Development of purified glycogen derivatives as siRNA nanovectors

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Abstract

Purified Glycogen (PG) is a highly hyper branched carbohydrate, characterized by high water solubility and very moderate increase in viscosity. The dendrimeric structure of PG, appropriately functionalized, makes it an alternative to current synthetic gene delivery agents. The present study explores the preparation of purified glycogen polycationic derivatives (PGPDs), developed and characterized starting from a single step reaction between PG and *N*,*N*-dialkylamino alkyl halides. Subsequently PGPDs were used for the complexation of a model siRNA nucleic acid, a transfection reagent siRNA and a fluorescein-labelled dsRNA oligomer. PGPDs-siRNA complexes were fully characterized by agarose gel electrophoresis and their efficacy was assessed by both confocal microscopy and transfection assays on breast and renal cancer cells. Results proved that PGPDs-siRNA complexes were efficient and not cytotoxic, maintaining their spherical and dendrimeric structure and, particularly, were able to effectively transfect the target cells by releasing the siRNA.

Keywords: Glycogen; siRNA; Nanocarriers; Gene Silencing; Cationic Polymers, Dendrimers, siRNA Delivery.

1. Introduction

Gene silencing is a general term that describes the epigenetic process of gene regulation, generally used to describe the "shutdown" of a gene by a mechanism other than genetic modification (Han, 2018). Post-transcriptional gene silencing is the result of the destruction or blocking of the mRNA of a gene. The destruction of the mRNA prevents the translation from forming a gene product. The common mechanism of post-transcriptional gene silencing is RNA interference (RNAi) (Agrawal et al., 2003). Therapy using RNAi is one of the most successful new frontiers of gene therapy, as it can act on almost all genes, influencing their behavior. Its highest therapeutic potential can be found in all the diseases that were previously difficult to treat, such as cancer (Pratt and MacRae, 2009). RNAi pathway is based on two steps: in the first step, the trigger RNA is processed into a short, interfering RNA (siRNA), while in the second step, siRNAs are loaded into the effector complex RNA-induced silencing complex (RISC).

siRNA molecules can be highly customizable, adapting perfectly to different genetic targets and leading to a decrease in gene expression. The use of siRNA has already been tested in clinical trials and is effective, however, a further step must be taken by developing a safe and efficient carrier for siRNA that is not of viral origin (Crooke et al., 2018). Delivery of siRNAs at the cellular level encounters numerous difficulties. Two main barriers are encountered during the siRNA delivery process: the extracellular barrier and the intracellular barriers, which are difficult to cross by siRNA because of its characteristics (strong anionic charge, hydrophilicity) (Kim et al., 2019). Considering the natural properties and length of siRNAs (21-23 nucleotides), they are easily recognized by endogenous enzymes in serum and are filtered out by the kidneys, giving a low half-life (Cheng and Lee, 2016). The blood-brain barrier and the immune recognition system prevent siRNA from entering the interior of diseased cells (Morad et al., 2019). Like other molecules, siRNAs have no power to escape the endosomal system, resulting in an additional challenge to reach the cytosol of cells (Du Rietz et al., 2020). Because the limited properties of siRNA hinder its ability to silence genes, many delivery tools have been devised for therapeutic siRNA. To date, siRNA transporter systems are divided into two parts: viral and non-viral. The vector studied should be able to complex the highest amount of siRNA avoiding enzymatic degradation, it should be easily manipulated so that, once the siRNA is complexed, it is able to cross the cell membranes and arrive safely on the cellular targets, releasing the siRNA so that it integrates in the RNAi pathway (Gao et al., 2021).

Numerous studies show that some of the most effective non-viral carriers are cationic polymers.

The most common synthetic polymers used as vectors for siRNA are polyethyleneimine (PEI) (Ndong Ntoutoume et al., 2017), poly(l-lysine) (PLL) (Kodama et al., 2017) and poly(lactic-co-glycolic acid) (PLGA) (De Rosa and Salzano, 2015), which allow numerous delivery applications. Most of these polymers have been shown to be very effective in *in vitro* studies, while there is concern about their use as vectors due to their marked cytotoxicity (as for PEI and PLL). For this reason, research is focused not only on finding a type of carrier that demonstrates the same effectiveness as the existing ones, but that at the same time is less cytotoxic, in order to meet the safety parameters in the drug administration. For this reason, polysaccharide-based delivery systems are seen as the most effective solution, considering their natural characteristics such as high biodegradability and biocompatibility and low values of immunogenicity and cytotoxicity (Mizrahy and Peer, 2012). The main characteristics required of the new polymers are low cost, the ability to maintain specific bioactivity and minimal collateral effects related to their use. The most used polysaccharide polymers of this type have cellulose, starch or glycogen as their basic structure (Wang et al., 2013). In previous studies we have evaluated different natural and synthetic polymers for drug delivery of small molecules, as Dextran (Lopalco et al., 2018), Chitosan (Perrone et al., 2018), Glycogen (Perrone et al., 2017a) and Hydroxypropyl-β-cyclodextrin complexes (Laquintana et al., 2019).

Several works have investigated how glycogen, properly functionalized, shows to be effective in the delivery of nucleic acids in biological tissues for different purposes (Liu et al., 2015) (Kim et al., 2019) (Lan et al., 2020). Based on these data we focused our paper on the applications of a highly purified glycogen-based polysaccharide vector, functionalized to act as a carrier for the delivery of nucleic acids for gene silencing therapy. Glycogen is characterized by a branched chain structure, interspersed with short linear chains. The length of the chains determines the structural properties of the glycogen. The following sections deal with the synthesis and engineering of a polysaccharide structure useful for pharmaceutical applications.

