Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting information for:

Squalene/Polyethylenimine – based non-viral vectors: synthesis and use in systems for sustained gene release

Geta David[a*], Lilia Clima[b], Manuela Calin[c], Cristina Ana Constantinescu[c], Mihaela Balan[b], Cristina Mariana Uritu[b,d], Bogdan C. Simionescu[a,b*]

[a] *Department of Natural and Synthetic Polymers, "Gh. Asachi" Technical University of Iasi, Iasi 700050, Romania*

[b] *"Petru Poni" Institute of Macromolecular Chemistry of Romanian Academy, Iasi 700487, Romania*

[c] *Institute of Cellular Biology and Pathology "Nicolae Simionescu" of Romanian Academy, Bucharest 050568, Romania*

[d] *Advanced Research and Development Center in Experimental Medicine, "Gr. T. Popa" University of Medicine and Pharmacy, Iasi 700115, Romania*

[*] *To whom correspondence should be addressed:* dgeta54@yahoo.com

bcsimion@icmpp.ro

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1. Experimental section

1.1. Materials

Squalene (Sq, ≥98%), N-bromosuccinimide (NBS) (99%), 2,2'-ethylenedioxy bis(ethylamine) (98%), N,N'-bis(acryloyl)cystamine (BAC, 99%), branched polyethylenimine (BPEI, Mw~ 1.8 kDa, 50 wt. % in H_2O), 1H-pyrazole-1-carboxamidine hydrochloride (99%), N,N-diisopropylethylamine (DIEA, 99.5%), (5-fluorescein isothiocyanate (FITC, ≥90%), trypsin, Triton X-100 – all purchased from Sigma - Aldrich, Germany – and periodic acid (99%, Alfa Aesar) were used as received.

Type I atelocollagen (AteCol, aqueous solution of 1.9 wt%, pH~2), nano-hydroxyapatite surface functionalized with polyethylenimine (HAp_{LPEI} – nanoplates of about 45.5 nm x 11.6 nm, Ca/P=1.57, with 4.5 wt% LPEI of 1.8 kDa, relative to inorganic material) and poly(ε-caprolactone) diisocyanate (PCL-DI, Mn ~ 2.5 kDa) were prepared according to literature.[1-3] Dimethylsilanediol hyaluronate (DMSHA) (aqueous solution of 0.3% dimethylsilanediol and 0.3% hyaluronan with Mw of 1.8–2.2 MDa) was supplied by EXSIMOL S.A.M. (Monaco). Phosphate-Buffered Saline (PBS) tablets for buffer solution (pH of 7.3–7.5) were purchased from Invitrogen.

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and the antibiotics for cell culture (penicillin G, neomycin, streptomycin) were obtained from Gibco BRL (USA). The cell culture plates were purchased from Eppendorf (Germany), the tissue culture flasks from Corning (USA), plasmid pEYFP from Clontech Laboratories Inc. (USA), *Escherichia Coli* host strain DH5α and HEK 293T cells (Human Embryonic Kidney 293T cell line) from American Type Culture Collection (ATCC, LGC Standards GmbH, Germany), HeLa cells from CLS-Cell-Lines-Services-

GmbH (Germany), salmon sperm DNA (SDNA-Deoxyribonucleic acid, low molecular weight from salmon sperm) from Sigma-Aldrich, the Plasmid NucleoBond Xtra MidiPrep kit from Macherey-Nagel GmbH & Co. (Germany), E.Z.N.A. Endo-free Plasmid Mini II kit from Omega Bio-Tek, Inc. (USA), MTS cell growth assay reagent (CellTiter 96® Aqueous One Solution Cell Proliferation Assay), 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT assay) from ThermoFisher Scientific and Bright-Glo(TM) Luciferase Assay System kit from Promega (USA).

MEM Alpha Eagle medium with UltraGlutamine, deoxyribonucleoside, and ribonucleosides, as well as penicillin–streptomycin–amphotericin B mixture were purchased from Lonza Verviers (Belgium).

Plasmids pCS2+MT-Luc (pLuc) encoding for firefly luciferase and pCS2+NLS-eGFP (pGFP) encoding an enhanced variant of green fluorescent protein were kindly provided by Dr. Adrian Salic from Harvard University, Boston.

