Biodegradable Starch Derivatives with Tuneable Charge Density – Synthesis, Characterization and Transfection Efficiency

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Abstract

Regioselective oxidation of water-soluble starch and conversion with alkyl diamines resulted in defined cationic starch derivatives. Those were assessed in their potential for polyplex formation, biocompatibility and transfection efficacy. The new polymers have the advantage of being biodegradable, being not cytotoxic at rather high concentrations (LC_{50}>400µg/ml) for C2 substitution and reach transfection efficiencies comparable to commercial transfection reagents. The polymer with the highest transfection efficacy is a C12 substituted polymer (degree of substitution = 30%) at N/P 3. The LC_{50} value of that highly modified polymer is still one order of magnitude lower than that of PEI 25kDa.

Key words: Polysaccharides, Polymeric Nanoparticles, plasmid DNA, Gene Therapy

Introduction

The delivery of nucleotides into cells is still a challenging task. Despite many research activities, there is still no gene delivery system available, that has a good transfection efficacy and satisfactory safety profile *in vivo*. Many polymeric materials have been and are still under investigation for gene delivery such as poly(ethylenimine) (PEI) [1], poly(dimethylaminoethyl methacrylate) [2], poly(amidoamine) (PAMAM) dendrimers [3], poly-lysin [4, 5] and chitosan [6–8]. Typical short-comings of the existing cationic polymers are a relatively low transfection efficacy [9], often caused by the cytotoxicity of high cationic charge density, as well as non-biodegradability.

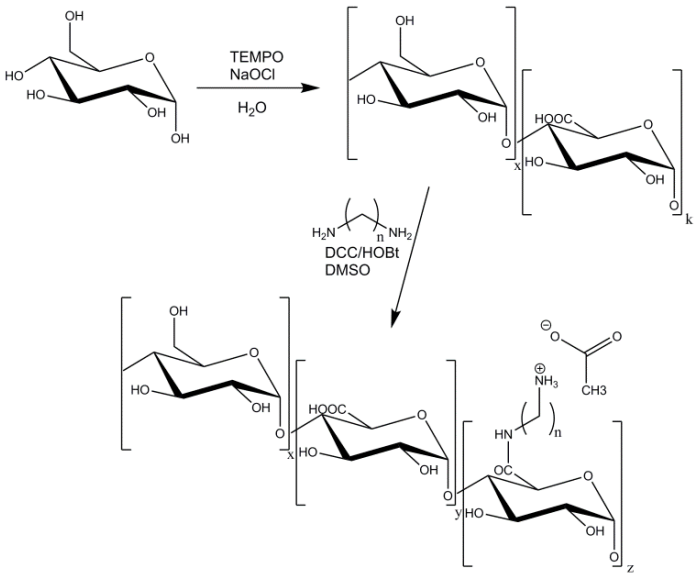
Cationic starch derivatives are commonly used in the paper industry [10] and for flocculation [11]. Regarding the excellent biocompatibility of starch, it surprises that the potential of cationic starch derivatives for gene delivery is only explored in recent times by few research groups. Amylopectin has been previously modified at random positions with cationic side chains and tested for nucleic acid delivery by Zhou et al. [12]. However in this process it was not possible to control the site and degree of substitution (DS) in the polymers. Our group has recently published the synthesis of starch with low molecular weight PEI side chains [13]. These cationic starch derivatives worked well for transfection and remained amylase cleavable, however had still a considerable level of cytotoxicity. The cationic surface functionalization of nanodendritic starch [14], cationic dendronization of amylose functionalized with azido groups and then conjugated with propargyl focal point PAMAM [15], water soluble starch with modified with quaternary amine groups [16] and conjugation of hydroxyethylstarch (HES) with 22 kDa L-PEI [17] are recently published strategies to use starch based excipients for nucleotide delivery.

Here it was our approach to construct a biodegradable polymer with tunable charge density with short diamine side chains on the starch. The aim was to achieve efficient delivery of pDNA with a much safer polymer. Our hypothesis was that tuning the charge density would allow to reach optimum nucleotide condensation and transfection efficacy with minimum cytotoxicity, and moreover keeping starch´s advantage of biodegradability. Further we also varied the length of the alkyl spacer to evaluate the impact of side chain flexibility.

