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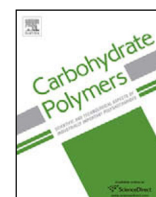

Synthesis of amphoteric curdlan derivatives for delivery of therapeutic nucleic acids

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A B S T R A C T

Cationic polymers are powerful carriers for intracellular delivery of therapeutic nucleic acids. However, the positively charged macromolecules have considerable cytotoxicity and often induce irreversible damages to the cells and tissues, which greatly negate the clinical application of such materials as drug delivery system. Herein, we report the synthesis of novel amphoteric polymers based on curdlan, and the evaluation of their cytotoxicity as well as the nucleic acid delivery efficiency. β-(1,3)-polyglucuronic acid, a TEMPO-oxidized derivative of curdlan, was chemically modified by conjugation of tetraethylenepentamine. The resulting amphoteric polymers, denoted tetraethylenepentamine-curdlan (TEPAC) polymers have the degree of substitution (DS) ranging from 25% to 48%. The result of MTT assay indicated that TEPAC polymers have negligible cytotoxicity on HeLa cells and A549 cells. The novel amphoteric polymers efficiently bound with plasmid DNA and delivered pcDNA-eGFP plasmid to 293T cells and induced expression of GFP 48 h after the transfection. Moreover, TEPAC polymers delivered siRNA to HeLa cells and HepG2 cells in high efficiency, and induced significant RNAi for the expression of an endogenous gene. Collectively, our data demonstrate that the novel curdlan-based amphoteric polymers are biocompatible and may provide a highly efficient system for the delivery of therapeutic nucleic acids.

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1. Introduction

Gene therapy potentiates the cure of mutation induced genetic disorders through one time DNA fixation. Traditional gene therapy procedure includes cloning of a copy of functional gene into a carrier vector and delivering the vector into the target cells in order to obtain persistent transcription and translation of a functional protein. Discovery and application of RNA interference (RNAi) greatly extended the gene therapy concept by providing powerful approaches to efficiently silence the disease related gene at post-transcriptional gene expression level. (Fire et al., 1998) Recently, the application of CRISPR/Cas9 gene editing technology is once again redefining the concept of gene therapy through providing powerful gene editing approaches including gene correction, deletion, insertion and so on. (Gilbert et al., 2013)

In vivo gene delivery requires efficient intracellular delivery systems. Two types of gene delivery systems have been reported so far: viral delivery system and non-viral delivery system. Initial studies revealed that viral delivery system based on retrovirus successfully delivered functional genes into target cells of patients and achieved sustained expression of functional gene. (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002) However, random insertion of the viral DNA in the genome of the host cells induced insertional mutagenesis which eventually led to cancer. (Baum, Kustikova, Modlich, Li, & Fehse, 2006; Hacein-Bey-Abina et al., 2008; Hacein-Bey-Abina et al., 2003) Meanwhile, severe immune responses associated with adenovirus based gene vector were also reported. (Raper et al., 2003) Although recent development of improved viral vectors may bypass those adverse side effects to some extents, the application of viral vectors in clinical trials should be closely monitored for unexpected side effects and outcomes.

Non-viral vectors have several advantages over viral vectors because they can be chemically synthesized from non-immunogenic materials, such as cationic liposomes, (Mintzer & Simanek, 2009) polymers, (Zhang et al., 2016) peptides (Raad, Teunissen, & Mastrobattista, 2014) and inorganic materials (Loh, Lee, Dou, & Deen, 2016). Gene delivery efficiency of non-viral vectors is usually not comparable to viral vectors. Moreover, some cationic molecule based vectors have considerable cytotoxicity. Nonetheless, clinical trials currently underway include non-viral vectors such as liposomes.

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Naturally occurring polymers are more biocompatible and less toxic compared to synthetic cationic polymers. Natural polysaccharides such as chitosan (Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008) and curdlan (Mochizuki & Sakurai, 2011) have shown great potentials for nucleic acid delivery. Our previous study has shown that chemically modified curdlan can efficiently deliver nucleic acids in vitro and in vivo. (Altangerel et al., 2016; Han et al., 2015) In the present work, we synthesized, for the first time, amphoteric curdlan derivatives bearing both cationic groups and anionic groups in varying ratios. We discovered in the study that the DNA binding ability and transfection efficiency of the novel amphoteric polymers, which are the most essential traits for non-viral vectors, depend on the substitution ratio between amine group and carboxyl group. The novel amphoteric polymers showed negligible cytotoxicity, extraordinary DNA binding ability and high nucleic acid transfer efficacy in vitro.

