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Fast, facile and ethidium bromide-free assay based on the use of adsorption indicator for the estimation of polyethylenimine to nucleic acid ratio of complete polyplex assembly for gene delivery

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ABSTRACT

A new method was developed for the estimation of polyethylenimine (PEI) to nucleic acid ratio at which the polyplex was completely formed. The assay relied on the attraction of dichlorofluoresceinate dye to adsorb on self-assembling particles as counterions, as induced by the surface charge of the polyplex which became positive once PEI associated equivalently with nucleic acid. This phenomenon resulted in the appearance of pink colored pellets of the polyplex after centrifugation. By the other means, sodium hydroxide solution might be added to free the adsorbed dye into the solution, producing conspicuous green fluorescence under UV light (366 nm). The assay was well applied to the polyplex formulations of PEI and plasmid DNA or siRNA with satisfactory detectability and gave the results in agreement with those from gel retardation method and zeta potential analysis. Importantly, the proposed method required no sophisticated instruments, time-consuming gel electrophoresis, carcinogenic ethidium bromide as well as costly dyes and the analysis could be 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 PEI to nucleic acid ratio for gene delivery.

1. 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. Among nonviral vectors which are safer alternatives to viral vectors, polyethylenimine (PEI) is considered to be one of the most effective and widely accepted cationic polymer for gene delivery [1,2]. It forms self-assembling polyelectrolyte complexes or polyplexes with nucleic acids via electrostatic interactions, and mediated transfection by forming discrete condensed particles, protecting DNA from nuclease degradation and facilitating the endocytosis and endolysosomal escape [3–7]. In most studies, it has been revealed that the optimal PEI 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 PEI turns out to be cytotoxic [8]. Accordingly, the investigation of PEI to nucleic acid ratio in terms of weight ratio or molar ratio of PEI nitrogen atoms to DNA phosphate (N/P ratio) that brings about the complete PEI/DNA complexation is usually a prerequisite step in the polyplex formulation protocols 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 [9] and the most commonly used gel retardation assay which is based on the loss of electrophoretic mobility of DNA when it binds a critical amount of cationic polymers [10,11]. Nevertheless, some methods need costly specialized instruments such as zeta sizer while some assays are tedious and time-consuming. For instance, many steps starting from gel casting, followed by electrophoretic run and final staining are required for gel retardation assay. Furthermore, a potent carcinogenic ethidium bromide [12,13] is commonly employed. Despite the current availability of less mutagenic alternatives for nucleic acid stains, most of them are significantly high-priced.

In the analytical chemistry, dichlorofluorescein (DCF) has been used as an adsorption indicator in Fajan's precipitate-forming titration of chloride using silver nitrate as a titrant [14–16]. In water, weakly acidic DCF dissociates to green dichlorofluoresceinate anions. Once the titration reaches the equivalence point and the excess of silver ion titrant adsorbs on silver chloride surface imparting a positive charge, anionic DCF ions are attracted to the

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particles as the counter ions, and undergo the color change to pink upon adsorption, representing the endpoint. In analogue to this phenomenon, nucleic acid which is anionic like chloride ion is titrated with various amounts of cationic PEI. When a critical amount of PEI 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.

Based on the aforementioned rationale, a new assay has been developed by using inexpensive DCF for the estimation of PEI to nucleic acid ratio at which the complete polyplex was formed. In this study, the effective size and amount of plasmid DNA, the molecular weight of PEI and the minimum concentration of DCF required for the assay were studied. In addition, the applicability of the method to the formulation of PEI/siRNA polyplex was investigated and demonstrated. Therefore, the assay is not only a green and low-cost alternative method for routine task in the formulation of PEI and nucleic acids, but also a fast technique useful for the screening of new polymers e.g. from the combinatorial synthesis prior to in vitro and in vivo evaluation of gene delivery performance [17–19].