Experimental

Cationic starch synthesis, purification and characterization

Synthesis of cationic starch derivatives was performed in a two-step procedure as shown in Scheme 1. The starting material was partially hydrolysed starch with a molecular weight (MW) > 1,500 kDa (AVEBE, Vendaam, NL). In the first step of the reaction the primary hydroxyl groups are oxidized with TEMPO/NaOCl to carboxylic acids.



Scheme 1 Synthesis of cationic starch derivatives with tuneable degree of substitution and variable spacer lengths with n = 2, 6, 12

The oxidation step was carried out as described by Bragd et al [18]. During this reaction the degree of substitution can be easily controlled by varying the amount of the oxidizing agent sodium hypochlorite between 0.25 and 2 equivalents per anhydroglucose unit. Coupling of the diamine compound was done using DCC/HOBt in DMSO. Briefly, the carboxylic acid of the polymer was converted into the free acid using a Dowex cation exchanger resin and then reacted with 1.5 equivalents of coupling reagents for activation. In a typical reaction 5 g of free acid were dissolved in 200 ml of DMSO. 1.5 equivalents of DCC and HOBt were added as solid and stirred at 50°C for 24h to activate the acid. After addition of the diamine the reaction mixture was stirred at 50°C for 2 days. The reaction mixture was diluted with water and acidified with acetic acid to pH = 5. Insoluble material was removed by filtration and the remaining yellow solution purified by tangential flow filtration using a Vivaflow 200 system (Sartorius) over a 5 kDa membrane (Hydrosart®, Sartorius) and characterized by NMR, UV, IR and SEC measurements. 1H-NMR spectra were collected using a Bruker Biospin spectrometer NMR Magnet System 400 MHz

Ultrashield plus in D_2O. IR measurements were performed in solid state (Spectrum 400 FT-IR spectrophotometer, Perkin Elmer). SEC measurements (Tosoh Bioscience EcoSEC) were done using SupremaMax columns 1000 (PSS, Mainz, Germany) at 35°C and 0.1% TFA in water as eluent.

Table 1 Synthesized cationic starch derivatives with ethylene diamine as ligand

DS (COOH) before reaction [*]	DS (amine) after reaction[**]	Conversion	Yield	MW[***]
5%	5%	96%	82% (6.9g)	n.d.
15%	15%	98%	77% (6.8g)	n.d.
22%	21%	97%	63% (5.8g)	27.6 kDa
26%	25%	94%	76% (3.5g)	42.8 kDa
38%	37%	96%	73% (8.0g)	92.9 kDa
44%	42%	95%	83% (5.0g)	63.5 kDa
54%	52%	96%	n.d.	83.4 kDa
62%	61%	97%	90% (11.0g)	91.6 kDa

[*] Molecular weight determined by Blumenkrantz assay [19]

[**] determined by trinitro toluene sulfonic acid (TNBS) assay [20]

[***] determined by SEC,

n.d.: not determined

Table 2 Synthesized cationic starch derivatives with alkyl diamine ligands at constant DS ≈ 30%

DS (COOH) before reaction	DS (amine) after reaction	Conversion	Yield	Ligand
32%	30%	96%	78% (6.8g)	C2
32%	30%	95%	85% (7.9g)	C6
30%	29%	97%	82% (9.6g)	C12

In-vitro assays for cytotoxicity and α-Amylase degradation of the polymers

The synthesized polymers were tested regarding their cytotoxicity on A549 and HEK293 cells. Standard test protocols such as MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich) ViaLight™ (Lonza), and Calcein staining (LIVE/DEAD® Viability/Cytotoxicity Kit, Thermo Fisher Scientific) were used. All toxicity studies were performed in full cell culture medium including 10% FBS without phenol red. The enzymatic degradability of synthesized starch derivatives was measured. In brief, to 40µl starch solution (2mg/ml) 40µl of α-amylase (Type I-A from porcine pancreas, Sigma-Aldrich) was added. The incubation at 37°C was

performed for various predefined time periods before stopping the reaction by 20µl 1M HCl addition. Quantification of polymeric starch was performed by iodine inclusion assay [21] adding 100µl of Iodine reagent and measuring the absorbance (E=580nm). The percentage of degradation per hour was calculated according to a calibration curve (R^2=0.9998) from native starch degradation.

