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The effect of molar mass and degree of hydroxyethylation on the controlled shielding and deshielding of hydroxyethyl starch-coated polyplexes

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ABSTRACT

PEGylation is currently the gold-standard in shielding cationic DNA-polyplexes against non-specific interaction with blood components. However, it reduces cellular uptake and transfection, in what is known as the "PEG-dilemma". In an approach to solve this problem we developed hydroxyethyl starch (HES)-shielded polyplexes which get deshielded under the action of alpha amylase (AA). In this study, the effect of molar mass and degree of hydroxyethylation on the shielding and deshielding of the polyplexes as well as their *in vivo* performance were investigated. For this purpose, a battery of HES −polyethylenimine (PEI) conjugates was synthesized, and their rate and extent of biodegradation were investigated using asymmetric flow-field flow fractionation (AF4) and quartz-crystal microbalance with dissipation (QCM-D). Additionally, the transfection efficiency of the polyplexes was tested in Neuro2A cells and tumor-bearing mice. AF4 and QCM results show a rapid degradation for HES with lower degrees of hydroxyethylation. Meanwhile, *in vitro* transfection experiments showed a better shielding for higher HES molar masses, as well as deshielding with a significant boost in transfection upon addition of AA. Finally, *in vivo* experiments showed that the biodegradable HES markedly reduced the non-specific lung transcription of the polyplexes, but maintained gene expression in the tumor, contrary to the non-degradable HES and PEG controls, which reduced both tumor and lung expression. This study shows that by controlling the molecular characteristics of HES it is possible to engineer the shielding and deshielding properties of the polyplexes for more efficient gene delivery.

1. Introduction

Designing carriers for safe and efficient delivery of therapeutic genes offers great potential for the treatment of many difficult-to-cure diseases, such as metastatic cancer. Although the use of viral carriers shows very high efficiency in gene transfection, their application is limited due to several safety concerns, most importantly the potential for flawed insertion of the virally-carried genetic material into the human genome, associated with the risk of developing cancer [1,2], as well as the high incidence of immunogenic responses to recombinant viruses [3–5] possibly leading to death [6]. Meanwhile, polymeric carriers are extensively studied as possible alternatives to viral carriers, with polyethylenimine (PEI) as the gold-standard, since it shows a high nucleic acid transfection efficiency [7]. The latter efficiency is due to 1) its high ability to compact DNA into nano-sized particles, 2) the high PEI-mediated cellular uptake due to its high surface charge, and 3) its capacity for endosomal release [7–9]. However, PEI-based polyplexes show non-specific interactions with blood components and cells, leading to aggregation and accumulation in the lung [10]. Despite the successful use of polyethylene glycol (PEG) to reduce these effects [11,12], PEGylation compromises the cellular uptake and endosomal release, leading to lowered transfection efficiency *in vitro* and *in vivo* [13], in what is known as the "PEG-dilemma" [14]. Accordingly, several groups developed sheddable PEG-coats by

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incorporating labile linkers, which lead to shedding the disturbing PEG molecules in response to several stimuli, such as temperature, pH, reducing environment or tumor-specific enzymes [15–22].

We previously described an alternative approach for the controlled shielding and enzymatically-catalyzed deshielding of polyplexes using hydroxyethyl starch (HES) and α-amylase (AA) [23]. In the later proof-of-concept study, the developed HES-decorated core–shell nanoparticles showed effective shielding and reduced transfection *in vitro*, as well as deshielding and activation after partial cleavage of the HES-coat by AA. In the current study, we investigate the effect of HES′ molecular characteristics, namely molar mass and degree of molar substitution of hydroxyethyl groups on the rate and extent of biodegradation, as well as the shielding and deshielding characteristics of the polyplexes. Additionally, gene expression was evaluated in tumor-bearing mice to show the feasibility of this approach *in vivo*. LPEI and PEG–PEI polymers served as controls in most of the experiments.

2. Experimental section

2.1. Materials

HES70[0.5] with an average molar mass (M_w, nominal value as provided by supplier, number after HES) of 70 kDa and a molar substitution (MS = the mean number of hydroxyethyl groups per glucose unit; nominal value as provided by supplier, number in square brackets) of 0.5 was kindly provided by Serumwerk Bernburg, Germany [23]. HES10[1.0], HES30[0.4], HES30[1.0], HES60[0.7], HES60[1.0] and HES60[1.3] were kindly provided by Fresenius Kabi, Friedberg, Germany. Linear polyethylenimine (LPEI) with an average molar mass of 22 kDa and the PEG20–PEI conjugate (PEG20: polyethylene glycol with an average molar mass of 20 kDa) were synthesized as described in Ref. [24]. α-amylase (AA) from porcine pancreas was bought from Sigma–Aldrich (Steinheim, Germany, catalog number A3176). Plasmid pCMVluc [25] was prepared by PlasmidFactory, Bielefeld, Germany. Phadebas® Amylase Test was purchased from Magle AB, Lund, Sweden. Other solvents and chemicals were reagent grade and were used as received.

