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Green, fast and cheap paper-based method for estimating equivalence ratio of cationic carriers to DNA in gene delivery formulations

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ABSTRACT

To achieve efficient and safe cationic carrier-mediated gene delivery for gene therapy, the optimal ratio of carrier to DNA in formulations is a key factor and it is usually determined prior to transfection experiments. In this work, a simplified drop-and-read assay was developed for the first time using paper as a platform to estimate the equivalence ratio of cationic carriers to negatively charged DNA. By spotting a series of complexes containing varied ratios of carrier to DNA on filter paper, then allowing them to dry and finally dropping yellowish-green anionic 2′,7′-dichlorofluorescein dye solution on top of the complexes, the equivalence point was detectable by the instant formation of stable pink spots as a result of the dye adsorption onto the positively charged complexes and free carriers. The method gave the same results as those determined by gel retardation assay and zeta potential measurement, however it allowed more rapid reporting of results in 5 min and required no tedious steps, harmful reagents or expensive instruments. By using paper instead of microcentrifuge tubes and omitting centrifugation, plasticware and electrical energy were no longer consumed and disposal of this degradable material was more environmentally friendly. With respect to analytical performance, filter paper inherently holding negative charge helped to trap and concentrate the complexes on the white background, enabling greater visibility of the colored spots and a lower required amount of DNA used for the assay. The method was successfully applied to estimate the equivalence ratios in a variety of gene delivery formulations containing different types of cationic carriers, i.e. polymers, dendrimers, liposomes and niosomes.

1. Introduction

Lab-on-paper is a powerful and attractive way to develop budget, user- and environmentally friendly assays since methods performed in this format are mainly designed for convenience, equipment-free operation and low consumption of samples/reagents as well as electrical power (Dou et al., 2015; Hu et al., 2014; Li et al., 2012; Sharma et al., 2018). From a general viewpoint, paper is an inexpensive, disposal and degradable material. As an analytical device, paper made typically from cellulose fibers possesses a high porosity and surface-to-volume ratio, thereby being capable of adsorbing and allowing liquids to penetrate by capillary flow without the need for external pieces of equipment such as pumps. In addition, the white background of paper enables obvious readouts of colored results with the naked eye. These advantages have highlighted paper as one of the most popular platforms for assays of various types of analytes including small molecules and biomolecules (Cate et al., 2015; Chen et al., 2012; Dungchai et al., 2010). For nucleic acid related work, paper-based tests and devices have been widely developed and used for the extraction, amplification and detection of the DNA/RNA of infectious pathogens (Magro et al., 2017; Rodriguez et al., 2015; Teengam et al., 2017; Zhang et al., 2014) and for the differentiation of DNA from different sources (Araújo et al., 2012). These devices are ideally suited for point-of-care diagnostics and meet the World Health Organization ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users) criteria (Lee et al., 2010; Martinez et al., 2010).

Apart from the use of nucleic acid for diagnostic purposes, the delivery of exogenous DNA or RNA into a patient's cells in order to correct gene expression – so-called “gene therapy” – is currently a potential strategy in medicine for the treatment of certain diseases e.g. genetic abnormalities, autoimmune disorders and cancers (Foldvari et al., 2016; Ibraheem et al., 2014). To achieve successful gene transfer and

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therapeutic outcomes, meaning that the transfection efficiency is high and no apparent toxicity is obtained, proper kinds and amounts of transfection reagents must be used for incorporation with the delivered genes. In gene transfection mediated by non-viral, cationic carriers, e.g. cationic polymers and cationic lipids where the carriers form small and compact complexes with negatively charged DNA via electrostatic interactions and help to protect the DNA from degradation and facilitate its cellular uptake and intracellular traffic into the nucleus, the charge ratio of the carriers to DNA has been found to correlate with transfection efficiency (Huang et al., 2013; Jin et al., 2014; Reed et al., 2006). At low carrier to DNA ratios, DNA condenses poorly resulting in large particles with the remaining negative charges entering cells inefficiently. Meanwhile, a ratio of carrier to DNA which is too high leads to the formation of extremely positively charged complexes as well as excessive free cationic carriers in the formulation which interact with negatively charged components of the cell membranes and unfavorably cause membrane disruption (Hunter, 2006; Lv et al., 2006). Practically, a particular ratio which produces a slightly positive net charge on the surface of complexes is desirable (Mansouri et al., 2006; Zhao et al., 2009) and therefore determined by physicochemical methods and optimized prior to transfection and biological evaluation.