2. Experimental

2.1. Materials

PEI with the molecular weigh (MW) of 25 kDa was purchased from Sigma-Aldrich (Munich, Germany). PEI with MW of 10 kDa, 1.8 kDa and 0.6 kDa were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2′,7′-Dichlorofluorescein was purchased from Fluka (Switzerland). Agarose was purchased from ISC Bioexpress, USA. The pEGFP-C2 plasmid DNA encoding green fluorescent protein (GFP) was obtained from Clontech, USA. The λDNA/*Hin*dIII was obtained from Promega, USA. pSVβ-Gal (Promega, Madison, WI) contained bacterial β-galactosidase gene under the control of SV40 promotor.

2.2. Preparation of plasmid DNA and siRNA

The plasmids pEGFP-C2 (4.7 kbp) and pSV-β-galactosidase (6.8 kbp) were amplified in *Escherichia coli* and purified by using the commercial plasmid midi kits (Qiagen, Hilden, Germany). The quality and quantity of purified pDNA were evaluted 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 (pH 7.5). The siRNA-EGFP was synthesized by using Ambion's Silencer™ siRNA Construction Kit. (Ambion, USA).

2.3. Formulation of PEI/pEGFP, PEI/pSV and PEI/siRNA polyplexes

The PEI/pEGFP and PEI/pSV polyplexes were prepared by adding the solution of PEI (in 1X Tris-acetate-EDTA (TAE) buffer) to the plasmid solution in 1.5 mL microcentrifuge tubes at the different weight ratios of 0, 0.01, 0.1, 0.25, 0.5, 0.75, 1 and 2. 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 1X TAE buffer (pH 8.3) to 30 μL. The PEI/siRNA polyplexes were prepared by using the same procedures and at the weight ratios as those for PEI/pDNA polyplexes. In this case, the dilutions were made by using 1X Tris-borate-EDTA (TBE) buffer (pH 8.3) which is commonly used for RNA works.

2.4. Estimation of the ratio of complete polyplex formation

2.4.1. Proposed method based on DCF dye adsorption

To find out the ratio at which the PEI associated equivalently with nucleic acid by using the proposed method, 5 μL of 0.075 mg mL^{-1} DCF solution prepared in 1X TAE buffer for PEI/pDNA polyplex experiments or in 1X TBE buffer for PEI/siRNA polyplex experiments was added into a series of polyplex solutions prepared by using varied PEI 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 μL of 0.01 N 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 with ease.

2.4.2. Agarose gel retardation method

To compare the proposed method with the currently used assays, the association of PEI 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 μg mL^{-1} ethidium bromide. The bands were visualized and photographed by a UV transilluminator using a GelDoc system. In the case of PEI/siRNA determination, gel electrophoresis was carried out in 1X TBE buffer at 100 V for 20 min.

2.4.3. Zeta potential analysis

The surface charge of the polyplex was measured by using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) at 25 °C. The polyplexes were diluted with sterile water to 1 mL at the time of measurement. The sample measurements were performed in triplicate.

3. Results and discussion

3.1. Principle of the proposed method

In this study, an alternative method for the estimation of PEI 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 Fig. 1, a series of polyplexes set up at varied weight ratios of PEI/nucleic acid were firstly prepared by adding the various amounts of PEI to the nucleic acid solutions with the fixed concentration. After the self-assembly of pDNA or siRNA with cationic PEI (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 PEI remained negatively charged and the adsorption of DCF on the surface of the particles would not happen. Once a critical amount of PEI associated equivalently to the plasmids causing the complete self-assembly complexation, the positive charge of PEI 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.

