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Conjugation of poly(ethylene glycol) to poly(lactide)-based polyelectrolytes: An effective method to modulate cytotoxicity in gene delivery

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ABSTRACT

Positively charged amphiphilic polymers comprising of poly(*N*,*N*-dimethylaminoethyl methacrylate) segments on both sides of a poly(L-lactic acid) segment was conjugated with poly(ethylene glycol) (DMA-PLLA-DMA@PEG). Efficient condensation of plasmid DNA by these polymers was shown. Cell toxicity studies demonstrated that the positively charged polymers with attached PEG exhibited much less cytotoxicity than polymers with no PEG in both HEK293 and Hela cell lines. PEGylation also resulted in polymers with enhanced haemo-compatibility. The positively charged polymers displayed very good DNA plasmid delivery efficiencies in both HEK293 and Hela cells.

1. Introduction

DNA plasmid therapy is a technique where DNA plasmids are delivered to cells to prevent or cure diseases. There are several ways in which the DNA plasmids could be delivered to the cells [1–3]. One such method is by the use of nanocarriers. It is not efficient to deliver DNA directly without the use of any form of medium as they are rapidly degraded in the presence of degradative enzymes in the blood serum [4]. Thus, the use of vectors is crucial for DNA plasmid delivery [5–7]. Vectors could be categorized into viral and non-viral vectors. Viral vectors rely on viruses to deliver the therapeutic DNA plasmid to cells. This is done through the replacement of a section of the genome of a virus with a therapeutic DNA plasmid [4,8–10]. The virus is used as a vehicle to transport the DNA plasmid into the cell. Studies have shown that the use of viruses as a vehicle to transport DNA plasmid is fairly efficient as a result of its ability to overcome cellular barriers and immune defense mechanism [5]. However, as a result of the uncertain nature of viruses, there is a possibility whereby the virus could transform and evolve into something harmful. Additionally, viruses are immunogenic which means that it could result in a dangerous immune reaction [11]. These disadvantages have led researchers to explore the use of non-viral vectors. Non-viral vectors could be further divided into 2 sub categories; lipids and polymers. The role of non-viral vectors is to protect the DNA plasmids and mediate cellular entry by forming lipoplexes/polyplexes. The use of positively charged polymers as a non-viral vector is extensively researched as a result of its massive potential for applications and treatment of diseases [12]. Some of these potentials are improved safety conditions, greater flexibility and more facile manufacturing. However, non-viral vectors have significantly lower DNA plasmid-transfer effectiveness as compared to viral vectors. The low DNA plasmid-transfer effectiveness is as a result of the inability of the polymers to overcome some of the barriers that exists during DNA plasmid-delivery. Hence, polymers could be custom designed to enhance its chances of overcoming these barriers during DNA plasmid delivery. Two decades of research on, in spite of excellent DNA plasmid delivery effectiveness by viral vectors, the safety profile of these viral vectors have not been entirely clear. These factors have pushed researchers to look for other forms of non-viral vectors, such as poly(ethyleneimine) (PEI), poly(dimethylaminoethyl methacrylate) (PDMAEMA), chitosan (CS), and poly(L-lysine) (PLL) [13–26]. PDMAEMA is especially interesting to researchers in this area as a result of its pH sensitivity, hydrophilicity, availability of the functional amine/ammonium moiety for complexation with acidic/anionic substances and ease of quaternisation [27,28]. With a pK_a of ~7.0 and PDMAEMA behaves as a weak base at pH values above 7, and at lower pH values,

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protonation of the amine groups occurs leading to a positively charged polyelectrolyte [29–37]. PDMAEMA can enter cells by endocytosis when it is used as a non-viral DNA vector [38–40]. In addition, PDMAEMA was also found to have bioactivities in antibacterial, haemostatic, and anticancer effect, making it a promising material for biomedical applications [41,42].

The co-polymerization of PDMAEMA with a hydrophobic polymer allows for the formation of amphiphilic polymers [29,43–47]. Self-assembly of amphiphilic polymers above the critical aggregation concentration (CAC) could be used to develop drug encapsulating polymeric micelles for biomedical applications [48–50]. However, the susceptibility of micelles to dilution is a challenge in applications. It is vital that the integrity of micelles is retained *in vivo* to prolong blood circulation times, accumulation at targeted sites, and controlled drug release [51,52]. The high hydrophobicity of PLA makes it a prime candidate as the hydrophobic segment for this polymer. PLLA, in particular, has been reported as a biodegradable segment in several micelle studies for drug delivery applications [35]. PLLA has also been investigated for the delivery of DNA plasmid material through polymerization with positively charged polymers such as PEI [53], PLL [54,55] and PDMAEMA [56–59]. Abebe et al. reported the preparation of three-layered micelles made up of poly(L-lactide)-*b*-polyethyleneimine-*b*-poly(L-lactide) (PLLA-PEI-PLLA) and poly(L-lactide)-*b*-poly(ethylene glycol)-*b*-poly(L-lactide) (PLLA-PEG-PLLA) [60]. The PLLA-PEI-PLLA was employed as a DNA plasmid carrier. Upon condensation with DNA, the polyplex was further loaded into an amphiphilic micelle solution made from PLLA-PEG-PLLA polymers to obtain an aqueous stable colloidal dispersion. Several reports have attempted to improve the DNA plasmid transfection capabilities of polymers by quaternisation of the tertiary amino groups. However, Domb et al. demonstrated that instead of improving the transfection effectiveness, quaternisation appeared to lower the transfection effectiveness instead [61]. Arro et al. have also highlighted the significant toxicity of quaternised ammonium moieties [62]. Xu et al. also demonstrated that the quaternisation of tertiary amino groups appeared to have a detrimental effect on the DNA plasmid transfection capabilities of the polymers [63]. It thus appears that simple quaternisation of amino groups, while enhancing the positive charges for enhanced complexation, leads to higher toxicity and thus leads to poorer DNA plasmid transfection efficiencies. In this work, we developed a biodegradable PLLA with inherent positively charged charges and possessing PEG as the colloidal stabilizer in one polymer chain. This polymer is developed by: (i) attaching a difunctional alkyl halide to the tertiary amino groups of the PDMAEMA blocks in the pH-sensitive PDMAEMA-PLA-PDMAEMA triblock polymers by quaternisation reaction and (ii) followed by PEGylation onto the quaternised PDMAEMA segments. Our hypothesis is that with the quaternisation of the amino groups, the DNA complexation will be enhanced. However, to mitigate toxicity effects, PEG could be introduced as a biocompatible polymer group. This approach is not easily achieved as direct quaternisation of the amino group using a halide terminated high molecular weight PEG. Therefore, we proposed a two-step approach where we first quaternise the amino group and endow it with an azide group. The azide group is then further reacted *via* the high yielding classical "click" reaction to result in the "PEG quaternised" amino group. We further test the effectiveness of these materials for DNA plasmid transfection in various cell lines.