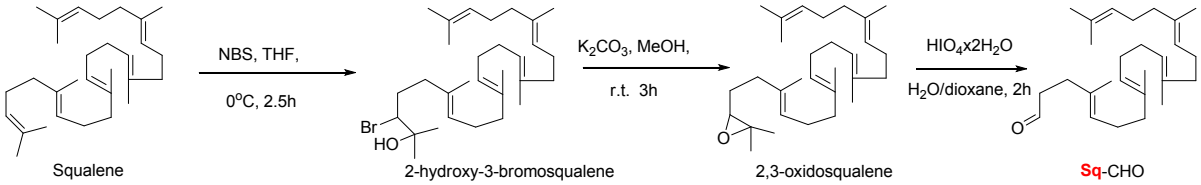
Commercially-available solvents (petroleum ether, diethyl ether (≥98%), methanol (99.8%), dimethylsulfoxid (DMSO, 99.8%), acetone (≥ 99.5%) – from Sigma-Aldrich) were used without further purification. Dichloromethane (DCM, 99.8%) was further dried by distillation in argon atmosphere on phosphorous pentoxide, stored in brown bottle, over molecular sieves, under inert atmosphere. Silica gel 60 (400-230 mesh, Roth), and Spectra/Por Biotech cellulose ester dialysis membranes (MWCO-1000 Da or 100-500 Da, Spectrum Laboratories) were used for purification steps.

Bidistilled, deionized or Millipore water was used for experiments and purification.

1.2. Synthesis

A. Sq-derivatives

Sq-CHO was synthesized in three steps (scheme S1) according to a previously described procedure.[4-6]



Scheme S1. Synthesis of Sq-CHO.[4]

Sq-CH=N-EO$_2$-NH$_2$. Schiff (imine) base chemistry was used. In brief: SqCHO (600 mg, 1.55 mmol) was dissolved in 2 mL freshly dried dichloromethane under inert atmosphere (Ar), in brown glassware and activated molecular sieves of 3 Å were added. To this, a solution of 2,2'-ethylenedioxy bis(ethylamine) (0.5 mL, 3.64 mmol) in dichloromethane (4 mL) was added dropwise and the resulted mixture was stirred for 2.5 h in the dark. The reaction mixture was filtered and washed with sodium bicarbonate saturated solution. The organic layer was collected, dried over anhydrous sodium sulfate, and evaporated to dryness. After resolving and separation the obtained product was dried overnight in vacuum desiccator (yield, 75%).

Sq-CH=N-EO$_2$-NH-BAC. The synthesis was performed by addition of the aminated compound Sq-CH=N-EO$_2$-NH$_2$ to N,N'-Bis(acryloyl)cystamine (BAC) according to an adapted procedure[7], where dichloromethane was used as solvent, and temperature and duration were selected after series of experiments. In a typical procedure, to a solution of Sq-CH=N-EO$_2$-NH$_2$ (360 mg, 0.7 mmol) in 1.5 mL of freshly dried dichloromethane, BAC (240 mg, 0.9 mmol) and hydroquinone (10 mg, 0.09 mmol), preliminary dissolved in 2 mL of dichloromethane, were added and the obtained suspension was maintained with occasional stirring for 12 h at 30 °C, and another 72 h at room temperature (under

stirring), under inert atmosphere, in the dark, until most of the solid was dissolved. Undissolved solid was eliminated by filtration and the organic layer was collected and evaporated to dryness. The obtained residue was washed with anhydrous methanol, dried in vacuum and then stored in desiccator at 8 ^{o}C (yield ~60%).

All three resulted Sq-based intermediates were subjected to spectral analysis (^{1}H-NMR, ^{13}C-NMR, FTIR) as shown in **Figures 1**, **1Sa**, **2Sa** and **Table 1S**. Thin-layer chromatography (TLC) was also used to check their purity.

B. Sq/BPEI conjugates

Sq-BPEI-NH$_2$. To attach BPEI to the Sq-CH=N-EO$_2$-NH-BAC, Michael addition was applied. The experiment was carried out as follows: BPEI (0.9 mL, 0.22 mmol) and Sq-CH=N-EO$_2$-NH-BAC (170 mg, 0.22 mmol) were dissolved in 1 mL DMSO/DCM (3:1) mixture and continuously stirred at 40 ^{o}C for 5 h, and for another 48 h at room temperature. After completion, the reaction mixture was dialyzed using Spectra/Por dialysis membrane (MWCO-1000 Da) against bidistilled water at 4 ^{0}C for 24 h and lyophilized (yield, 70%)

