The Thai Journal of Pharmaceutical Sciences

Volume 38
Issue 0 *supplement issue*

Article 38

1-1-2013

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Samarwadee Plianwong.

Praneet Opanasopit

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Samarwadee Plianwong[1], Praneet Opanasopit[1], Tanasait Ngawhirunpat [1] and Theerasak Rojanarata[1]*

[1]*Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand*
**Corresponding author: teerasak@su.ac.th, rtheerasak@yahoo.com*

KEYWORDS: chitosan, chitosan pyridine, dichlorofluorescein, weight ratio, polyplex

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INTRODUCTION

Gene therapy is currently a promising approach to the treatment of diseases. It is carried out by transferring genetic materials to target cells in order to compensate for defective genes or produce therapeutic proteins. For successful delivery and expression of exogenous genes, suitable types and amounts of vectors are usually required[1)]. Chitosan (CS) and its derivative ex. chitosan pyridine (CS-Py) are commonly used as cationic polymeric carriers for gene delivery[2)]. Once the amino groups are protonated, CS and CS-Py binds and condenses negatively charged DNA into smaller particles. The optimal cationic polymers to DNA ratio has a significant correlation to the complete formation of polyplexes and the resulting transfection efficiency[1,3)] since too low amounts of polymers cannot efficiently compact DNA and neutralize the negative charge whereas a significant excess of cationic polymers turn out to be cytotoxic[4)]. Thus, the complete polymers/DNA complexes usually investigated prior to in vitro and in vivo transfection experiments. For this purpose, a range of techniques have been used to monitor the self-assembly process e.g. light scattering, the inhibition of ethidium bromide fluorescence, zeta potential measurement[5)] and the most commonly used gel electrophoresis. Nevertheless, some methods need costly specialized instruments and time- consuming. Furthermore, a potent carcinogenic ethidium bromide[6,7)] is commonly employed in gel electrophoresis method. Despite the current availability of less mutagenic alternatives for nucleic acid stains, most of them are significantly high-priced. From these rationales, a new assay has been developed by using inexpensive dichlorofluorescein (DCF) for the estimation of CS and CS-Py to DNA ratio at which the complete polyplex was formed. DCF has been used as an adsorption indicator in Fajan's precipitate-forming titration of chloride using silver nitrate as a titrant[8)]. In analogue to this phenomenon, DNA which is anionic like chloride ion is titrated with various amounts of cationic CS or CS-Py. When a critical amount of CS or CS-Py is added to completely form the complex particles with nucleic acids and totally masks the negative charge, the surface charge of the polyplexes becomes positive and DFC anions are attracted to adsorb on the particles as counterions. In the previous study, we developed the dye adsorption based method for determining the complete polyplex formation of polyethylenimine (PEI), the synthetic cationic polymer with nucleic acid. The results showed the successful estimation of PEI/pDNA and PEI/siRNA polyplex formation. In addition, the assay is also a green and low-cost alternative method for routine task in the formulation of PEI and nucleic acids[9)]. In this study, we developed the proposed method for the estimation of the natural cationic polymer, CS and its derivative, CS-Py to nucleic acid ratio at which the complete polyplex was formed.

MATERIALS AND METHODS

Preparation of plasmid DNA The plasmids pEGFP-C2 (4.7 kbp) was amplified in *Escherichia coli* and purified by using the commercial plasmid midi kits (Qiagen, Hilden, Germany).The quality and quantity of purified pDNA were evaluated by the optical density at 260 nm and 280 nm and by agarose gel electrophoresis. The purified plasmid was resuspended and kept in Tris-EDTA (TE) buffer (pH7.5).

Formulation of CS/pEGFP and CS-Py/pEGFP polyplexes The CS/pEGFP and CS-Py/pEGFP polyplexes were prepared by adding the solution of CS or CS-Py (in 0.01 mM hydrochloric acid) to the plasmid solution in 1.5 mL microcentrifuge tubes at the different weight ratios of 0, 0.1, 0.5, 1, 5, 10, 15 and 20. The mixtures were gently mixed and further incubated at room temperature for 30 min, sufficiently for the complex formation. Then, the polyplex solutions were diluted with water to 30 mL.