2. Experimental section

2.1. Preparation of PDMAEMA-PLLA-PDMAEMA (DMA-PLLA-DMA) triblock polymers

Triblock polymers of PDMAEMA-PLLA-PDMAEMA were prepared by ATRP. In the synthesis process, the [PLLA-diBr] / [DMAEMA] / [CuBr] / [HMTETA] molar fraction was 1 / 1000 / 1 / 2. In one example, PLLA-diBr, DMAEMA and 1,4-dioxane were placed into a round bottom flask filled with nitrogen. The ligand, HMTETA and copper bromide were added under N_2. Polymerization proceeded at 60 °C. The molecular weight was monitored by Gel permeation chromatography (GPC). Upon completion, the reaction was stopped by diluting the reaction mixture with THF and exposing it to air. Purification was carried out by passing the polymer solution through an Al_2O_3 column. The eluent was concentrated, precipitated in ether and dried.

2.2. Conjugation of PEG to DMA-PLLA-DMA polymers

PEG conjugated DMA-PLLA-DMA (DMA-PLLA-DMA@PEG) polymers were made in a one-pot synthesis approach using AEBP as difunctional linking agent. Typically, 1.0 g of DMA-PLLA-DMA polymer was dissolved in 10 mL of DMF containing 0.072 mL of AEBP. The molar fraction between AEBP and DMAEMA was fixed at 1:10, aiming to achieve an extent of 10% quaternisation in the quaternised DMA-PLLA-DMA@N_3. During the synthesis, the above mixture was firstly stirring at 50 °C for 48 h and cooled to ambient temperature thereafter. After that, equal mole of propargyl-PEG and PMDETA to AEBP were added into the flask under nitrogen atmosphere and the mixture was further purged with nitrogen for another 30 min. The click reaction was catalyzed by adding of CuBr (0.07 g). After 24 h, the PEG conjugated polymer DMA-PLLA-DMA@PEG was purified by dialysis. To completely remove the excess PEG, the dialysis was carried out against deionized water for 48 h with the MWCO 1000 of the dialysis membrane. The final product was harvested by freeze drying with the yield of 68%.

2.3. Methods and characterizations

The molecular structure of the polymers and intermediate products were characterized by *Nuclear Magnetic Resonance* (Bruker AV-400). The solvent peaks of DMF at δ 8.03, 2.92 and 2.75 ppm, $CHCl_3$ at δ 7.3 ppm, and H_2O at δ 4.8 ppm were used as reference in the determination of chemical shifts of the polymers (Fig. S1). The proton integral regions in Fig. S1 were used to evaluate the chemical compositions of the polymers. *Gel Permeation Chromatography* (GPC, Shimadzu SCL-10A and LC-8A system) was used to determine the molecular information (Mw and PDI) of the polymers. DMF containing 0.1 M of LiBr was used as the eluent and the flow rate was 1.0 mL/min. The molecular weight difference before and after the linked reaction was used to calculate the percentage of PEG linked to the DMA-PLLA-DMA@PEG polymers.

2.4. Preparation of the polymer/pDNA polyplexes

The plasmid DNA (pDNA) was amplified according to previously reported protocol and its purity was assessed by agarose gel electrophoresis [28]. The purified pDNA was dissolved in Tris-EDTA (TE) buffer as a stock solution. The concentration of pDNA was assessed by absorption at 260 nm. For the polyplexes preparation, the polymers were mixed with pDNA solution according to preassessed molar fractions of nitrogen (N) in polymer to phosphate (P) in pDNA (*i.e.* N/P fractions). After 30 min incubation, the freshly prepared polymer/pDNA polyplexes were used for gene transfection evaluation.

The particle sizes of the polymer/pDNA polyplexes and surface charge of the polymer solutions at various pH were characterized using a Zetasizer Nano ZS (Malvern Instruments), with a laser light wavelength of 633 nm at 173°. The morphological observation of the polymer/pDNA polyplexes was carried out using transmission electron microscopy (TEM, Philips EM300) operated at 300 kV. Typically, the TEM samples were made by directly depositing one drop of polyplex solution (N/P = 10) stained with osmium tetroxide (OsO_4) onto a 200 mesh carbon coated TEM grid. Samples were kept for 24 h before TEM imaging.