Sq-BPEI-G. Guanidinylation[8] of Sq-BPEI-NH$_2$ was performed by mixing a solution prepared from Sq-BPEI-NH$_2$ (200 mg, 72 μmol) and 10 mL deionized water with a solution prepared from 1H-Pyrazole-1-carboxamidine hydrochloride (33.6 mg, 0.23 mmol) in 5 mL deionized water. N,N-diisopropylethylamine (40 μL, 0.229 mmol) was added to the mixture and the obtained solution was stirred overnight at room temperature. After completion, the reaction mixture was dialyzed using Spectra/Por dialysis membrane (MWCO-100-500 Da cut-off) against bidistilled water at 4 ^{0}C for 24 h and lyophilized (FreeZone Freeze Dry System, Labconco, USA), giving 95 mg of product (yield, 41%).

Sq-BPEI-FITC. FITC labeling[9] was accomplished by the following procedure: 14 mL of FITC solution in DMSO (conc 1mg/mL, 36 μmol) were added to a solution prepared from Sq-BPEI-NH_2 (200 mg, 72 μmol) in 5 mL PBS. The mixture was incubated for 3 h at 5-8^{o}C, then at room temperature for 48 h, in the dark. After completion, the reaction mixture was dialyzed using Spectra/Por dialysis membrane (MWCO-1000 Da cut-off) against bidistilled water at 4 ^{o}C for 24 h, and the process was repeated until orange color disappeared in water. The washed concentrated intense orange solution was lyophilized to obtain the product in 80% yield.

The structures of the conjugates were confirmed by ^{1}HNMR (**Figure 2**), ^{13}C-NMR (**Figure S1b**) and FTIR (**Figure S2b, Table S1**) registrations.

C. Hybrid cryogel preparation

Hybrid cryogel $CH_{10}P_{10}HAp_{25}$-15 was prepared according to literature.[10] Briefly, the procedure supposes mixing AteCol and DMSHA solution after bringing at pH of about 6.5, followed by addition of LPEI functionalized nHAp (in aqueous dispersion) and subsequent inclusion of the appropriate amount of crosslinker (PCL-DI) in acetone/DMSO mixture (2:3 v/v, 2 v% organic solvent/total aqueous dispersion) containing a stabilizer (4 wt% Triton X-100 relative to PCL-DI). The adopted formulation finally includes 10% DMSHA, 10% PCL-DI relative to AteCol and 25 wt% nHAp relative to total solid content. The solid concentration of the homogenized (by stirring and sonication) dispersion was of 0.9 wt%. After degassing in vacuum, the mixture was subjected to cryogelation in quartz vessels, being submitted to freezing for 15 h at -18 °C and 6 h at -12 °C, followed by UV irradiation in frozen state for 15 min, stepwise thawing and rinsing with water. The stable, elastic product was then lyophilized.

1.3. Characterization

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance III 400 instrument operated at 400.1 and 100.6 MHz for ^{1}H and ^{13}C nuclei, respectively, at room temperature. Chemical shifts were reported in ppm, and referred to tetramethylsilane (TMS) as internal standard. ATR-FTIR spectra were registered on a Bruker Vertex 70. The size and shape of the conjugates and their self-assembling constructs in water were studied with a transmission electron microscope (Hitachi High-Tech HT7700, working also in STEM mode). The matrices morphology before and after loading with polyplexes was directly observed (without sputter coating by conducting matter) using a SEM – Quanta 200 scanning electron microscope, equipped with energy-dispersive X-ray spectroscopy (EDX) module working in low vacuum mode.

For fluorescence microscopy studies a Leica DMI 3000B fluorescence inverted microscope, or an Olympus IX81 microscope equipped with fluorescence filter cube for FITC/GFP was used, depending on experiment, i.e. for evaluation of *in vitro* transfection efficiency for free polyplexes or polyplexes included in the hybrid 3D matrix, respectively.

The kinetics study was performed by using an UV-6300 PC spectrophotometer for the released SDNA evaluation (λ=260 nm), and a Horiba FluoroMax - 4 spectrofluorometer for investigation of FITC labeled vectors delivery (λex: 485 nm; λem: 515 nm).

1.4. Preparation of plasmid DNA

Plasmids pCS2+MT-Luc (pLuc) encoding for firefly luciferase and pCS2+NLS-eGFP (pGFP) encoding an enhanced variant of green fluorescent protein were amplified in *E. Coli* DH5α. The plasmids were extracted and purified with E.Z.N.A. Endo-free Plasmid

Mini II kit (Omega Bio-Tek, Inc.). The concentration of plasmids was determined with DS-11+ spectrophotometer (DeNovix Inc., Wilmington, DE).