2.1.1. Synthesis and characterization of HES–PEI conjugates

The different HES molecules were used to synthesize a library of HES–PEI conjugates, which were characterized according to Ref. [23]. PEI was attached to HES via Schiff's base formation between HES′ reducing ending group and PEI's amino groups, followed by reductive amination. Briefly, 50 mg linear PEI were mixed with HES in 150 mM PBS buffer (pH 7.4). After 2 h, 59.8 mg of $NaBH_3CN$ were added for reductive amination over 20 h. Ion exchange chromatography was carried out to remove unbound HES using a cation-exchange column (Bio-Rad Macro-Prep high S HR 10/10, Hercules, CA, USA) and fractionated using a sodium chloride gradient from 0.5 M to 3.0 M NaCl concentration in 20 mM HEPES, pH 7.3. The collected fractions were dialyzed against highly purified water (Cellu Sep T1, nominal MWCO 3500 Da, Membrane Filtration Products Inc, Seguin, TX, USA), then lyophilized. HES–PEI copolymers were characterized by ^{1}H NMR, colorimetry (using copper assay), and SEC as previously described [23].

2.1.2. Investigation of the biodegradation of HES homopolymers with AA using AF4-MALS

Several HES homopolymers differing in their molar mass and molar substitution were dissolved at a concentration of 5 mg/mL in PBS pH 7.4. 100 μL sample volume was injected into the AF4 channel assembly, with a regenerated cellulose ultrafiltration membrane (Wyatt Technology, cut-off 5 kDa). All samples containing pancreatic AA were adjusted to an enzyme activity of 100 U/L using the Phadebas® Amylase Test. Mixtures were incubated at 25 °C, and samples were withdrawn at time points 0, 0.5, 1, 2, 4, 6, and 24 h. To stop the enzymatic degradation of HES, samples were heated to 99 °C for 3 min. All samples and controls (without AA) were treated under aseptic conditions with sterile filtration to prevent possible degradation caused by microbial contamination. The reduction in the molar mass of HES was followed by the Wyatt Eclipse 2 AF4 system (Wyatt Technology Corp., Santa Barbara, CA) in combination with MALS (DAWN EOS MALS, Wyatt Technology Corp.)- and RI-detection (Agilent Technologies, Palo Alto, CA).

2.1.3. Quartz-crystal microbalance with dissipation (QCM-D)

The Q-Sense E4 instrument (Q-Sense, Gothenburg, Sweden) was used for the investigations on the enzymatic degradation of HES in the different HES–PEI conjugates. Prior to each measurement, the silica-coated QCM-D sensor crystals (QSX 303, Q-Sense) were washed with 2% SDS solution and treated with oxygen plasma (0.4 mbar, 150 W) for 45 min (TePla 100 System, Feldkirchen, Germany) to decontaminate the crystal surface. The system was operated at 25 °C in the flow mode, interrupted by phases of no flow. A single QCM-D run comprised the following five steps: 1) system rinsing with buffer (15 min), 2) polymer adsorption onto the SiO_2 sensor (5 min sample flow, 10 min no flow), 3) system rinsing with buffer under flow (15 min), 4) start of enzymatic degradation by supplementation of α-amylase (5 min sample flow, 55 min no flow), and 5) system rinsing with buffer under flow (15 min). A battery of HES–PEI copolymers was tested with the special focus on HES′ molar mass and degree of hydroxyethylation (HES30[0.4]–PEI, HES30[1.0]–PEI, HES60[0.7]–PEI, HES60[1.0]–PEI, HES60[1.3]–PEI and HES70[0.5]–PEI). LPEI and PEG20–PEI served as controls. All polymers were applied at a concentration of 100 μg/mL (based on LPEI) in HBG pH 7.1. The enzyme activity was set to 100 and 300 U/L (according to Phadebas® Amylase Test). To rule out polymer desorption, and to prove the enzyme-specific degradation, bovine serum albumin (BSA) was applied instead of AA as a negative control. The Sauerbrey equation [26] was used to follow the adsorbed and desorbed mass onto the silica-coated quartz crystal. Changes in the mass Δm [ng/cm^2] on the quartz surface are defined as:

$$\Delta m = \frac{-C \times \Delta f}{n}$$

whereas C is the mass-sensitivity constant (17.7 ng Hz^{-1} cm^{-2} for the 5 MHz quartz crystal), Δf [Hz] is the resonance frequency and $n = 1, 3, 5, 7$ is the overtone number. In the present analysis, the low overtone number 3 was used to avoid underestimation of the mass. QSoft 4.01 software was used for data acquisition, QTools for data analysis (both from Q-Sense, Sweden).