Estimation of the ratio of complete polyplex formation

Proposed method based on DCF dye adsorption The ratio which the CS or CS-Py associated equivalently with nucleic acid was found by using the proposed method. Five µL of 0.2 mg mL^{-1} DCF solution was added into a series of polyplex solutions prepared by using varied CS or CS-Py to nucleic

acid ratios. After gently mixed, the solutions were centrifuged at 20,000 rpm for 5 min to precipitate the polyplexes at the bottom of the reaction tubes. The pellets were washed twice by using sterile water and briefly centrifuged to remove the unadsorbed DCF in the supernatant. The point of complete polyplex formation was seen by the formation of pink colored smear or pellets of DCF-adsorbed polyplex. By the other means, 30 mL of 0.01N NaOH was added to the pellets to free the adsorbed DCF from the polyplex. Upon the exposure to UV light at 366 nm, the reaction solutions emitted green fluorescent light which could be observed.

Spectrophotometry method The complete CS/pEGFP and CS-Py/pEGFP polyplex formation was examined by measuring the absorbance values of unadsorbed DCF in the supernatant. The absorbance values were measured at 504 nm by a cuvetteless drop-based NanoVuePlas spectrophotometer (GE healthcare, UK), against a sterile water blank.

Agarose gel retardation method To compare the proposed method with the currently used assays the association of CS or CS-Py and nucleic acid was also examined by gel retardation method, using. 0.8% agarose gel. The electrophoresis of DNA polyplex was carried out in 1X TAE buffer at 100 V for 45 min. Subsequently, the gel was stained with 0.5 mg mL^{-1} ethidium bromide. The bands were visualized and photo graphed by a UV transilluminator using a GelDoc system.

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RESULTS

Principle of the proposed method The estimation of CS or CS-Py to nucleic acid ratio of complete polyplex formation was developed based on the adsorption of common, safe and inexpensive dye onto the surface of the polyplex. As illustrated in Figure 1, a series of polyplexes set up at varied weight ratios of CS or CS-Py/nucleic acid were firstly prepared by adding the various amounts of CS or CS-Py to the nucleic acid solutions with the fixed concentration. After the self-assembly of pDNA with cationic CS or CS-Py (A), anionic DCF with green color was added to the solution (B). At this step, the nucleic acid which did not completely form polyplexes because of the inadequate amount of CS or CS-Py remained negatively charged and the adsorption of DCF on the surface of the particles would not happen. Since a critical amount of CS or CS-Py associated equivalently to the plasmids causing the complete self-assembly complexation, the positive charge of CS or CS-Py attracted DCF onto the particles as counterions (C). This resulted in the appearance of pink colored smear or pellets which could be visualized after spinning down these particles. By the other means, the adsorbed dye could be released into the solution by the addition of sodium hydroxide solution and the green fluorescence could be observed under UV light at 366 nm.

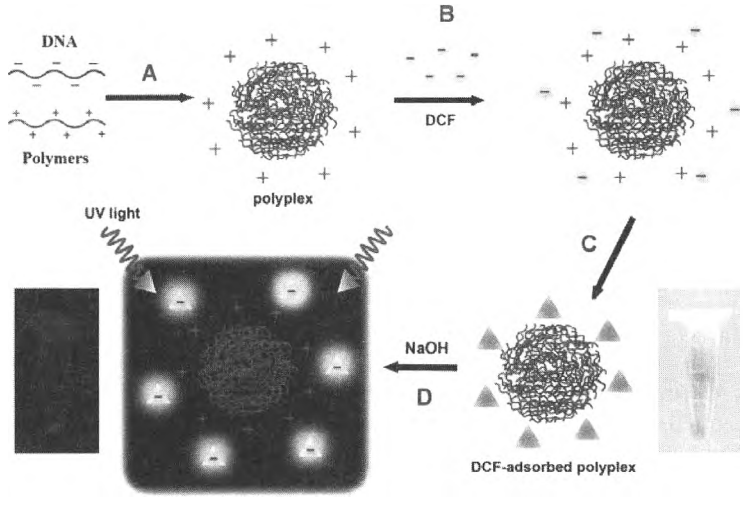


Figure 1 Method for the estimation of polymer to nucleic acid ratio of the complete polyplex formation based on dye adsorption.