The excipient Polglumyt®, a Purified Glycogen (PG) has been studied in our laboratories as a very efficient vector of genetic material after chemical modification, as it can be designed to comply with safety parameters in the administration of siRNA (Russo et al., 2013). PG is a glycogen derivative extracted from mussels, composed of D-glucose molecules linked by α (1→4) bonds with branches every 5-10 glucose units linked by α (1→6) bonds (Bertoldo et al., 2013). In nature the glycogen is always bound with a protein, the Glycogenin, and the quality of the commercial glycogen depends on the presence of protein residues. PG, as a highly purified form of glycogen, shows a small amount of this protein. The hyperbranched spherical macromolecular structure gives

PG the same chemical-physical properties as synthetic dendrimers. PG has a high solubility in water (30% p/v) accompanied by a low increase in the viscosity of the solution. Pharmacologically, PG has no direct activity on biological structures and is devoid of effects such as increased blood pressure and heart rate (Perrone et al., 2017b).

The present work describes the preparation of Purified Glycogen Polycationic Derivatives (PGPDs), as attractive hyperbranched nanocarriers for complexation of nucleic acids. We have developed and characterized the structure of the derivatives following the derivatization of Purified Glycogen with *N*,*N*-dialkylamino alkyl halides. In addition, the samples have been characterized regarding morphological characteristics and biological interactions. Purified Glycogen polycationic derivatives can be classified into four classes according to the type of amine substitutes introduced in the polymer chains: DiEthylAminoEthyil-PG (DEAE-PG); DiMethylAminoEthyl-PG (DMAE-PG); DiMethylAminoPropyl-PG (DMAP-PG); 2-hydroxyPropyltriMethylAmmonium-PG (2-OH-PTMA-PG). PG polycationic derivatives are prepared by a single-step synthetic process by reacting an alkylamino alkyl chloride with PG in an alkaline aqueous medium (Richard et al., 2013). In this work we have focused our attention on the possibility of intervening through gene therapy on two different types of cancer particularly difficult to treat, and that would benefit from the use of this innovative therapy: breast cancer and renal cancer.

Breast cancer occurs as an uncontrolled formation of tissue within the mammary gland (tissue), consisting of abnormally growing cells that are no longer subject to the mechanisms of cell apoptosis. One of the most recent strategies for breast cancer gene therapy is the use of gene silencing (Khan et al., 2019), for example acting through gene silencing systems directly on p38 mitogen-activated protein kinases (MAPK) (Doğaner et al., 2014) and hdm2 oncogene (Liu et al., 2004), that plays a pivotal role in tumorigenesis of breast tumour.

Renal cell carcinoma is a macro class containing heterogeneous types of tumours, which involve numerous genetic and molecular alterations underlying major histological subtypes (Shuch et al., 2015). Renal cell carcinoma can be categorized as a sporadic or hereditary event, but both forms are associated with structural alterations involving the short arm of chromosome 3 (Srinivasan et al., 2015). Many gene modifications have been associated with renal cell carcinoma. Somatic mutations or epigenetic alterations of *VHL*, a tumour-suppressor gene, are seen in most of clear-cell subtypes (Brugarolas, 2013) (Ibragimova et al., 2013) ("Comprehensive molecular characterization of clear cell renal cell carcinoma," 2013). In the state of research, the most widely used gene silencing therapy for renal cancer focuses on the Erc/mesothelin target, which is a tumor-specific gene expressed in the Eker (*Tsc2* mutant) rat model of hereditary renal cancer (Imamura et al., 2008).

2. Materials and methods

2.1. *Chemicals*

Purified Glycogen (PG, Polglumyt®, from Mythilus Edulis and Mythilus Gallo Provincialis, average mol wt 2.5 ± 0.1 x 10^6 Da) was gifted by A.C.R.A.F. Angelini (Pomezia, ITALY), while 2-Chloro-*N*,*N*-diethylethylamine hydrochloride (DEAE), *N*,*N*-Dimethylaminoethyl chloride hydrochloride (DMAE), *N*-(3-Dimethylaminopropyl) hydrochloride (DMAP) and (3-Chloro-2-hydroxypropyl)trimethylammonium chloride solution (2-OH-PTMA) were purchased by Sigma-Aldrich Merck (Darmstadt, GERMANY). The siRNA filaments of type Luc2-siRNAsens (GCU-AUG-GGC-UGA-AUA-CAAA-A99) and Luc2-siRNAantisens (UUU-GUA-UUC-AGC-CCA-UAG-C99) used were purchased from Kaneka Eurogentec S.A. (Liège, BELGIUM). The Optifect Transfection Reagent® was purchased by Thermo Fisher Scientific (Waltham, US-MA, USA). The AllStars Hs Cell Death siRNA were purchased by Qiagen (Hilden, GERMANY). All other chemicals were analytical grade and were used without further purification.