Plasmids pEYFP-C1, after amplification in *E. Coli* DH5α in the presence of ampicillin, were purified using NucleoBond Xtra MidiPrep kit. Plasmid DNA concentrations were determined by UV spectrophotometry with NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

1.5. Agarose gel electrophoresis

Complexes of various N/P ratios were prepared by mixing the synthesized Sq/BPEI conjugates with SDNA at appropriate weight ratios in deionized water. BPEI (1.8)/SDNA was used as control system. After a 10 min incubation period, the complexation ability was investigated by agarose gel electrophoresis retardation assay (1% agarose gel, staining with ethidium bromide), the measurements being carried out at 90 V, at pH=7.4, in 1M TAE buffer (Tris-Acetate-EDTA) for 90 min. The resulted DNA bands were visualized under UV trans-illuminator.

1.6. Cell cultures

HeLa cells were maintained in tissue culture flasks with MEM Alpha Eagle medium with UltraGlutamine, deoxyribonucleoside, and ribonucleosides supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–amphotericin B mixture.

HEK 293T cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL neomycin/mL) at 37 ºC in a humidified incubator, in 5% CO_2 atmosphere.

1.7. *In vitro* transfection efficiency for polyplexes formed with Sq/BPEI vectors

HeLa cells were seeded (10^4 cells/well) in 96-well white opaque microplates (PerkinElmer) and cultured overnight. After 24 hours the medium was replaced with 100 μL/well solution of polyplexes in complete culture medium. The polyplex solution was prepared by mixing 500 ng pLuc with the corresponding amount of polymer in pure water (Millipore) to achieve the desired N/P ratios followed by an incubation step at room temperature for one hour and adding complete cell culture medium up to 100 μL. Bright -Glo(TM) Luciferase Assay System kit was employed according to manufacturer protocol 48 hours post transfection. In short, 100 μL Bright - Glo solution were added to each well with a multichannel pipette and generated light was measured after 4 minutes on a multimode microplate reader (EnSight, PerkinElmer). For qualitative assessment of transfection, cells were transfected as described above with the exception of using pGFP plasmid. Plates with transfected cells were investigated 48 hours post transfection using Leica DMI 3000B fluorescence inverted microscope fitted with GFP filtercube.

1.8. Cytotoxicity assay

Cytotoxicity of polyplexes against HeLa cells was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay - MTS tetrazolium compound. HeLa cells were seeded (10^4 cells per well) in 96 well transparent plates, in 100 μL complete medium 24 hours before treatment. The next day, cells were treated with polyplexes made with pLuc as described above and incubated for another 44 hours. Finally, 20 μL of MTS reagent were added to each well, and the plates were incubated at 37 °C for another 4 h. Absorbance at 490 nm was recorded with a multimodal multiplate reader (EnSight,

PerkinElmer) and cell viability was expressed as percentage of absorbance from the transfected cells compared to the absorbance of control, non-treated cells.

XTT assay[10] was used to assess the viability of HEK 293T cells after incubation in the presence of polyplexes of plasmid DNA and Sq-based conjugates (with N/P ratios of 20 for Sq-BPEI-NH_2, and of 15 for Sq-BPEI-G), free or embedded into matrices, as compared to cells alone or exposed to free plasmid (in the absence or presence of matrix). HEK 293T cells were previously seeded in 24-well plates at a density of 50.000 cells/well, 24 hours before incubation with polyplexes embedded or not into matrices, free plasmid and blank matrix. These were then subjected to incubation with polyplexes in the before mentioned conditions for 1 or 2 days. At the end of each incubation period the culture medium was replaced with XTT solution (1 mg/ml in culture medium without phenol red) for 2 hours at 37 °C and 5 % CO_2, and the optical absorbance was measured at 450 nm using a microplate reader (TECAN GENios). The results were expressed as fold change over control (cells exposed to culture medium).

1.9. *In vitro* transfection studies

1.9.1. Polyplex formation and loading into matrices

The formation of polyplexes was accomplished by mixing for 30 minutes at 37 ºC plasmid DNA (pEYFP) and Sq/BPEI conjugates in water, N/P ratios of 20 for Sq-BPEI-NH_2 and of 15 for Sq-BPEI-G, using a fixed amount of 10, 15 and 20 μg plasmid DNA.