CS or CS-Py/pDNA polyplex formulations by DCF dye adsorption method The method was applied to the estimation of the CS or CS-Py to pEGFP ratio at which both components equivalently, giving rise to the complete polyplex formation. As seen in Figure 2, the assembling reactions with the adequate CS/pEGFP or CS-Py/pEGFP ratios of 5 and higher produced pink pellets or green fluorescence under visible light or UV light, respectively whereas those comprising the sub-optimal CS/pEGFP or CS-

Py/pEGFP ratios did not showed pink pellets and no light emission. Based on this rationale, the lowest CS/pEGFP or CS-Py/pEGFP ratio that gave pink colored pellets or green fluorescence represented the equivalent ratio for the complete polyplex formation. In this case, both CS/pEGFP and CS-Py/pEGFP showed the same optimal ratio which was found at 5.

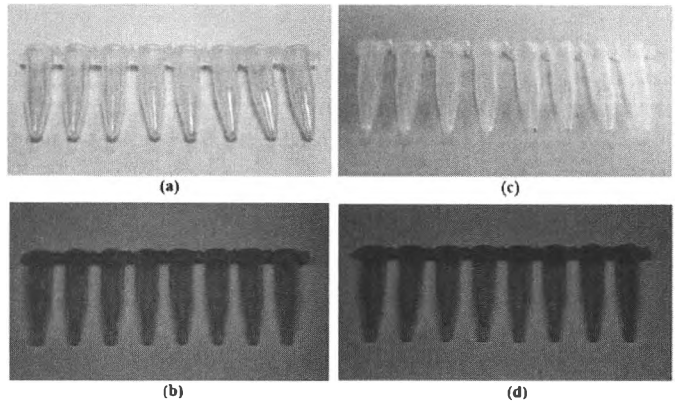


Figure 2 The estimation of CS/pEGFP ((a) and (b)) and CS-Py/pEGFP ((c) and (d)) ratio of the complete polyplex formation by using DCF adsorption method as detected by the formation of pink pellets ((a) and (c)) or the fluorescence emission under UV light ((c) and (d)).

CS or CS-Py/pDNA polyplex formations by spectrophotometry method To understand the fundamental aspects of the adsorption phenomenon, we have monitored the concentrations of DCF in CS/pEGFP and CS-Py/pEGFP self-assembly reactions by measuring the absorbance values of free DCF at 504 nm. As shown in Figure 3, the concentrations of DFC in the solutions decreased abruptly at the CS/pEGFP and CS-Py/pEGFP ratio of 5 due to the adsorption of dye onto the polyplex particles which were formed completely at this point.