Nanoplex formation and characterization

Formation of nanoplexes occurs upon mixing of pDNA (pGL3, Promega; encoding luciferase protein) solution and the polymer solution. The cationic starch derivative was dissolved at a concentration of 10 g/l in 10 mM HEPES buffer (pH 6.8, 5% glucose). This solution was added to the solution of pDNA (in water, concentration = 1g/l) and followed by immediate vortexing for 30s. Polyplexes were incubated for 30 min at R.T. before use. The trinitro toluene sulfonic acid assay allows determination of the amount of primary amine groups in the polymer and therefore the calculation of the MW per primary amine [20]. This MW was used for calculation of the N/P ratio.

In-vitro assay for and transfection

For transfection studies A549 (DSMZ #ACC107) and HEK293 cells (ATCC-CRL-1573) were used. A549 and HEK293 cells were seeded with seeding densities of $5x10^4$ cells/well and $2.5x10^4$ cells/well, respectively in 24-well plate and grown till 70% confluence. Cells were incubated with the nanoplexes, at different N/P ratios, in serum-free medium for 4h, than cells were washed twice and incubated for further 44h in fresh cell culture medium. Luciferase expression was measured using the Luciferase kit provided by Promega as well as the total protein content (BCA Assay, Sigma-Aldrich) for normalization.

Results

Cationic starch derivatives

The oxidation reaction allows to easily controlling the DS by varying the amount of the NaOCl / anhydroglucose unit. In the second step we coupled a diamine for introduction of the positive charge. This reaction proceeds with almost complete conversion ≥94%, allowing therefore the adjustment of the charge density of the final product in a controlled way. The polymer products ranging from 5-62% DS and their characteristics are listed in Table 1.

Two series of cationic starch derivative were synthesized. In the first set of polymers, ethylendiamine as ligand was coupled to starch to yield materials with the same ligand in different DS. In the second set of polymers we kept the DS constant and coupled alkyl diamines with different alkyl chain lengths, i.e. two, six and twelve methylene groups. Their characteristics are summarized in Table 2.

Cytotoxicity assays

Results of the cytotoxicity assays from the cationic starch derivatives are summarized in Table 3. As expected the LC_{50} values increase with increasing charge density. With increasing degree of modification more cationic charges are introduced leading to an increased cytotoxicity but remaining moderate compared to PEI 25kDa. While the C6 spacer does not increase cytotoxicity, coupling of C12 alkyl spacer leads to an increased toxicity, possibly reflecting some increased perturbation of cellular membrane structures. Thus, it can be stated, that all synthesized polymers are less toxic than the standard branched 25 kDa PEI for which a toxicity of LC_{50} between 10-20µg/ml were reported [22, 23].

Table 3 LC_{50} of polymers on A549 and HEK293 in mg/ml

DS	Ligand	LC_{50} A549 MTT	LC_{50} A549 ViaLight	LC_{50} HEK293 MTT	LC_{50} HEK293 Calcein staining
21%	C2	> 5	> 5	> 5	> 5
42%	C2	≈ 2.0	2.9 ± 0.5	≈ 2.5	≈ 4
61%	C2	0.4 ± 0.03	0.5 ± 0.02	0.4 ± 0.02	1.0 ± 0.4
30%	C2	2.9 ± 0.4	1.3 ± 0.4	≈ 1	2.1 ± 0.2
30%	C6	> 5	> 4	≈ 0.5	4.3 ± 0.4
29%	C12	0.2 ± 0.0	0.1 ± 0.0	< 0.2	< 0.2

Enzymatic degradability by α-Amylase

Incubation of cationic starch derivatives with α-amylase proved that all starch derivatives are biodegradable and that the speed of degradation is dependent from the DS and spacer length (Figure 1).