2.2. *Methods*

^{1}H-NMR spectra were recorded on a Varian Mercury 300 MHz instrument. Chemical shifts were expressed in ppm and referenced by solvent using the residual protic peak (D_2O, 4.80 ppm). FT-IR spectra were sampled in KBr pellets and were recorded on a Perkin Elmer 1600 FT-IR spectrophotometer (Spectrum One). The signals have been reported as λ (nm). In parenthesis the intensity of the peaks: vs (very strong), s (strong), m (medium), w (weak). Freeze-drying was performed using Christ Alpha 1-4 LSC, and all samples were dried for 24 h under reduced pressure (0.016 mbar) at −60 °C. DSC thermograms were acquired by a Mettler Toledo DSC 822e (Stare 202 System) equipped with a thermal analysis automatic program and using indium as internal standard. The data were processed using Microsoft Excel 2010 or GraphPad Software.

2.3. *Synthesis of Purified Glycogen Polycationic Derivatives (PGPDs)*

The Purified Glycogen Polycationic Derivatives (PGPDs) were prepared according to the synthetic procedures reported.

In a 2-necked flask, equipped with a magnetic stirrer and reflux cooler, 10 g of Purified Glycogen (61.73 mmol of glucose) were dissolved in 124 mL of NaOH 1N solution. When the solubilization

was complete, the temperature was brought to 70 °C and left under stirring for 2 hours. For the preparation of DEAE-PG the suitable base DEAE (1 to 124 mmol) was added and was left under stirring overnight at 70 °C. The day after the heating was interrupted, it was brought to room temperature and the reaction raw product was slowly dripped into 400 mL of acetone. After dripping, the suspension obtained was kept under stirring for about half an hour. Then, the stirring was interrupted, left to settle, the supernatant was decanted, and the obtained precipitate was washed twice with acetone (200 mL). The obtained solid was filtered, dissolved in 200 mL of distilled water, neutralized with a 1N HCl solution and dialyzed (15,000 cut-off tubes of regenerated cellulose) against distilled water up to constant conductivity. The obtained solution was filtered at 0.45 μm, concentrated in vacuum and lyophilized. The so-prepared sample was stored at 4 °C until further use.

DMAE-PG, DMAP-PG and PTMA-PG were prepared following the same procedure above reported using DMAE (1- 246 mmol), DMAP (1- 246 mmol), PTMA (1- 246 mmol), respectively, as base.

2.4. *Synthesis of FITC-PGPDs*

In a two-necked flask, equipped with a magnetic stirrer, 500 mg of PGPDS are solubilized in 10 mL of distilled water. 2 mL of 1N NaOH are added, and the mixture is left under stirring at room temperature for 1.5 hours. 36 mg of fluorescein isothiocyanate (FITC), solubilized in 0.3 mL of DMSO, are added and the mixture is left under stirring at room temperature for one night. The next day 20 mL of acetone are poured into the reaction flask and after 30 minutes of stirring the polymer is left to deposit, the supernatant is decanted, and the precipitate is washed twice with 20 mL of acetone. The precipitate is solubilized in 10 mL of distilled water and is dialyzed against distilled water (12-14 KDa MW cut-off tubes of cellulose mixed esters). Once dialysis is completed, it is filtered at 0.45 μm and is freeze-dried.

2.5. *Characterizations of PGPDs*

The structure of PGPDs were fully characterized by FT-IR, ^{1}H-NMR.

DEAE-PG: %Yield (w/w): 9.2 g (85%). IR (KBr): 3320 cm^{-1}(m), 2930 cm^{-1}(w); 1650 cm^{-1} (w); 1415 cm^{-1} (w); 1365 cm^{-1} (w); 1150 cm^{-1} (m); 1080 cm^{-1} (s); 995 cm^{-1} (vs); 930 cm^{-1} (m); 850 cm^{-1}

(m); 760 cm^{-1} (m). ^{1}H-NMR (D_2O) δ: 1.8-2.1 (m, CH_3); 2.5-3.0 (m, CH_2-NR_2); 3.0-3.4 (m, CH_2-NR_3^+ "tandem"); 3.5-4.0 (m, COH, glucose unit); 5.0-5.5 (m, CH anomeric).

DMAP-PG: %Yield (w/w): 8.7 g (80%). IR (KBr): 3315 cm^{-1}(m), 2930 cm^{-1}(w); 1640 cm^{-1} (w); 1410 cm^{-1} (w); 1360 cm^{-1} (w); 1240 cm^{-1} (w); 1150 cm^{-1} (m); 1080 cm^{-1} (m); 995 cm^{-1} (vs); 930 cm^{-1} (m); 850 cm^{-1} (m); 770 cm^{-1} (m). ^{1}H-NMR (D_2O) δ: 1.4-1.6 (m, CH_2); 2.0-2.3 (m, CH_3); 2.5-3.0 (m, CH_2-NR_2); 3.0-3.4 (m, CH_2-NR_3^+ "tandem"); 3.5-4.0 (m, COH, glucose unit); 5.0-5.5 (m, CH anomeric).

DMAE-PG: %Yield (w/w): 8.5 g (79%). IR (KBr): 3315 cm^{-1}(m), 2930 cm^{-1}(w); 1650 cm^{-1} (w); 1410 cm^{-1} (w); 1360 cm^{-1} (w); 1250 cm^{-1} (w); 1150 cm^{-1} (m); 1080 cm^{-1} (m); 1015 cm^{-1} (vs); 996 cm^{-1} (vs); 930 cm^{-1} (m); 850 cm^{-1} (m); 760 cm^{-1} (m). ^{1}H-NMR (D_2O) δ: 2.2-2.3 (m, CH_3); 2.5-3.0 (m, CH_2-NR_2); 3.0-3.4 (m, CH_2-NR_3^+ "tandem"); 3.5-4.0 (m, COH, glucose unit); 5.0-5.5 (m, CH anomeric).