The formed polyplexes were loaded into cylindrical pieces of $CH_{10}P_{10}/HAp_{25}$-15 hybrid matrix (~ 1.0 mg weight, with a diameter of 0.5 cm and 0.2 cm in width), by hydration of matrices in cell culture medium containing polyplexes for 24 h, at 4 ºC. The amount of plasmid DNA encapsulated into the matrices was determined by measuring the

free pDNA that remained in the medium at the end of the incubation time using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples with 15 μg plasmid DNA were used for investigations, all pEYFP being loaded in the matrix.

1.9.2. *In vitro* transfection activity of the polyplexes released from matrices

HEK 293T cells were seeded into 6-cm diameter Petri dishes at a density of 200.000 cells/dish in order to achieve about 70 % confluence 24 h after seeding. The hybrid matrices $CH_{10}P_{10}/HAp_{25}$-15 loaded with Sq-BPEI-NH_2/pEYFP or Sq-BPEI-G/pEYFP polyplexes were placed above the cells and the expression over time of yellow-green fluorescent protein (encoded by pEYFP-C1 plasmid) was followed using an Olympus IX81 microscope equipped with fluorescence filter cube for FITC/GFP. To investigate the expression of fluorescent protein at time intervals longer than 5 days, the cells exposed to matrices were detached from the culture dishes (using 1.25 ‰ Trypsin) every week, and split at a 1:5 ratio in new culture dishes, left for 4 hours to adhere, and then the matrices were positioned again above the cells. Cells incubated with free Sq-BPEI-NH_2/pEYFP or Sq-BPEI-G/pEYFP polyplexes were used as transfection controls. In a previous study, the transfection efficiency of free pEYFP plasmid loaded or not into $CH_{10}P_{10}/HAp_{25}$-15 was followed and the data showed no transfection.[11]

1.10. Release of polyplexes from the 3D hybrid matrix

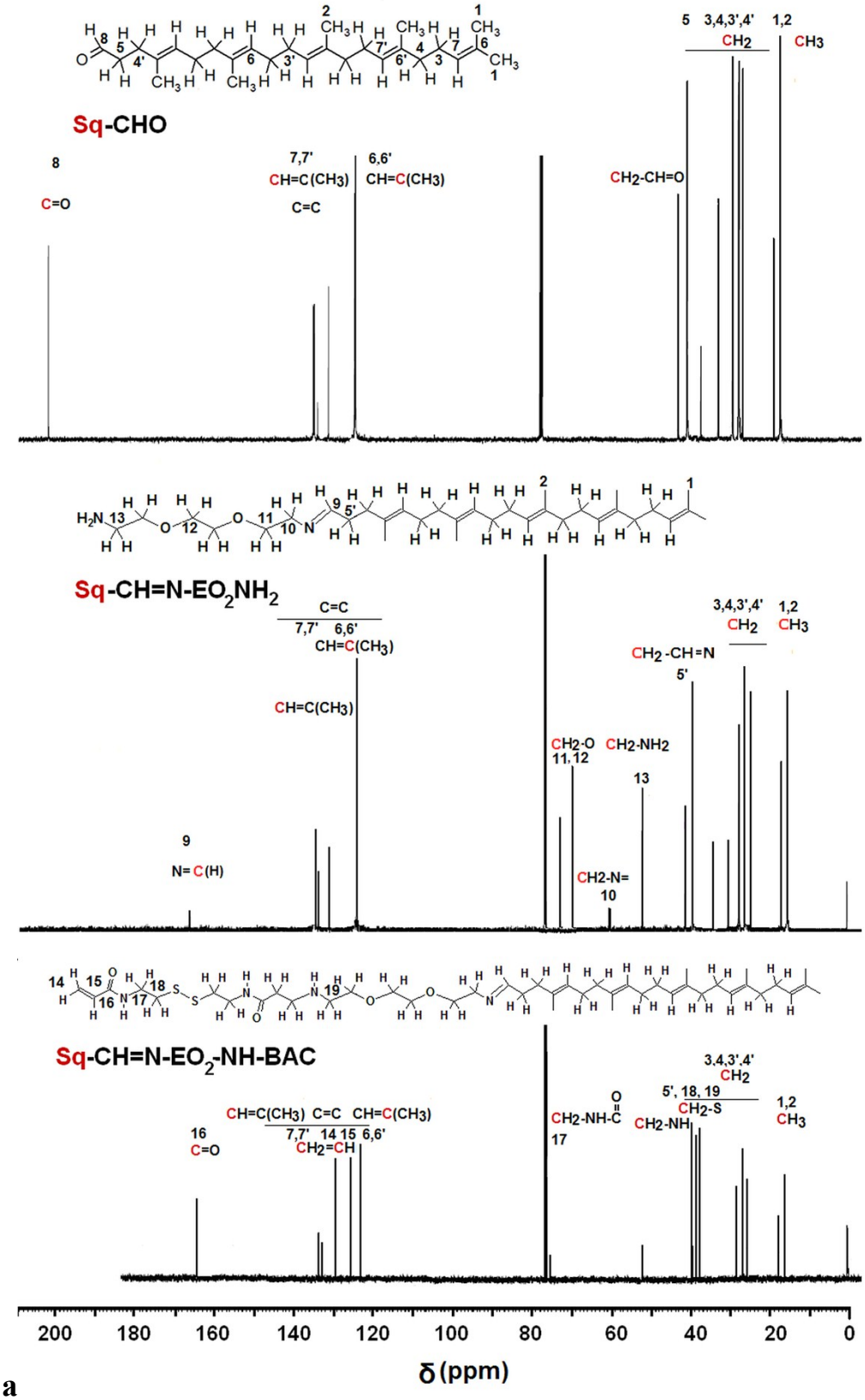
SDNA polyplexes with the synthesized vectors were prepared at a N/P ratio of 10 by slow addition of SDNA to the aqueous solutions of Sq/BPEI conjugates, under mild vortexing. The mixture was incubated for 1h at room temperature, in the dark. Weighted $CH_{10}P_{10}/HAp_{25}$-15 cryogel samples were embedded in the appropriate amount of aqueous