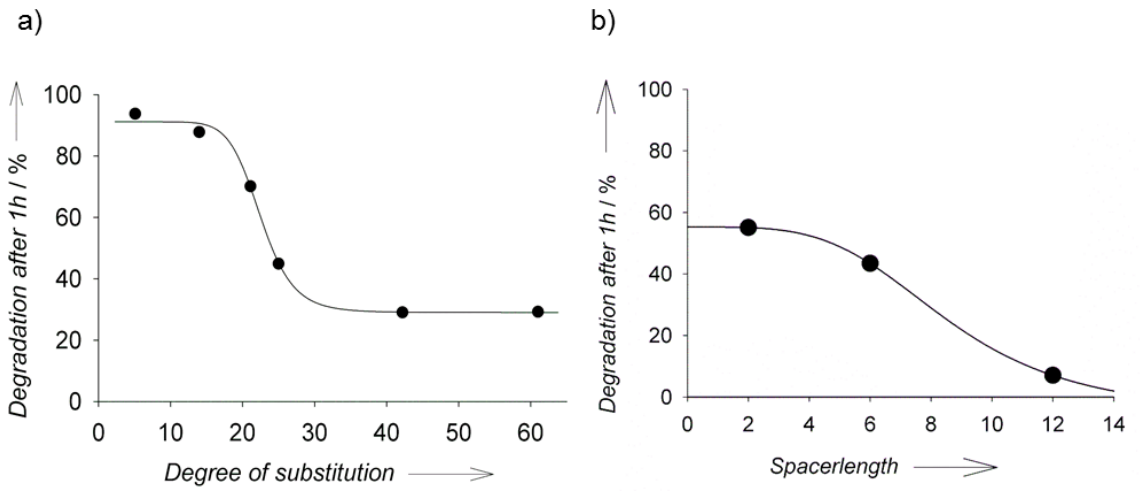


Fig. 1 Starch degradation by amylase after 1h a) variation of DS, b) variation of spacer length (constant DS~30%)

Polyplex formation with pDNA

The synthesized polymers were used for polyplex formation with pDNA. We could show that the ability to form polyplexes is clearly dependent from the DS which corresponds directly to the charge density of the polymer. If the DS is ≤15%, the polymer did not form polyplexes as shown by agarose gel electrophoresis (appendix Fig A1). With increasing DS ≥21%, which means at least every fifth glucose unit is bearing a cationic charge, complexation of pGL3, measured as all pDNA remaining in the agarose gel pocket, is complete at N/P ratio 1 – 2 (appendix Fig A1).

The polyplex size is mainly dependent from the charge density of the used polymer. As shown in Figure 2, the influence of the N/P ratio is less pronounced. Whenever an excess of polymer is used, so that the zeta potential of the resulting polyplexes is positive the particle size is rather constant also with increasing N/P ratio. At N/P ratio close to 1:1, resulting in a zeta potential close to zero, the nanoplexes cannot be stabilized by charge and tend to agglomerate.