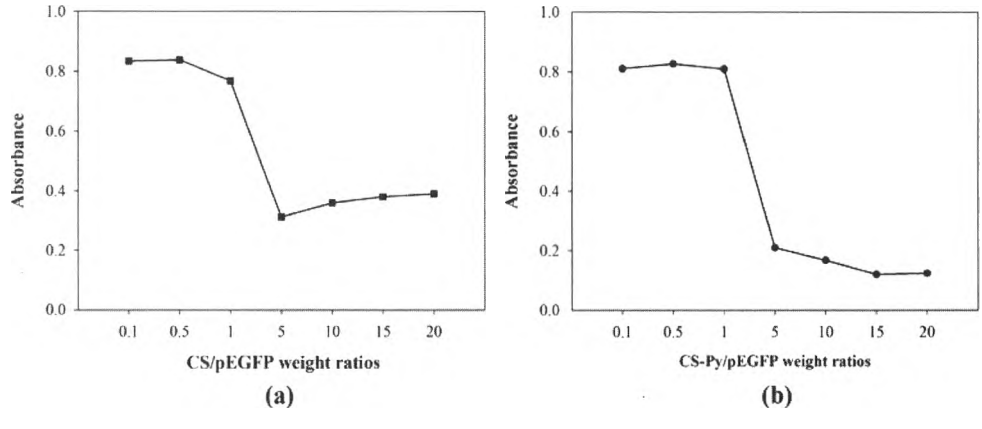
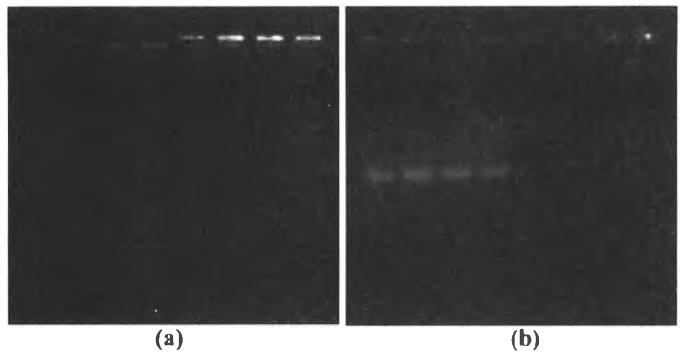


Figure 3 Absorbance values of free DCF in the solutions at different CS/pEGFP (a) and CS-Py/pEGFP (b) weight ratios.

Agarose gel electrophoresis To compare the proposed method with the conventional assays, gel retardation experiment confirmed that pEGFP was absolutely retained in the wells at the CS/pEGFP and CS-Py/pEGFP weight ratios of 5 and higher (Figure 4) which were totally in agreement with the results obtained from the proposed method.



(a) (b)

Figure 4 Gel electrophoresis assay of CS/pEGFP (a) and CS-Py/pEGFP (b) polyplexes prepared at weight ratio of 0, 0.1, 0.5, 1, 5, 10, 15 and 20 (from left to right).

DISCUSSION

In this study, a new assay has been developed for the estimation of CS and CS-Py to pDNA ratio at which the complete polyplexes are formed by the use of DCF dye. This method could determine by three means, visualization, fluorescence and spectrophotometry. From the principle of the propose method, an inadequate amount of CS and CS-Py did not completely form polyplexes with pDNA and remained negatively charged thus the adsorption of DCF on the surface of the particles would not happen. Once the amount of CS and CS-Py was equivalently to the plasmids, the positive charge of the polymers attracted DCF onto the particles and the pink smear or pellets are visible at the bottom of tubes after spinning down. An alternative detection method, sodium hydroxide solution was added to release the adsorbed dye into the solution and produced green fluorescence under UV light (366 nm). In both cases of CS and CS-Py, the optimal weight ratio was found to be 5. For detection by spectrophotometry, the concentrations of free DFC in the solutions decreased abruptly due to the adsorption of dye onto the polyplex particles formed completely point. Compared with gel electrophoresis experiment, the complete polyplex formation was found at the same weight ratio as the proposed method. These results suggested that the dye adsorption-based method was a reliable alternative method for monitoring the equivalence point for the optimal self-assembly between plasmid DNA with CS and CS-py.

CONCLUSION

Using the analog principle to the detection method for the end point of chloride titrimetry, a dye adsorption based method was developed and applied to the estimation of the ratio representing the complete polyplex formation between CS/pDNA and CS-Py/pDNA. The method gave comparable results to currently used assays i.e. gel electrophoresis. However, it required no sophisticated instruments, time-consuming, carcinogenic ethidium bromide and costly new generation of fluorescent nucleic acid staining dyes. Moreover, the proposed method could be usually accomplished within less than 10 min. Hence, it was a fast, facile, cost- effective and safe for operator alternative method, suited for the investigation of the optimal CS and its derivative such as CS-Py to pDNA ratio for gene delivery.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of this study by Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program scholarship (PHD/0069/2553) for Samarwadee Plianwong and by Faculty of Pharmacy, Silpakorn University, Thailand.

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