SDNA solution or polyplex dispersion, to reach 5 wt% SDNA relative to hybrid matrix. The resulted mixture was incubated in the dark for 1h, with mild occasional stirring, at room temperature, to allow penetration into the macroporous matrix, and then lyophylized. To study the release kinetics, similar amounts of naked matrix (control sample), matrix loaded with SDNA only, and matrices loaded with polyplexes (formed by SDNA with Sq-BPEI-G or Sq-BPEI-FITC) were immersed in deionized water (0.01 g/mL) and incubated at 37 ^{0}C, in an oven (under static conditions - I) or on a shaker at 200 rpm (experiment under dynamic conditions - II). At different time-points 0.1 mL supernatant was removed from the Eppendorf with sample and replaced with fresh water. Each sample was analysed for the amount and integrity of released SDNA, by UV-Vis (determinations against the blank specimen containing similar amount of cryogel only) and gel-electrophoresis investigations, respectively. After 1000 h of tracking delivery, for selected samples, the release media was replaced by 0.1% SDS, to determine the amount of SDNA still remaining associated with the matrix. The samples containing SDNA /Sq-BPEI-FITC polyplex, after dilution (x100), were also subjected to fluorescence determination (Horiba FluoroMax - 4 spectrofluorometer). The cumulative release percent of SDNA was calculated by dividing the amount released at a given time by the initial loaded amount.

1.11. Statistical analysis

All experiments were performed in triplicate and data are expressed as mean ± SD. The differences were considered statistically significant when $p < 0.05$. Statistical analyses for cytotoxicity and Luciferase assays were performed with GraphPad Prism version 6.04 for Windows (Graphpad Software, Inc., San Diego, CA).