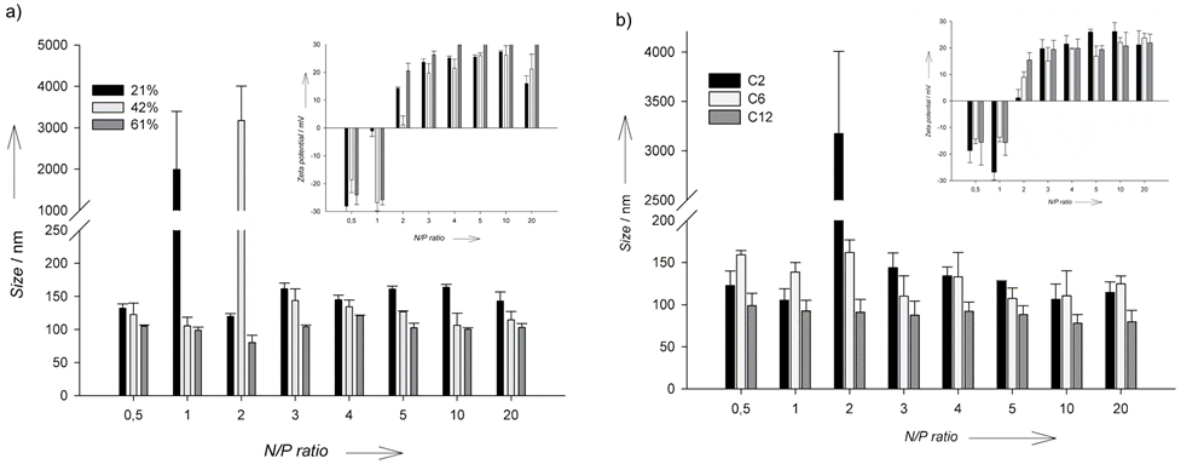


Fig. 2 Size and zeta potential (small diagram) of nanoplexes at N/P ratios 0.5-20, A) Variation of DS of polymer (20, 40 and 60%.), B) Variation of spacer length (C2, C6, C12)

The polyplex size could be varied via the DS or the alkyl chain spacer length. The higher the charge density of the used polymer was, the smaller the resulting polyplexes were due to tighter DNA binding. Additionally a longer spacer group allows on the one hand a higher flexibility of the cationic charge leading to smaller nanoplexes and on the other hand a stronger binding caused by additional hydrophobic interactions.

Transfection efficacy

The ability of the cationic starch nanoplexes to transfect cells was tested on two different cell lines, A549 (adenocarcinoma, human alveolar epithelial cell line) and HEK293 (human embryonic kidney cell line).

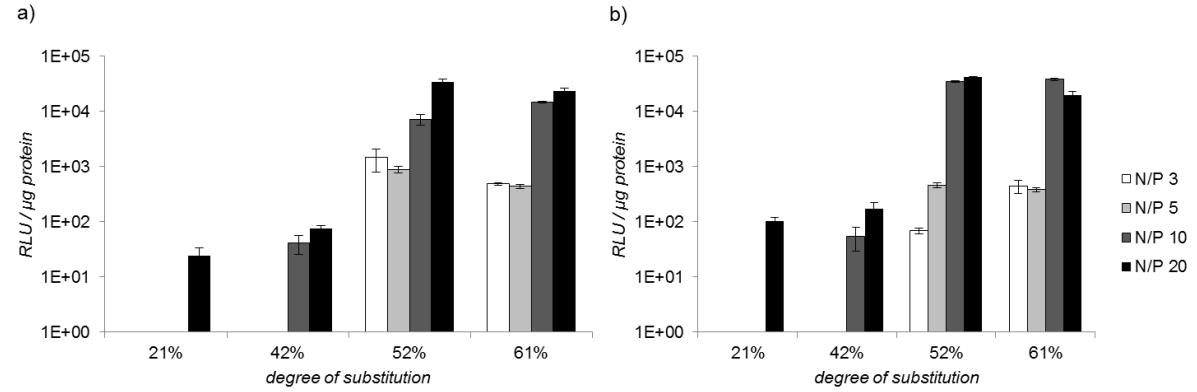


Fig. 3 Luciferase expression normalized to protein content as a function of degree of substitution and N/P ratio a) in A549 cells and b) in HEK293 cells (mean ± SD; n=3)

We could observe that in general the luciferase expression was higher when using a higher N/P ratio (see Figure 3a), except for the polymer with DS = 21%. HEK293 cells were showing a comparable trend but were easier to transfect (Fig 3b). Differences in transfectability between the two cell lines A459 and HEK293, have already been observed earlier [12].

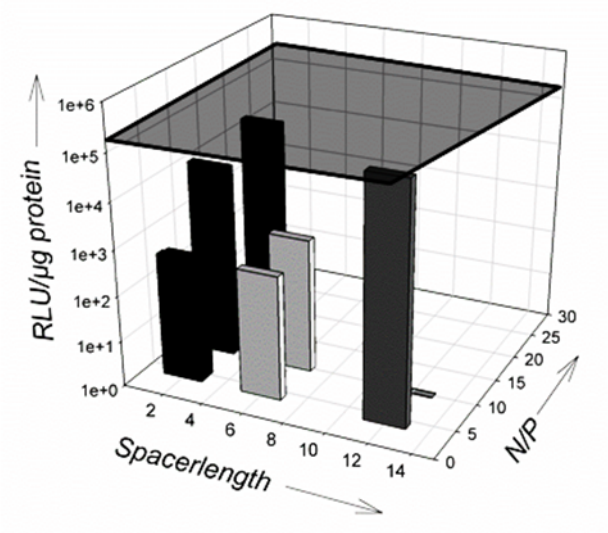


Fig. 4 Luciferase expression in A549 cells normalized to protein content as a function of the spacer length and N/P ratio. DS = 30% and 29% for C12. Results represent the mean of three samples.