PTMA-PG: %Yield (w/w): 8.9 g (82%). IR (KBr): 3310 cm^{-1}(m), 2930 cm^{-1}(w); 1650 cm^{-1} (w); 1415 cm^{-1} (w); 1360 cm^{-1} (w); 1250 cm^{-1} (w); 1210 cm^{-1} (w); 1150 cm^{-1} (m); 1090 cm^{-1} (m); 1015 cm^{-1} (vs); 975 cm^{-1} (vs); 925 cm^{-1} (m); 845 cm^{-1} (m); 760 cm^{-1} (m); 715 cm^{-1} (m). ^{1}H-NMR (D_2O) δ: 2.5-3.0 (m, CH_2-NR_2); 3.0-3.4 (m, CH_2-NR_3^+ "tandem"); 3.5-4.0 (m, COH); 5.0-5.5 (m, CH anomeric).

2.6. *PGPDs Size Exclusion Chromatography Analysis*

The average molecular weights and molecular weight distributions of the PGPDs were evaluated by size exclusion chromatography (SEC) using a Waters Associates (Milford, MA, USA) Model 1515 HPLC isocratic pump and with Waters Breeze software to process chromatographic data. HPLC was equipped with an Agilent PL Aquagel-OH column 60 (300 x 7.5 mm, 8 µm) and a Waters 2414 differential RID detector. As mobile phase, an aqueous solvent containing 50 mM NH_4NO_3 and 0.02% NaN_3 was used at 30 °C with a flow rate of 1.0 mL/min. This aqueous solvent is innovative and more effective than the previously adopted method based on the organic eluent DMSO/LiBr (Wang et al., 2021). In particular, NH_4NO_3 was used to increase the ionic strength of the mobile phase reducing the interactions between the PGPDs and the column. Whereas, NaN_3 was used as antimicrobial agent. Test solutions were prepared in water at a concentration of 3 mg/mL, and 20 µL samples were injected onto the column. Pullulan standards (Polymer Standards Services, Mainz, Germany) were used to generate subsequently the calibration curve.

2.7. *Determination of the Substitution Degree of residue groups of bases in the PGPDs.*

The determination of the substitution degree (SD) of residue groups of bases was conducted by ^{1}H-NMR. The SD has been defined as the number of nitrogen-containing groups present on the polymer backbone for each repeating chain. It was evaluated by transforming the amino groups into the corresponding hydrochloride ions, using ion exchange resin (DOWEX). We weighed exactly 15 mg of sample, dissolved in 1 mL of D_2O. The two ^{1}H-NMRs, performed on the same sample of PGPDs before and after resin treatment, are then compared to determine, on the base of the signal integral, the difference in terms of nitrogen bases present between tertiary and tandem quaternary groups and the total value of degree of substitution.

2.8. PGPDs Dynamic Light Scattering (DLS) and ζ – Potential

The average hydrodynamic diameter (Z-average), hydrodynamic diameter number (number PSD), size distribution (polydispersity index, PDI) and ζ - potential of the PGPDs were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The DLS study was conducted after suitable dilution of the PGPDs samples in distilled water starting from 1 mg/mL PGPDs solutions. From these solutions 100 μL were subsequently taken and combined with 900 μL of distilled water to give a final concentration of 0.1 mg/mL. Measurements of ζ - potential of PG derivatives were performed using the Laser Doppler Velocimetry (LDV) technique, carried out starting from 1 mg/mL solutions of PGPDs in distilled water. From these solutions 100 μL were subsequently taken and combined with 900 μL of a 0.1 mg/mL solution of phosphate buffer (pH = 4.5) to give a final concentration of 0.1 mg/mL. We therefore evaluated the change in ζ - potential value based on the change in pH. We made this analysis to verify the behavior of our samples at any organic pH condition. The pH values taken into consideration are in range from 5.5 to 7.5, which is the pH range that can be observed at the level of different tumor tissues. We have repeated the analyzes of DLS and ζ - potential on the samples of PGPDs-FITC, starting from 1 mg/mL PGPDs solutions in distilled water. From these solutions 100 μL were subsequently taken and combined with 900 μL of distilled water, in case of DLS, and in 900 μL of phosphate buffer (pH = 4.5), in case of ζ - Potential, to give a final concentration of 0.1 mg/mL. After successful complexation of the PGPDs samples with siRNA, ζ - potential analysis was repeated maintaining the same initial analysis conditions.

2.9. *PGPDs pK_a evaluation*

PGPDs samples were transformed into hydrochloride using a solution of 0.1 N HCl (1.0 mmol) and the solution was kept in constant agitation for 2 hours, checking that the pH was stable at a value of about 4, and subsequently titrated with NaOH. The titration was monitored by pH variation. 100 mg of polymer hydrochloride were dissolved in 100 mL of distilled water, the solution was stirred overnight at room temperature. The following day, the solution was degassed by bubbling He for 15 min, so it was titrated with 0.01 N NaOH. The addition of the titrant was done by adding constant volume of 100 µl by micropipette and the titration was monitored with a pH meter (SevenExcellence Multiparameter, Mettler Toledo). The values of titrant were corrected using distilled water, treated under the same conditions, as blank sample.

