oligonucleotides used to make PEGA X-DNA

Strand	Base Sequence
X1	5'-NH_2- CGACCGATGAATAGCGGTCAGATCCGTACCTACTCG-3'
X2	5'-NH_2- CGAGTAGGTACGGATCTGCGTATTGCGAACGACTCG-3'
X3	5'-NH_2- CGAGTCGTTCGCAATACGGCTGTACGTATGGTCTCG-3'
X4	5'-NH_2- CGAGACCATACGTACAGCACCGCTATTCATCGGTCG-3'

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