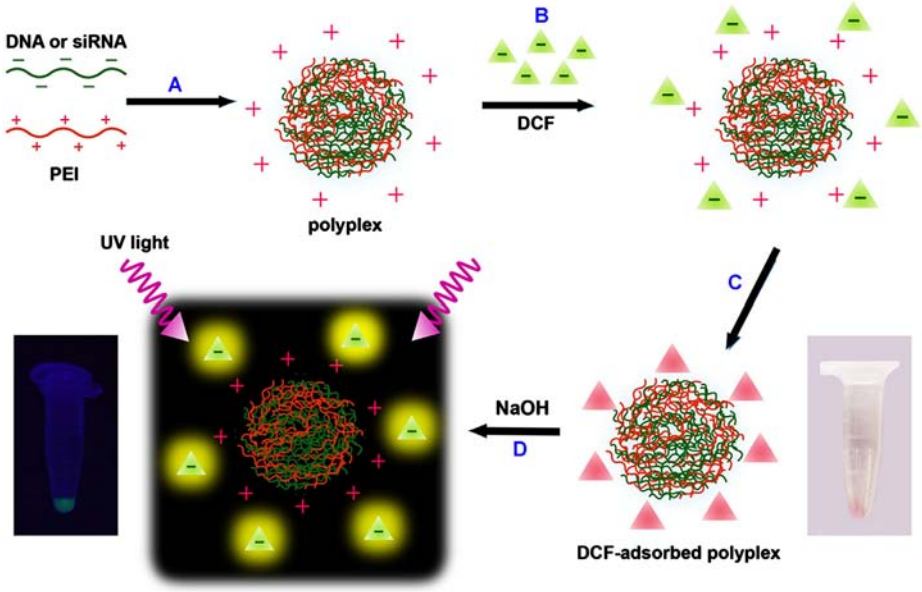

Fig. 1. Method for the estimation of PEI to nucleic acid ratio of the complete polyplex formation based on dye adsorption. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Application with PEI/pDNA polyplex formulations

As "proof of concept", the method was applied to the estimation of the PEI (25 kDa) to pEGFP ratio at which both components associated equivalently, giving rise to the complete polyplex formation. As seen in Fig. 2, the assembling reactions with the adequate PEI/pEGFP ratios of 0.5 and higher produced pink pellets or green fluorescence under visible light or UV light, respectively whereas those comprising the sub-optimal PEI/pEGFP ratios showed white pellets and no light emission. Based on this rationale, the lowest PEI/pEGFP ratio that gave pink colored pellets or green fluorescence represented the equivalent ratio for the complete polyplex formation. In this case, the optimal PEI/pEGFP ratio was thus found to be 0.5.

To understand the fundamental aspects of the adsorption phenomenon, we have monitored the concentrations of DCF in PEI/pEGFP self-assembly reactions by measuring the absorbance values of free DCF at 504 nm. As shown in Fig. 3, the concentrations of DFC in the solutions decreased abruptly at the PEI/pEGFP ratio of 0.5 due to the adsorption of dye onto the polyplex particles which were formed completely at this point.

To compare the proposed method with the previously established assays, the analysis of surface charge was performed and it revealed that the zeta potential of self-assembling particles gradually increased with the higher amount of PEI used and the values became positive when the PEI/pEGFP ratio reached 0.5 (Fig. 4). Similarly, gel retardation experiment confirmed that pEGFP was absolutely retained in the wells at the PEI/pEGFP weight ratios of 0.5 and higher (Fig. 5) which were totally in agreement with the results obtained from the proposed method. These results suggest 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 PEI.

3.3. Investigation of parameters effective for the proposed method

Since the detectability and applicability of the proposed method might depend on the plasmids, PEI and DCF used in the assay, the parameters involving these factors were studied in order to find out the minimal limits which still enabled the effective assay.