2. Additional figures



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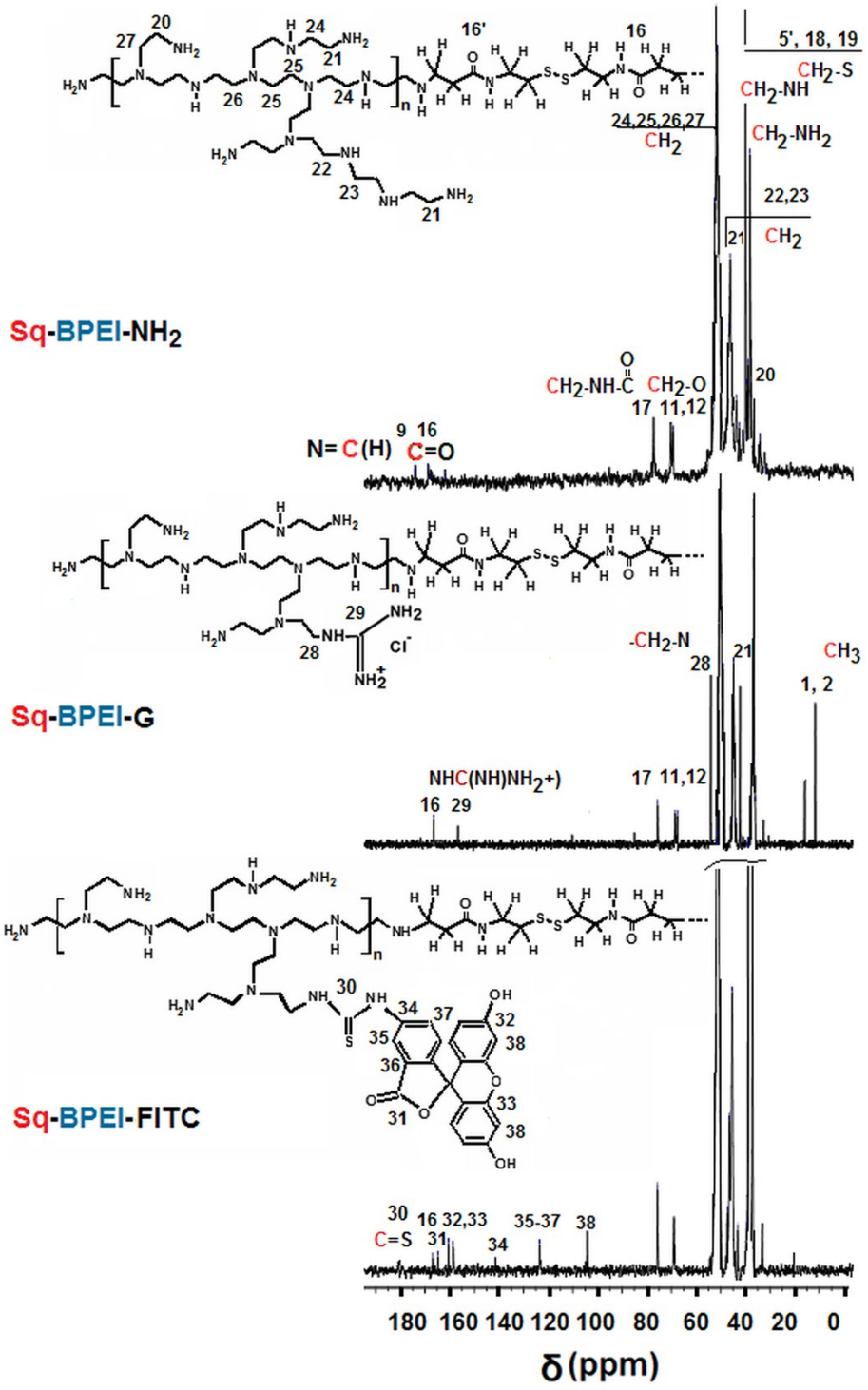


b

Figure S1. Structure confirmation by ^{13}C NMR spectra for: (a) SqCHO, Sq-CH=N-EO_2-NH_2 and Sq-CH=N-EO_2-NH-BAC ($CDCl_3$) and (b) Sq/BPEI conjugates (D_2O).

