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Biocompatible polymeric nanocomplexes as an intracellular stimuli-sensitive prodrug for type-2 diabetes combination therapy

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ABSTRACT

Combination therapy is usually considered as a promising strategy owing to its advantages such as reduced doses, minimized side effects and improved therapeutic efficiency in a variety of diseases including diabetes. Here we synthesized a new highly intracellular stimuli-sensitive chitosan-*graft*-metformin (CS-MET) prodrug by imine reaction between oxidative chitosan and metformin for type 2 diabetes (T2D) therapy. Hypothetically, CS-MET functions dually as an anti-diabetes prodrug as well as a gene delivery vector without superfluous materials. CS-MET formed nanocomplexes with therapeutic gene through electrostatic interactions and entered cells by Organic Cation Transporter (OCT)-independent endocytosis. The incorporation of metformin into chitosan has been found to increase endosomal escape via the proton sponge effect. When vector carrying a short-hairpin RNA (shRNA) silencing sterol regulatory element-binding protein (SREBP), a major transcription factor involved in *de novo* lipogenisis, it reduced the SREBP mRNA and proteins efficiently. Furthermore, by intraperitoneal injection, CS-MET/shSREBP nanocomplexes effectively knocked down SREBP in livers of western-type diet (WD)-induced obese C57BL/6J mice, markedly reversed insulin resistance and alleviated the fatty liver phenotype without obvious toxic effects. Thus we were able to show that the intracellular stimuli-sensitive CS-MET prodrug renders a potential platform to increase the anti-diabetes activity with synergistic enhancement of gene therapy.

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1. Introduction

Given the rise of sedentary lifestyles and obesity combined with genetic predisposition, diabetes represents one of the most prevalent metabolic diseases and is becoming a serious threat to global health [1,2]. Type 2 diabetes (T2D), accounting for more than 90% of all diagnosed diabetes cases, is characterized by insulin resistance in peripheral tissues and impaired glucose homeostasis. Current therapeutic treatments include publicity for education, meal plan, enhancement of physical activity, drug treatment, condition

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monitoring and so on. As T2D is polygenic in origin, drugs selective for single molecular targets are, not surprisingly, less dominant than combination therapy. As a cornerstone treatment for T2D, metformin treatment was applied for most of T2D patients attaining glycemic control [3–5]. There are still needs for additional treatment, since the combination therapy was often superior to metformin monotherapy [3,6].

With the increasing knowledge on the biochemical and cellular level, gene therapy has become a promising strategy for treating T2D [7–13]. However, the application got stuck owing to low delivery efficiency and safety issues [9,14]. To achieve successful gene therapy, development of safe and efficient gene delivery systems could be one of the most straightforward solutions. Recently a number of non-viral gene carriers have been developed due to their merits such as ease of preparation, high stability, good safety and facilitated modification [15–17]. As a natural non-toxic polysaccharide, chitosan is a biocompatible polymer and has been widely used as a non-viral vector [18,19]. Nevertheless, the usage is restricted due to low transfection efficiency [20]. In order to enhance the transfection efficiency, we intended to graft metformin to oxidized chitosan by an imine reaction to prepare a new carrier, namely metformin grafted chitosan delivery system (CS-MET). The grafted metormin contributes amines with pKa values spanning the physiological pH range to CS-MET, which is beneficial to endosomal escape and to enhance transfection efficiency [21].

Complex diseases such as diabetes, cardiovascular diseases manifest themselves in a way of multiple gene mutations combined with environmental factors. Choose right candidate for gene therapy is vitally essential. SREBP, a sterol regulatory element-binding protein, plays a central role in cholesterol and fatty acid metabolism [22,23]. Many reports indicate that inhibition of SREBP either by small molecules or gene disruption renders health benefits to animals with hyperlipidemia and greatly lowers the risk of T2D [24–26]. Thus, an shRNA construct silencing SREBP was applied in this study as a T2D gene therapy tool.

We hypothesized that by using biocompatible prodrug CS-MET as a gene delivery system, the synergistic effects of both metformin and SREBP silencing could be achieved, thus leads to better efficacy, as shown in Fig. 1. Indeed, the expression of SREBP (and other relative lipogenic) mRNAs and proteins are reduced in CS-MET/shSREBP treated western-type diet fed C57BL/6j mice, indicative of efficient gene delivery; we further demonstrated that CS-MET provided an effective platform for improving glucose and lipids homeostasis in animal models without obvious toxic effect.