2. Experimental section

2.1. Materials and methods

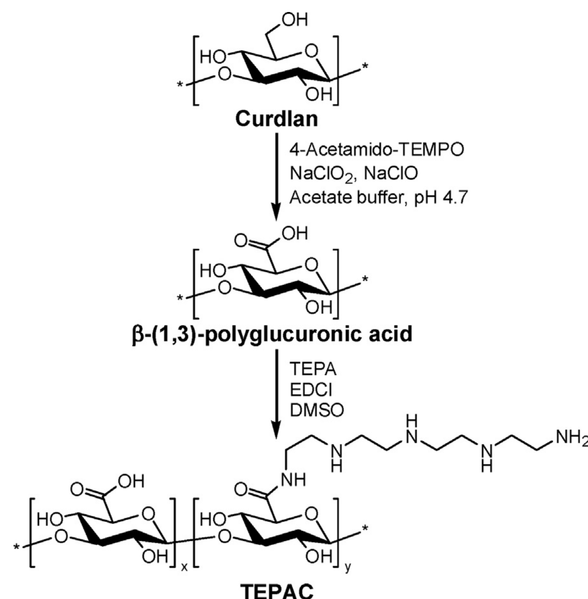
4-Acetamido-TEMPO, tetraethylenepentamine (TEPA), N,N-dimethylethylenediamine, N,N-diethylethylenediamine, sodium chlorite ($NaClO_2$) and sodium hypochlorite (NaClO) solution were purchase from Aladdin (Shanghai, China). Curdlan was purchased from Wako Pure Chemical Industries, ltd. (Osaka, Japan). Dimethyl sulfoxide (DMSO) was dried over 4 A^o molecular sieve. Dialysis tube (cut-off molecular weight (MWCO) of 3500; Spectrum Laboratories, Inc.) was used for the purification of curdlan derivatives. Gel permeation chromatography (GPC) was performed on a Waters HPLC instrument equipped with Ultrahydrogel^{TM} 500 (7.8 × 300 mm) and Ultrahydrogel^{TM} 250 (7.8 × 300 mm) columns (Waters) using PBS buffer (pH7.2) as eluent. ^{13}C NMR was recorded on a Brucker 500 NMR spectrometer. Chemical shifts (δ = 0 ppm) were referred to TMS with the residual proton of the deuterated solvent.

2.2. Synthesis of β-(1,3)-polyglucuronic acid sodium salt

β-(1,3)-polyglucuronic acid sodium salt was synthesized according to the previous report. (Watanabe, Tamura, Saito, Habu, & Isogai, 2014) Briefly, curdlan (500 mg) was suspended and stirred in 10 mL 0.2 M acetate buffer (pH 4.7) containing 96 mg (0.45 mmol) 4-acetamido-TEMPO and 680 mg (7.5 mmol) $NaClO_2$. After 10 min, 620 μL (1.5 mmol) NaClO was added and stirring was continued for 2 days at 35 °C. Then, 20 mL ethanol was poured into the reaction flask and the precipitate was collected and washed for 5 times with ethanol. The solid was dried at 50 °C under reduced pressure to give white powdery β-(1,3)-polyglucuronic acid sodium salt (576.3 mg). Yield: 94.5%. For conjugation of various amines, β-(1,3)-polyglucuronic acid was prepared as following: A solution of β-(1,3)-polyglucuronic acid sodium salt (PGA-Na, 200 mg) in pure water (5.0 mL) was applied to an ion exchange chromatography (H type) to give protonated product (150 mg). Yield: 85%.

2.3. Synthesis of TEPAC polymers

β-(1,3)-polyglucuronic acid (50 mg, 0.28 mmol) was dissolved in dimethyl sulfoxide (DMSO, 2 mL) under nitrogen protection. Then 1-*E*thyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 544 mg, 2.8 mmol) was added and the solution was stirred for 15 min. TEPA (808 μL, 4.26 mmol) was added and the reaction mixture was stirred for 40 h. The product was purified by dialysis (MWCO 3500) against pure water and freeze dried to give TEPAC-1 (60 mg) in 77.8% yield. TEPAC-2 and TEPAC-3 were synthesized in the same way by lowering feeding ratio of TEPA.



Scheme 1. Synthesis of amphoteric curdlan derivatives TEPAC polymers.

2.4. Electrophoretic mobility shift assay

2.0 μL of pcDNA-eGFP plasmid (445 ng/μL) was mixed with stock solutions of TEPAC polymer and 6AC-100 polymer (6-amino-6-deoxy-curdlan). The mixtures were pre-incubated for at r.t for 20 min before analyzing gel mobility shift by agarose gel electrophoresis (2%). The gel was stained using ethidium bromide (0.5 μg/mL) and the image was captured using a Gel Logic 212 PRO imaging system (Carestream, Toronto, Canada).

2.5. Buffering efficiency

The buffering capacity of polymers was determined by acid-base titration. TEPAC polymers were dissolved in 15 mL dH_2O and the pH of the solution was increased to 10.0 with 1 M NaOH, followed by titrating with 0.1 M HCl. Buffer capacity (β) was calculated as previously described, using the following equation: $\beta = \Delta Amol/\Delta pH$ (ΔA mol is the change of the moles of acid added). (Fischer et al., 2002; Shi et al., 2013)

2.6. Measurement of particle size and zeta potential

TEPAC polymer/pcDNA-eGFP complexes were prepared at different weight ratio and the complexes were incubated for 20 min at room temperature. The particle size and zeta potential were measured on a Zetasizer Nano S instrument (Malvern Instrument, UK).

2.7. Cell culture and cytotoxicity measurement

293T cells, A549 cells, HeLa cells, HepG2 cells were cultured in DMEM/High glucose with 10% FBS containing 100 IU/mL penicillin, 100 μg/mL streptomycin under 5% CO_2 atmosphere at 37 °C. The cytotoxicity of TEPAC polymers was evaluated using MTT assay. Cells were planted at a density of 1.0×10^4 cells per well in 96-well plates. After the confluency was reached 70%, the culture medium was replaced with 100 μL medium containing different concentrations of TEPAC polymers solutions arranging from 0.2 μL to 3.0 μL of stock solution (2.0 mg/mL). 24 h later, cell viability was assayed using the MTT test.

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