2.10. *PGPDs-siRNA complex synthesis*

12 µM of DMAE-PG were prepared by dissolving 20 mg of the compound in 1 mL of free nuclease water and making a 1:1000 dilution. 0.1 mM of siRNA were prepared by dissolving 250 nmol of siRNA in 2.50 mL of free nuclease water. From this solution 20 µL were taken and placed in 480 µL of nuclease free water. The siRNA (2 nmol) and PG (6 nmol) were mixed so that the molar ratio is 1:3 (siRNA: PG) in a total volume of 500 µL. (scaled up or down as required). The sample was vortexed for 1 minute and incubated at RT for 10 minutes.

2.11. *PGPDs – siRNA agarose gel electrophoresis*

Agarose 1% m/V was prepared by adding 1 g of agarose powder to 100 mL of TBE buffer. The agarose was dissolved by boiling. The agarose was left to cool before adding 5 µL of a 2.5 mg/mL ethidium bromide solution under the fume hood. They were mixed and decanted into a tray (without bubbles) until the agarose solidified. The comb was removed from the tray. The samples were loaded into the wells (4 µL of loading dye for each sample, the maximum loading volume is 20 µL) at final concentration of 1% m/V. Electrophoresis was performed at 120 V for 15-20 minutes. Image analysis was performed using the MF-ChemiBIS gel imaging system. The gel was then stored in a Saran film (transparent).

2.12. *MDA-MB-231-luc2 cell lines*

MDA-MB-231-luc2 cell lines, expressing luciferase Luc2 gene and Green Fluorescent Protein (GFP) gene, were obtained from Bioware Caliper Life Science. These cells serve as a new tool to detect the efficacy of the drug in vitro and in vivo with high sensitivity in experimental models of metastasis and orthotopic models of breast cancer (in mammary fat pad) (Hu et al., 2011). Cells are maintained in EMEM medium supplemented with 10% of Fetal Bovine Serum (FBS) and 0.5% of Penicillin-Streptomycin mix and subcultured twice a week and divided by 4 after trypsinization. The cell lines used of type MDA-MB-231 and MDA-MB-231-Luc2 were provided by UMR CNRS 8612 Institut Galien Paris-Saclay.

2.13. *PGPDs-siRNA MTT test*

Cells were sedimented in a 96-well plate with a concentration of 7500 cells/well. After 24 h, cells were treated with the different samples and analyzed successively at 24, 48, and 72 h. For the MTT test, 6 different concentrations of PGPDs-siRNA were examined: 1 mg/mL; 0.5 mg/mL; 0.1 mg/mL; 0.05 mg/mL; 0.01 mg/mL; 0.005 mg/mL. Purified Glycogen and siRNA were incubated under the same conditions as a negative and positive control, respectively. The MTT solution was prepared at a concentration of 5 mg/mL in PBS. The powder was well homogenized, and the vial was covered to protect it from light. The solution was filtered with a syringe and a 0.2 µm filter. 20 µL of MTT solution was added to each well (containing 200 µL of drug solution) to have a final concentration of 0.5 mg/mL. The solution was incubated at 37°C for 1 to 4 hours (crystal formation was continuously monitored). The supernatant containing MTT (220 µL) was removed with the multichannel pipette. 200 µL of DMSO per well was added to solubilize the formazan crystals, and the plate was placed under slow agitation (no more than 150 rpm): the plate was kept on the agitator until the crystals were completely dissolved. The absorbance was read at 570 nm with the plate reader.

2.14. *PGPDs – siRNA confocal*

For Confocal Microscopy, the MDA-MB-231 cells were sedimented with the PGPDs-siRNA complex treated with FITC and its behavior was analyzed for an incubation time of 3h and 24h. The plates were subsequently read on an Axio Observer Z1 Zeiss / Inverted microscope, which mounts an HSm Axiocam MSm lens with a 63x Pixel camera size. The samples were read at 470 nm using

a Halogen Nanolamp (12 V) as contrast and set a Filter EM BP 505 - 550 nm. The image analysis was performed using Zeiss ZenLite software.

2.15. *HK2 cell lines*

HK2, an immortalized PTEC line from normal adult human kidney, was obtained by Department of Emergency and Organ Transplantation from American Type Culture Collection (ATCC, Manassas, VA, USA) (Ryan et al., 1994). Cells were grown to confluence in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin, 1% streptomycin. For passage, confluent cells were washed with phosphate-buffered saline (PBS) 1×, removed with 0.05% trypsin/0.02% ethylenediamine-tetra acetic acid (EDTA) in PBS, and plated in DMEM/F12. The cell lines used of type HK2 were provided by Department of Emergency and Organ Transplantation, University of Bari.

2.16. *PGPDs-siRNA Transfection Test with Hs Cell Death siRNA*

Hs cell death transfection assay was performed using Optifect Lipofectamine as a positive control of the occurred transfection, according to the manufacturers' instructions, and PGPDs as transfection reagents. For this purpose, all PGPDs were diluted with water to the final concentration of 150 nM and incubated with Hs cell death siRNA 50 nM for 20 min before adding the complexes to HK2 cells. Cell viability was observed after 24 hours, 48 hours, and 72 hours. At the end of the experiment, cells were fixed in 4% PFA and then stained with haematoxylin. Images of the cells were collected by light microscopy.

2.17. *Statistic Analysis*

All data are presented as mean ± S.D. The statistical analysis was accomplished using one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc tests (GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$*.