For the estimation of the ratio, gel retardation assay and zeta potential analysis are the two most commonly used methods (Amin et al., 2016; He et al., 2013; Honary and Zahir, 2013; Plianwong et al., 2013a). The first one relies on the electrophoresis of complexes at varying carrier to DNA ratios. At a ratio where the carriers form equivalent complexes by charge with DNA resulting in neutral particles, no migration of DNA to the gel is observed. Despite being regarded as an accustomed technique in many laboratories, a gel retardation assay consists of many time-consuming steps, i.e. gel casting, sample running and final staining. Moreover, ethidium bromide which is used as a DNA dye is carcinogenic and harmful to the environment (Singer et al., 1999). In zeta potential analysis, the surface charges of complexes are measured, usually by dynamic light scattering optical devices. The equivalence ratio is indicated by a zero or slightly positive zeta potential measurement value. This method is convenient and capable of reporting quantitative results, however the major drawback is the high-cost of the equipment involved. Previously, we reported a new method based on dye adsorption (Plianwong et al., 2013b). In this assay, yellowish-green, anionic 2′,7′-dichlorofluorescein dye is added to the carrier/DNA complexes in microcentrifuge tubes. After centrifugation of the mixture to sediment, the equivalence ratio is indicated by the formation of pink pellets as a result of the dye's color change from green to pink once it is adsorbed onto the positively charged complexes. This method was shown to give accurate results with a rapid analysis time of less than 10 min when tested with a cationic polymer, namely polyethyleneimine (PEI), without the use of expensive zetasizer, tedious gel electrophoresis or harmful dye. Though promising, some weak points, i.e. consumption of electrical energy in the centrifugation step and release of plastic tube waste after the assay, were identified when considering the green analytical chemistry metrics (Gałuszka et al., 2013; Tobiszewski, 2016). In addition, the observation of the faint pink color of the pellets or smears through the walls of the tubes was sometimes unclear.

In the work reported here, we detail the further development of a greener version of the 2′,7′-dichlorofluorescein-based method by performing the assay on filter paper, not only to exclude the use of plastic tubes and the centrifugation step, but also to facilitate the readout of results. Moreover, besides the cationic polymer PEI, other types of gene carriers including cationic dendrimers, cationic liposomes and cationic niosomes were used to assess the applicability of the proposed method.

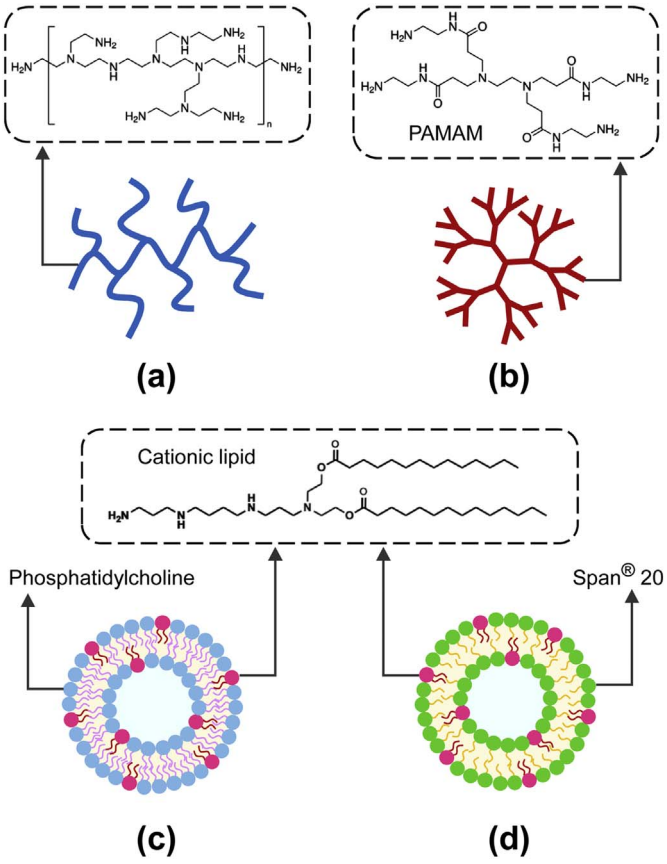

Fig. 1. Cationic carriers for gene delivery used in this study: (a) PEI cationic polymer, (b) SuperFect® cationic dendrimer, (c) cationic liposome, and (d) cationic niosome.

2. Experimental

2.1. Materials

Filter papers were purchased from Whatman International Ltd. (Maidstone, England). 2′,7′-Dichlorofluorescein was obtained from Fluka (Buchs, Switzerland). Branched PEI (molecular weight of 25 kDa) cationic polymer was purchased from Sigma-Aldrich (Munich, Germany) and SuperFect™ cationic dendrimer was commercially obtained from Qiagen (Hilden, Germany). The cationic lipid (Fig. 1) used for the preparation of cationic liposomes and niosomes was kindly provided by Dr. Boon-ek Yingyongnarongkul, Faculty of Science, Ramkhamhaeng University, Thailand.

Plasmid DNA encoding enhanced green fluorescent protein (pEGFP-C2) was cloned in *Escherichia coli* and purified using plasmid midi kits (Qiagen, Hilden, Germany). After purification, the size (4.7 kbp) was verified by digestion by the *Eco*RI restriction enzyme. The quality and quantity of plasmid were assessed by its optical density at 260 nm and 280 nm. The purified plasmid was kept in a Tris-EDTA buffer (pH 7.5) at 4 °C.

2.2. Preparation of cationic carriers and formulation of carrier/DNA complexes

Four types of cationic carriers were used in this study (Fig. 1). PEI (25 kDa), which is a highly branched polymer with primary, secondary and tertiary amine groups and is widely accepted as the gold standard in gene delivery (Patnaik and Gupta, 2013), was employed as the representative cationic polymer. SuperFect® was used as the cationic dendrimer. This transfection reagent is fractured or activated generation 6, poly(amidoamine) (PAMAM) dendrimer with a tree-like