2.1.4. Preparation of HESylated polyplexes

Naked LPEI-polyplexes (nPx) were prepared by mixing of PEI to the plasmid pCMVluc (pDNA) to a final DNA concentration of 20 μg/mL in HBG pH 7.4 at N/P ratio of 6.0, then incubated at room temperature (RT) for 30 min prior to analysis. For instance, naked polyplexes (nPx) were composed of 20 μg DNA and 16 μg PEI. HESylated polyplexes (and PEGylated control particles) were produced in the same fashion as nPx, with the exception that the unmodified PEI was partially replaced by HES–PEI or PEG–PEI, e.g. HES70[0.5]–PEI complexes with DNA at the molar ratio of HES–PEI to free PEI of 10:90 were made of 20 μg DNA, and a mixture of 14.4 μg free PEI and an amount of 8.475 μg HES70[0.5]–PEI equivalent 1.6 μg PEI. Similarly, polyplexes for *in vivo* experiments were made at a final DNA concentration of 200 μg/mL and N/P ratio 6.0.

2.1.5. Treatment of polyplexes with pancreatic α-amylase (AA)

The effect of pancreatic AA on the biophysical properties of polyplexes with HES60 decoration was investigated. HES60-decorated polyplexes were generated at the DNA concentration of 20 μg/mL in HBG pH 6.0, and at the N/P ratio of 6.0. HESylated polyplexes were prepared at the molar ratio 10:90 of PEI-conjugates to free PEI. After 30 min incubation of the polyplexes at room temperature, 100 μL of AA stock solution (amylase activity 1000 U/L) was added to 900 μL polyplex solution to give a final AA activity of 100 U/L, mixed intensively, and the resulting AA-polyplex mixture was analyzed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) instantly after combination of the enzyme and substrate. Analysis of particle size and zeta-potential of PEI/DNA complexes was performed at time points 0, 0.25, 0.5, 1, 2, 4, and 6 h holding the polyplexes at 37 °C. Measurements of the particle size and the zeta-potential were conducted in semi-micro PMMA disposable cuvettes (Brand, Wertheim, Germany) and in folded capillary cells (Malvern Instruments, Worcestershire, United Kingdom), respectively.

2.1.6. Cell culture experiments

Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Life Technologies (Karlsruhe, Germany). Cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. Murine neuroblastoma, Neuro2A (ATCC CCl-131, purchased from DSMZ, Braunschweig, Germany) was cultured in Dulbecco's Modified Eagle Medium (DMEM). DMEM was supplemented with 10% FCS, 4 mM stable glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.1.7. In vitro luciferase reporter gene expression studies

In vitro pDNA transfection efficiency was evaluated in murine Neuro2A cells. Experiments were performed in 96 well plates by seeding 1×10^4 cells per well in 100 μL medium 24 h prior to transfection. Directly before transfection, the medium was exchanged against 90 μL fresh medium with/without pancreatic AA (100 U/L). An amount of 10 μL polyplex solution (N/P 6.0, 20 μg/mL DNA concentration, 10% and 25% molar ratio of conjugate to free PEI) was added to the cells. 4 h after transfection, the medium was replaced by fresh medium with/without AA. 24 h after pDNA transfection, the cells were treated with 100 μL cell lysis buffer (25 mM Tris pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100). Luciferase activity in 35 μL cell lysate was measured in white 96 well plates using a luciferase assay kit (100 μL Luciferase Assay buffer, Promega, Mannheim, Germany) on a luminometer for 10 s (Centro LB 960 instrument, Berthold, Bad Wildbad, Germany).

2.1.8. Metabolic activity of transfected cells

The cellular metabolic activity after pDNA transfection was evaluated using MTT assay. Cells were seeded and transfected as explained above. 24 h after transfection, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich, Germany) was dissolved in phosphate buffered saline at 5 mg/mL,