2. Materials and methods

2.1. Materials

Metformin (MET) and chitosan (CS) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolm; MTS] was obtained from promega (promega, USA). PEI 25K was obtained from Wako (Osaka, Japan). Dulbeco's modified eagle medium (DMEM) was obtained from KeyGEN Biotech (KeyGEN, China) and fetal bovine serum (FBS) was obtained from HyClone (Thermo Fisher Scientific, USA). Trypsin-EDTA solution (0.25%) was obtained from Gibco (Burlington, ON, Canada). All other chemicals were analytical grade and applied without further purification. To generate shRNA targeting GFP, forward primer 5′-GATCCGGCTACGTCCAGG AGCGCAttcaagagaTGCGCTCCTGGACGTAGCCTTTTTTGGAAA-3′ and reverse primer 5′-AGCTTTTCCAAAAAAGGCTACGTCCAGGAGCGCAtctcttgaaTGCGCTCCTGGACGTAGCCG-3′ were annealed and clone into pSilencer. Hygro vector with BamH I-Hind III sites. To generate shRNA targeting SREBP, forward primer 5′-GATCCGGAGCCATGGATTGCACTTttcaagagaAAGTGCAATCCATGGCTCCTTTTTTGGAAA-3′ and reverse primer 5′-AGCTTTTCCAAAAAAGGAGCCATGG ATTGCACTTtctcttgaaAAGTGCAATCCATGGCTCCG-3′ were annealed and clone into pSilencer. Hygro vector with BamH I-Hind III sites. Plasmids were propagated in *Escherichia coli*, extracted by the alkali lysis technique, and purified by a E.Z.N.A.® Fastfilter Endo-free Plasmid Maxi kit (Omega, USA).

2.2. Synthesis of CS-MET prodrug

CS-MET was synthesized by an imine reaction between oxidative chitosan and metformin. Briefly, chitosan was first oxidized with different amounts of periodate at different reaction temperatures for different time to synthesis chitosans with different oxidation degrees under N_2 protection. The oxidative chitosans further reacted with metformin, generating a series of CS-MET.

2.3. Protection and release assay

Protection and release of shRNA in CS-MET complexes were measured using Gel electrophoresis assay. Briefly, 1 μL of DNase I (2 units) or PBS in DNase/Mg^{2+} digestion buffer (50 mM Tris–HCl, pH 7.6 and 10 mM $MgCl_2$) was added to 4 μL of CS-MET complex solution (CS-MET:shRNA ratio 20:1, w/w) or to 0.2 μg of naked plasmid DNA, and incubated at 37 °C with shaking at 100 rpm for 30 min. For DNase I inactivation, all the samples were treated with 4 μL EDTA (250 mM) for 10 min at 65 °C and mixed with 1% sodium dodecyl sulfate (SDS), dissolved in 1 M NaOH at a final volume of 18 μL. Then the samples were incubated for 2 h at room temperature, and electrophoresis was performed in 1% agarose gel with TAE running buffer for 20 min at 100 V. The image was captured through gel image system (Tanon 1600, China).

2.4. Cell culture

A549 (human lung carcinoma cells), Hepa 1-6 (mice hepatoma cells), SMCC-7721 (human hepatoma cells), HepG2 (human hepatoma cells) and HL-7702 (human liver cells) cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone) containing 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin. All cells were conditioned at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. In vitro cytotoxicity

The cytotoxicity of polymers against SMCC-7721, Hepa 1-6, HL-7702 and A549 was evaluated by MTS assay. The cells were seeded into 96-well plates (1×10^4 cells/well) and incubated overnight. CS-MET, chitosan, metformin and PEI 25K polymers at predetermined concentration (5–100 μg/mL) were added to each well and incubated for 24 h. For MTS assay, 20 μL of Cell Titer 96® A_{queous} One Solution Reagent was added and incubated for extra 4 h. Absorbance was measured at 490 nm using an ELISA plate reader (Thermo Scientific Multiskan Go, USA). Cell viability was calculated by $(Abs_{sample} - Abs_{blank})/(Abs_{control} - Abs_{blank}) \times 100\%$.

2.6. In vitro transfection efficiency studies

In vitro transfection efficiency was evaluated both by luciferase activity (pGL3) and GFP gene expression assays. Briefly, SMCC-7721, Hepa 1-6, HL-7702 and A549 cells were seeded in 24-well plates at an initial density of 1×10^5 cells/well. After 18 h incubation, the medium was replaced with serum-free media containing different polymer/pGL3 (1 μg) complexes weight ratio (CS-MET: 20:1; CS: 30:1; PEI 25K: 1:1; CS: 20:1; Lipofectamine®2000: according to

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