3. Results and Discussions

3.1. *Synthesis and purification of Purified Glycogen Polycationic Derivatives (PGPDs)*

A general summary of the preparation of PGPDs has been given in the scheme. In short, PG's aqueous solution was treated with the suitable reactive in the presence of NaOH at 70°C. The reaction stock was then purified by dialysis and cryodrying. The synthetic process leads to two different types of substitution: a "single" tertiary amine group, which should ensure the binding with nucleic acids, and a "tandem" group, consisting of a quaternary amine with a second alkylamine halide, which should promote endosomal escape with a proton sponge effect. For each compound, different derivatives were prepared with different degrees of substitution, varying the reagent concentration, as shown in **Table 1**. PGPDs are synthesized in good to excellent yield (ca 80-85 %) with good reproducibility.

<< Insert Figure 1>>

3.2. *PGPDs Size Exclusion Chromatography (SEC)*

Table 1 and Supplementary data **Figure S1** shows the data collected by the SEC. The samples have an Mw value between 2,32 x 10^3 kDa and 2,42 x 10^3 kDa, comparable with the PG base value of 2,30 x 10^3 kDa. The Polydispersity of the samples has a value close to 1.00. As we can see from the results shown in the **Table 1**, the samples do not show a significant increase in weight after the functionalization. And do not show a significant difference between the various PGPDs samples. The polydispersity index is close to one and indicates a uniformity of molecular weight measurements.

Table 1. Stoichiometric ratios between PG and base were used, average molecular weights and polydispersity of PG and PGPDs.

PGPDS	**MN** (10^3 KDa)[a]	**MW** (10^3 KDa)[b]	**MP** (10^3 KDa)[c]	**POLYDISPERSITY** (MW/MN)
PG	2.30	2.30	2.30	1.01
DEAE 1 mmol	2.39	2.40	2.40	1.01
DEAE 15 mmol	2.32	2.32	2.32	1.01
DEAE 31 mmol	2.33	2.33	2.32	1.01

DEAE 62 mmol	2.41	2.42	2.41	1.01
DEAE 124 mmol	2.33	2.33	2.33	1.01
DMAE 1 mmol	2.36	2.36	2.36	1.01
DMAE 31 mmol	2.33	2.33	2.32	1.01
DMAE 62 mmol	2.32	2.32	2.32	1.01
DMAE 124 mmol	2.40	2.40	2.42	1.01
DMAE 246 mmol	2.33	2.33	2.33	1.01
DMAP 1 mmol	2.38	2.38	2.45	1.01
DMAP 31 mmol	2.33	2.33	2.32	1.01
DMAP 62 mmol	2.33	2.33	2.32	1.01
DMAP 124 mmol	2.41	2.41	2.42	1.01
DMAP 246 mmol	2.41	2.41	2.41	1.01
PTMA 1 mmol	2.38	2.38	2.37	1.01
PTMA 31 mmol	2.33	2.33	2.32	1.01
PTMA 62 mmol	2.32	2.32	2.32	1.01
PTMA 124 mmol	2.41	2.41	2.42	1.01
PTMA 246 mmol	2.39	2.39	2.39	1.01

[a]Number average (MN) molecular weight; [b]Weight average (MW) molecular weight; [c]Molar mass at the peak maximum (MP); Each value was reported as (media ± s.d.). [a,b,c,]S.d. = ± 0.20 x 10^3 kDa.

3.3. *Determination of the Substitution Degree of residue groups of bases in the PGPDs*

The degree of substitution (SD) was determined by ^{1}H-NMR, based on the chemical shift of the alkyl aminic chain (Supplementary data **Figures S2-S6**). For example, for the DMAE derivate, the CH_2 signal adjacent to the tertiary amine group resonates at δ = 2.0-2.5, while the same CH_2 when bound to the quaternary amine group undergoes a shielding resonating at δ = 3.0-3.4 (**Figure 2**). After resin treatment, we have an increase of the signal at δ = 3.0-3.4, from the integral of this value we can determine the degree of overall substitution (**Table 2**). Instead, from the difference in signal given by the resin treatment, we can determine the number of quaternary "tandem" groups. Finally, the values related to the single and tandem groups, thus the degree of derivatization, were determined through the ratio of the integrals related to the peaks referred to CH_2 in α position to the tertiary and quaternary amine groups, respectively. The data concerning the degree of substitution are shown in **Table 2**.

Table 2: PGPDs Substitution Degree data

SAMPLE	SUBSTITUTION DEGREE	SINGLE GROUPS*	TANDEM GROUPS*
DEAE 15 mmol	0.05	0.05	0.00
DEAE 31 mmol	0.20	0.16	0.02
DEAE 62 mmol	0.35	0.28	0.06
DEAE 124 mmol	0.71	0.45	0.20
DMAP 31 mmol	0.17	0.08	0.04
DMAP 62 mmol	0.18	0.08	0.07
DMAP 124 mmol	0.36	0.08	0.14
DMAP 246 mmol	0.57	0.10	0.20
DMAE 31 mmol	0.12	0.07	0.04
DMAE 62 mmol	0.21	0.09	0.05
DMAE 124 mmol	0.36	0.12	0.13
DMAE 246 mmol	0.42	0.01	0.29
PTMA 31 mmol	0.09	0.09	-
PTMA 62 mmol	0.15	0.15	-
PTMA 124 mmol	0.39	0.39	-
PTMA 246 mmol	0.45	0.45	-

*Groups per glucose unit

<<Insert Figure 2>>

3.4. *PGPDs Dynamic Light Scattering*

Purified Glycogen Polycationic derivatives show a Gaussian distribution of measurements, between 40 and 80 nm in diameter, and a polydispersity index of 0.2-0.3, as shown in **Table 3**. As we can see in **Table 3**, for each PGPDs we can see that an increase in initial reagent concentration corresponds to a slight increase in the value of Z-average (diameter) and the polydispersity index under the 0.3 indicates that we are close to a monodisperse sample (Supplementary data **Figure S7**).