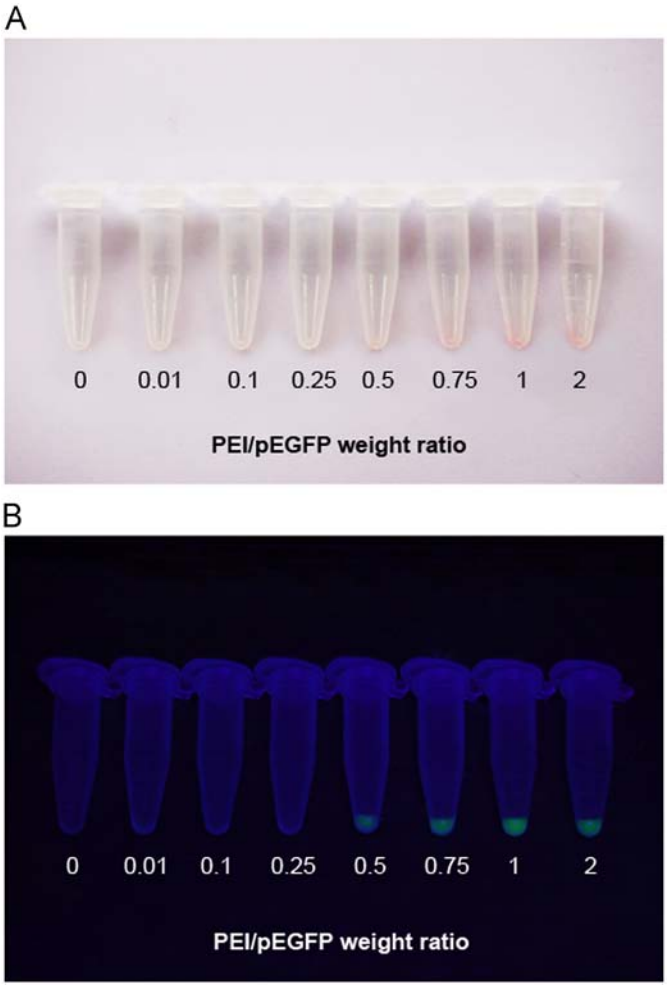

Fig. 2. The estimation of PEI (25 kDa)/pEGFP ratio of the complete polyplex formation by using DCF adsorption method as detected by the formation of pink pellets (A) or the fluorescence emission under UV light (B).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

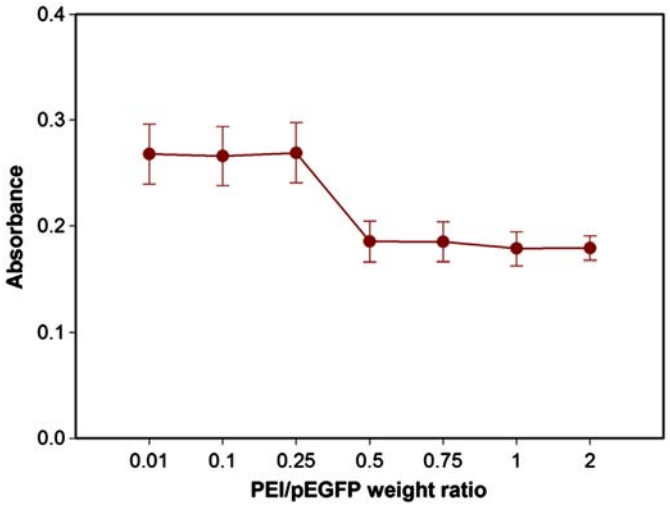

Fig. 3. Absorbance values of free DCF in the solutions at different PEI/pEGFP weight ratios.

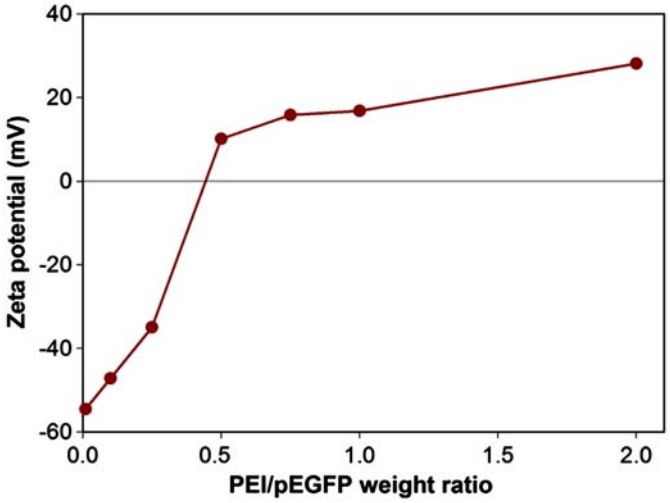

Fig. 4. Zeta potential of PEI (25 kDa)/pEGFP polyplexes prepared at different weight ratios.