The transfection efficacy can be improved by using starch derivatives with a longer hydrophobic side chain. The material with the dodecyl spacer shows a transfection efficacy well comparable with the transfection reagent JetPrime (Polyplus-transfection® SA; Illkirch, France) as shown in Figure 4, marked by gray plane. Notably, maximal transfection was already observed at an N/P ratio of 3, i.e. using a small amount of polymer in the nanoplex.

Discussion

Cationic polymers are known to form polyplexes with pDNA at different N/P ratios. The polyplex stability is a known key parameter for such complexes as both the too early release cause degradation and inefficient transport across the cell membrane, or too tight binding even after transfer into the cytoplasm prevents translation. A

tuneable charge density is therefore favorable. Cationic carbohydrates are known transfection reagents with chitosan and cationic dextran as most used polymers. Our main goal was to improve the biocompatibility. We used the biopolymer starch as starting material for our synthesis. Regarding the long-term safety of novel gene delivery materials biodegradability is a clear advantage. Besides by chemical hydrolysis, starch is a polysaccharide with degradability by human enzymes. Within the amylotic enzymes α-amylase is the key enzyme for starch degradation and was therefore selected to demonstrate the maintenance of enzymatic degradability after derivatization. Main sources of α-amylase are the pancreas and the salivary glands, resulting in its presence in saliva and pancreatic juice, and to smaller extent in blood serum. Some minor α-amylase activity is also present in other tissues including normal lung, adipose tissue and skeletal muscle and there is some documentation of hyperamylasemia in tumors or nonmalignant pulmonary disorders [24, 25]. As expected an increasing DS as well the introduction of a longer alkyl spacer yields in a slower speed of degradation due to suboptimal accuracy of fit in the active site of the enzyme. As shown in Figure 1 there is a dramatic change in degradation speed at a degree of substitution at 20%. This finding may be due to the fact that starting from that DS every fifth or more monomer units is substituted, or in other words at least one functional group per helix turn in the unmodified polymer. Our hypothesis is that due to the high modification, the conformational structure of the polymer undergoes a change in that way that the formation of a helical structure is not possible anymore. Furthermore, the modification with longer side chains slows down the degradation process as the longer hydrophobic alkyl chain have a greater influence on the properties of the polymer than the short ones. A possible explanation for that is again the disruption of the helical structure of amylose due to a high substitution meaning at least one cationic group introduced per turn of the helix or even more. In contrast the modification with low MW PEI at approximately every 8-9th glucose unit [13] left enough regions of original starch structure to maintain a fair enzymatic degradation. The transfection studies were performed in serum-free cell culture medium as the aim was to develop this system for local (e.g. pulmonary) application. Further studies would be needed to see if the transfection efficiency remains in full serum, which would qualify this delivery system also for intra venous administration.

Comparison of cytotoxicity in form of LC_{50} values against other cationic polymers show that the ethylendiamine starches are good biocompatible for polymers of such

MW range. The approach to shorten the side chain in comparison to the 0.8kDa PEI was successful in enhancing the safety profile but on the price of a high DS needed for maintenance of transfection, which reduced the enzymatic degradability.

Conclusion

Our hypothesis was to synthesize a new class of polymers with tuneable charge density to obtain a material that is biodegradable, shows a limited cytotoxicity and is able to condense pDNA for transfection studies. We succeeded in developing a reproducible stable and easy manufacturing process for cationic starch derivatives with tuneable spacer length and charge density. The polymers maintain the enzymatic degradability, and show DS- and spacer-length-dependent increase in cytotoxicity which is overall moderate in comparison to many other cationic polymers. Best derivatives are able to transfect cells in the same order of magnitude than commercial reagents.

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Conflict of interest

All authors declare that they have no conflict of interest.

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