Table 3: PGPDs Diameter, ζ - Potential Values and ζ – Potential in function of pH.

PGPDS	DIAMETER (nm)	PDI	ZP (mV)	ZP (mV) in function of pH			
				5.5	6	6.7	7.5
PG	40.71 ± 0.60	0.20	- 2.00 ± 0.30				
DEAE 1 mmol	56.84 ± 0.41	0.20	- 2.50 ± 2.20				

DEAE 15 mmol	56.72 ± 0.51	0.30	+ 3.90 ± 0.60				
DEAE 31 mmol	57.22 ± 0.22	0.20	+ 5.90 ± 1.00				
DEAE 62 mmol	59.34 ± 0.43	0.30	+ 12.50 ± 2.80	5.90 ± 2.80	4.00 ± 1.90	0.80 ± 0.20	0.30 ± 0.10
DEAE 124 mmol	63.90 ± 1.81	0.20	+ 43.90 ± 0.80				
DMAE 1 mmol	34.23 ± 2.24	0.20	- 2.00 ± 2.30				
DMAE 31 mmol	38.74 ± 2.20	0.20	+ 4.80 ± 1.00				
DMAE 62 mmol	43.52 ± 1.21	0.20	+ 5.60 ± 0.50				
DMAE 124 mmol	46.20 ± 0.52	0.20	+ 12.00 ± 1.10	5.60 ± 1.40	4.30 ± 0.10	-0.90 ± 0.30	-3.70 ± 1.20
DMAE 246 mmol	49.21 ± 1.02	0.20	+ 23.40 ± 1.90				
DMAP 1 mmol	37.73 ± 1.21	0.30	- 1.80 ± 1.80				
DMAP 31 mmol	33.92 ± 2.33	0.30	+ 6.80 ± 0.30				
DMAP 62 mmol	36.44 ± 0.60	0.20	+ 8.00 ± 0.10				
DMAP 124 mmol	39.21 ± 0.54	0.30	+ 12.30 ± 1.30	5.00 ± 1.40	3.20 ± 1.10	-0.10 ± 0.50	-3.80 ± 1.00
DMAP 246 mmol	42.62 ± 1.40	0.20	+ 15.30 ± 1.90				
PTMA 1 mmol	41.90 ± 0.32	0.20	- 0.30 ± 0.30				
PTMA 31 mmol	42.12 ± 0.11	0.20	+ 5.70 ± 0.20				
PTMA 62 mmol	48.43 ± 2.43	0.30	+ 7.00 ± 0.20				
PTMA 124 mmol	49.43 ± 2.71	0.30	+ 12.20 ± 3.40	4.60 ± 0.70	0.80 ± 0.40	-1.40 ± 0.40	-4.80 ± 1.10
PTMA 246 mmol	50.24 ± 1.30	0.30	+ 19.90 ± 0.50				

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3.5. ζ - *Potential*

The values of the different samples shown a range of measurements from -9.00 to +44.00 mV in relation to concentration of N, N-dialkylamino alkyl halides (**Table 3**). We therefore evaluated the change in ζ - potential value based on change in pH. The pH values taken into consideration are in range from 5.50 to 7.50 (**Table 3**). The PG has a ζ - potential value of -2.00 mV. For all PGPDs samples we can see that the increase in the starting reagent concentration corresponds to an increase in the value of ζ - potential, which goes from a negative value found in the sample at a lower concentration to positive values up to +44 mV, indicative values for the stability of nanoparticle dispersions in water. A positive ζ - potential value this high makes the sample suitable for nucleic acid complexation and subsequent initiation of the proton sponge effect at the cellular level. In addition, we can see how the increase in pH value corresponds to a decrease in ζ - potential value. From the ζ - potential analysis performed on the higher concentration PGPDs samples after complexation with siRNA, we have seen how the ζ - potential value decreased, mainly due to the binding of the cationic surface of the polymer with siRNA (Supplementary data **Table S1**).

3.6. *Evaluation of the buffer capacity by titration studies*

Figure 3 (B) shows the titration curves of DEAE, DMAE, DMAP and PTMA cationic polymers for comparative purposes. The derivatives with the highest degree of substitution have been chosen as models. All derivatives, except PTMA, show a curve with two points of inflection, from which it is possible to calculate a pK_a1 (in a range of about 6.0-7.0), and a less pronounced pK_a2 at a higher pH value (in a range of about 8.5-10.0) (Supplementary data **Table S2**). The buffer capacity has been evaluated to verify that the cationic polymers have characteristics that induce the proton sponge effect. Titration studies, carried out on PGPDs, have identified a distribution of pK_a in a range close to or just below the physiological pH which gives cationic polymers a high buffering capacity. pK_a values close to physiological pH are useful to give cationic polymers the positive charge necessary for nucleic acid complexation. pK_a values below the physiological pH are useful to ensure the release of complexes from endosomes into the cytoplasm (by the proton sponge). The two pK_a are attributable to the presence of the two basic centers, as shown in **Figure 3** (A).