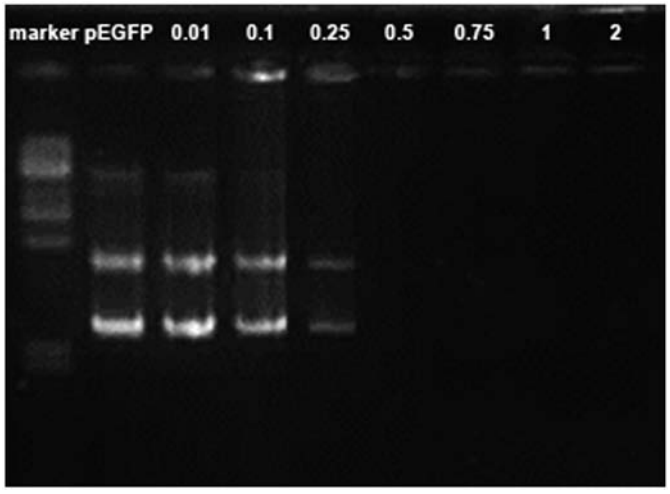

Fig. 5. Gel retardation assay of PEI (25 kDa)/pEGFP polyplexes prepared at different weight ratios.

3.3.1. Concentration of plasmid DNA and dye

The effect of pEGFP concentration on the detection limit was examined by varying the amount of pEGFP at 5 μg, 2.5 μg, 1 μg, 0.5 μg and 0.25 μg. It was found that as low as 0.5 μg of pEGFP (in 30 μL reaction) combined with 0.075 mg mL^{-1} DCF enabled the effective detection of pink color on the pellets and green fluorescence emitted from the solutions.

3.3.2. Type of plasmid DNA

Besides pEGFP, the proposed method well applied to the assay when the different plasmids with the different size i.e. pSV (6.8 kbp) was used. The results from the dye adsorption-based method showed the complete PEI/pSV polyplex formation at the weight ratio of 0.5 when 0.5 μg of pSV was used. This ratio was exactly the same as those obtained from gel retardation and zeta potential determination method, indicating the applicability of the method to other types of plasmids.

3.3.3. Molecular weight of PEI

In regardless of the polymer, the investigation was performed by using PEI with different molecular weights (25 kDa, 10 kDa, 1.8 kDa and 0.6 kDa) since several works have reported that the molecular weight of polymer influenced on the pDNA condensation and the transfection efficiency [5,20]. From the experiments, 25 kDa-PEI and 10 kDa-PEI were capable of giving the detectable pink pellets and the green fluorescence when they formed the polyplexes with 0.5 μg pEGFP and assayed with 0.075 mg mL^{-1} DCF. However, for PEI with the lower molecular weights i.e. 1.8 kDa and 0.6 kDa, although the gel electrophoresis method could show the disappearance of pDNA bands, the dye adsorption-based method did not give the acceptably clear results. According to the surface charge determination experiments, the polyplexes formed by using 1.8 kDa-PEI and 0.6 kDa-PEI showed much less positive zeta potential than those formed by using 25 kDa-PEI and 10 kDa-PEI. Furthermore, their radius sizes were extraordinarily large, indicating the loose association between the polymers and pDNA. Therefore, it was possible that weakly positive surface charge could not well attract the dye and the PEI/pDNA polyplexes formed were not efficiently pelleted under the centrifugation condition used in the assay. As PEIs with low molecular weight (<2 kDa) have been previously demonstrated for their lacks of efficient capability of condensing pDNA and thus rarely used for gene delivery [21–23], this inability of the proposed method dealing with such PEIs should not be considered a practical limitation.