<<Insert Figure 3>>

3.7. *PGPDs-siRNA Agarose Gel Electrophoresis*

To verify the ability of PGPDs derivatives to form complexes with siRNA we incubated DMAE - PG, using different complexing ratios with siRNA. The DMAE derivative was chosen at the highest molar concentration because it has a high surface charge (as we can see in **Table 3**) and minimal cytotoxicity even at high concentrations (as shown later in **Figure 5**). This derivative has two different basic centers: the “single” amine group, which should guarantee the binding with nucleic acids, and the amine of the “tandem” group, which should promote endosomal escape by proton sponge effect. Analyzing the obtained data (**Figure 4**) we can see that the siRNA was almost completely retained by the polymer that slows down its run on the gel, in fact, the final signal of the wells is much less intense than free siRNA. In the samples 1:5 and 1:10 instead, we can notice that although there is an increase in the amount of polymer, it loses its effectiveness and lets the siRNA run on the gel, behaving exactly like the free siRNA. This could be due to aggregation phenomena that affect the polymer when used at high doses, leading to the loss of efficacy. In these samples, the best behavior was obtained from the samples with molar ratio 1:2, 1:3 and 1:4 (siRNA: PG). Evaluating this data, we decided to use the sample with N/P ratio equal to 1:3 as reference sample for the following tests on PGPDs functionalized with siRNA.

<< Insert Figure 4>>

3.8. *PGPDs-siRNA MTT test*

In **Figure 5** we can see the graphs concerning the cytotoxicity measurements, carried out by MTT, of siRNA, Purified Glycogen, DEAE, DMAE, DMAP and PTMA derivatives at incubation times of 24h, 48h and 72h. The MTT assay was performed on MDA-MB-231-luc2 cell culture. Analyzing the values obtained at the MTT test of PGPDs-siRNA samples we can see that some of these are completely non cytotoxic, even at higher concentration, as in the samples DMAE and DMAP, which always report cell viability values comparable to those obtained in the Control. Other samples, as DEAE and PTMA show slight cytotoxicity, on which the IC_{50} values could be calculated (**Figure 5**). For Purified Glycogen sample and all the PGPDs samples, we can see an increase in cell viability compared to the positive control, especially at lower PGPDs concentrations up to 48 h of incubation. This is due to the absence of cytotoxicity of the polymer on MDA-MB-231-luc2 cells and especially to the massive glycosidic component that characterizes the structure of the compounds and therefore could provide nutrient to the cells in culture.

<<Insert Figure 5>>

3.9. *PGPDs-siRNA Confocal Microscopy*

Confocal microscopy images were obtained at incubation times of 3h (**Figure 6 A**) and 24h (**Figure 6 B**) using DMAE-FITC sample complexed with siRNA on a population of MDA-MB-231 cells. From the images obtained by confocal microscopy we can see how, for the samples analyzed after an incubation time of 3h (**Figure 6 A**), we can see a cloud containing our functionalized polymer outside the cell with formation of scattered polymer agglomerates close to the cell barrier. This makes us understand how at 3h, the entry phase of the polymer functionalized with siRNA into the cell begins. Analyzing instead the images obtained at 24h incubation time (**Figure 6 B**), we can see that the cloud outside the cell has now disappeared, as well as the polymer agglomerates close to the cell barrier. Agglomerates of polymer that have instead moved inside the cell, have become much more numerous and we can also see how they tend to distribute themselves towards the cell-core wall. This experiment demonstrates that we have complete entry of the PGPDs-siRNA complex into the tumor cell within 24h.

<<Insert Figure 6>>

3.10. *PGPDs-siRNA Transfection Test with Hs Cell Death siRNA*

To evaluate the transfection efficacy of PGPDs, we performed transfection experiments with Hs cell death siRNA, a control siRNA targeting ubiquitously expressed human genes that are essential to cell survival. After 72 hours of transfection, we observed the specific reduction of cell viability induced by Hs Cell Death transfected with Lipofectamine, compared to basal condition (positive control). Interestingly, we observed that PGPDs were also able to induce HK2 cell death, with DEAE and DMAE samples showing the better efficiency compared to other conditions (**Figure 7**).

<<Insert Figure 7>>

4. Conclusions

In this work new cationic derivatives of the biocompatible polymer PG have been successfully prepared using different *N*,*N*-dialkylamino alkyl halides, for the delivery of nucleic acids as a model for breast and renal cancer cell gene silencing therapy. The complexes between the PGPDs and siRNA have been used for biological studies, such as Agarose Gel Electrophoresis, MTT test, Confocal microscopy and Transfection test, in order to evaluate the *in vitro* efficacy of intracellular delivery. From the several assays performed we can observe that the compounds result, moreover, not cytotoxic for the cell lines and at the right N/P ratio are able to efficiently complex the siRNA. In conclusion, PGPDs-siRNA complexes show that they maintain their spherical and dendrimeric structure, that they can cross cellular barriers and act directly on the nucleus of the different cell lines. The percentage of nucleic acid that is released into the cell should be further increased. For this reason, their complexation process has to be improved by eliminating the aggregation phenomena of the polymer and increasing the Z potential value of the complexes in order to trigger the proton sponge effect inside the cell and cause the release of siRNA.

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