3.4. Application with PEI/siRNA polyplex formulations

Apart from plasmid DNA, the delivery of short-interfering RNA (siRNA), 21–23 nucleotide fragments of double stranded RNA, has offered great options for gene therapy. This strategy has recently gained more attention because siRNA produces the efficient and specific gene silencing in the cytosol and does not require the transport into the nucleus [24]. In this study, the proposed method was further applied for the determination of the equivalent ratio of siRNA and PEI complexation. By using EGFP-siRNA, the dye adsorption-based method revealed that EGFP-siRNA completely formed the polyplex with PEI at PEI/siRNA weight ratio of 1:1 and higher (Fig. 6A). The lowest amount of siRNA and DCF that produced visible pink pellets and green fluorescence were 1 μg and 0.075 mg mL^{-1}, respectively. This ratio was slightly different from that obtained from gel electrophoresis method which exhibited a ratio of the complete PEI/siRNA complexation at 0.5. It was probable that the migration of polyplexes in the gel electrophoresis assay depended on not only charge but also structural properties e.g. size of the complexes and thus might not be always the

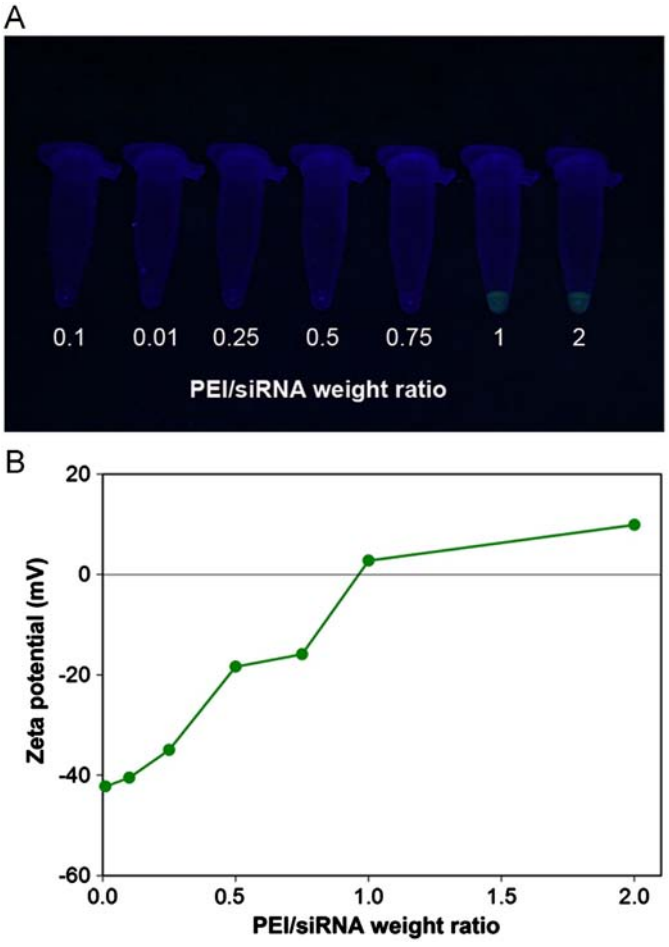

Fig. 6. The estimation of PEI (25 kDa)/EGFP-siRNA ratio of the complete polyplex formation by using DCF dye as detected by fluorescence under UV light (A) and the zeta potential of the polyplexes prepared at different weight ratios (B).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

best indication for the formation of discrete polyplexes [9,25,26]. Nonetheless, compared with the results from the surface charge analysis, the ratio from the proposed method was found to be the same first point where the zeta potential of PEI/siRNA polyplex became positive (Fig. 6B). From these results, the dye adsorption-based method could also be an effective alternative method for the estimation of PEI to siRNA ratio of complete polyplex assembly.

4. Conclusion

The dye adsorption-based method was developed and applied to the estimation of the ratio representing the complete polyplex formation between PEI/pDNA as well as PEI/siRNA. The method gave comparable results to currently used assays i.e. gel retardation method and zeta potential analysis. However, it required no sophisticated instruments, time-consuming gel electrophoresis, carcinogenic ethidium bromide as well as costly new-generation of fluorescent nucleic acid staining dyes. Moreover, the analysis 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 PEI to nucleic acid ratio for gene delivery.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.073.

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