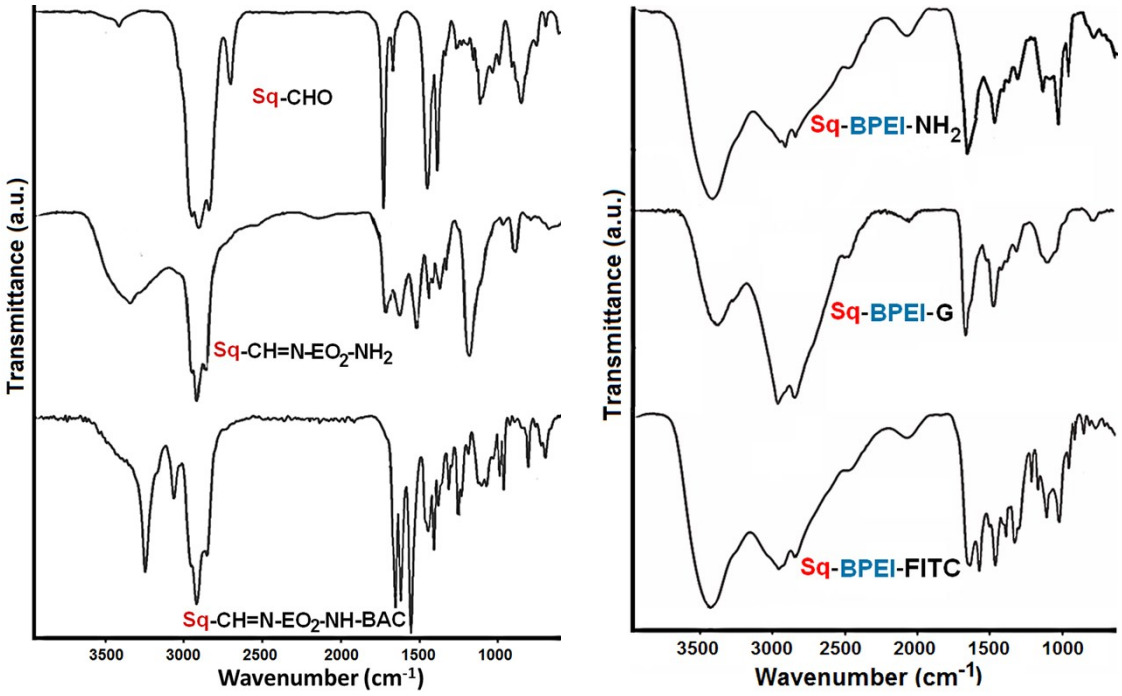


Figure S2. FTIR spectra of the (a) synthesized Sq derivatives (intermediates) and (b) final Sq/BPEI conjugates.

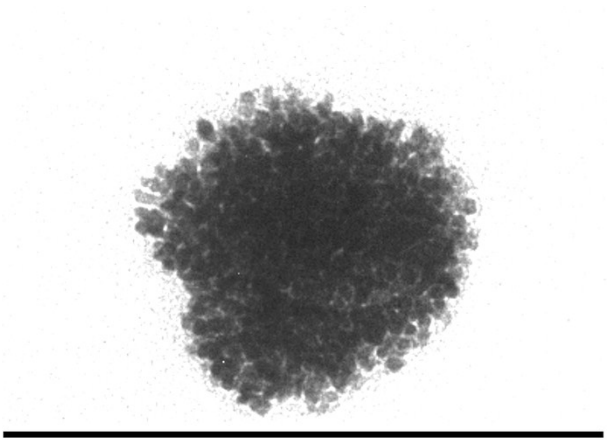


Figure S3. Enlarged TEM image for multimolecular micelle. Sample: Sq-BPEI-G. Bar: 200 nm.

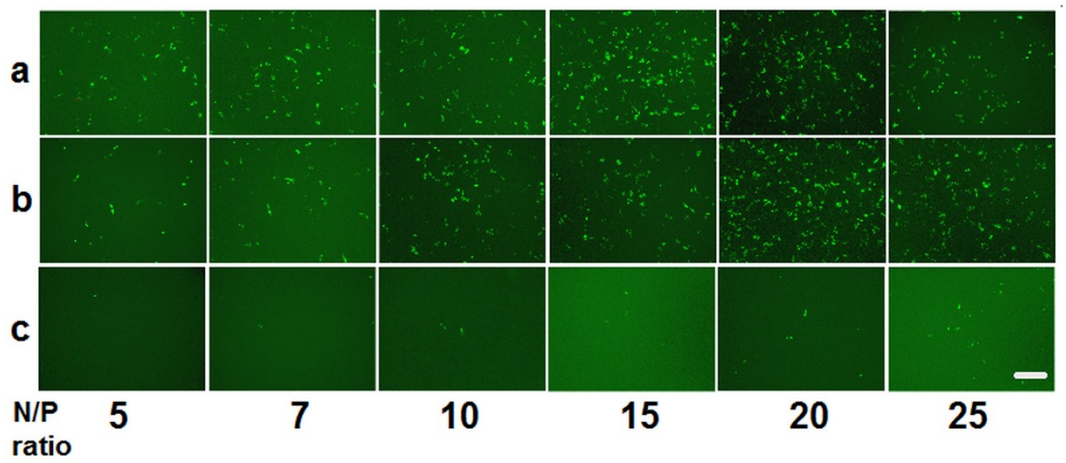


Figure S4. Fluorescence microscopy of HeLa cells transfected with polyplexes made with pGFP and (a) Sq-BPEI-G, (b) Sq-BPEI-NH_2, (c) BPEI. The plates with treated cells were investigated 48 hours post transfection. Scale bar 500 µm.

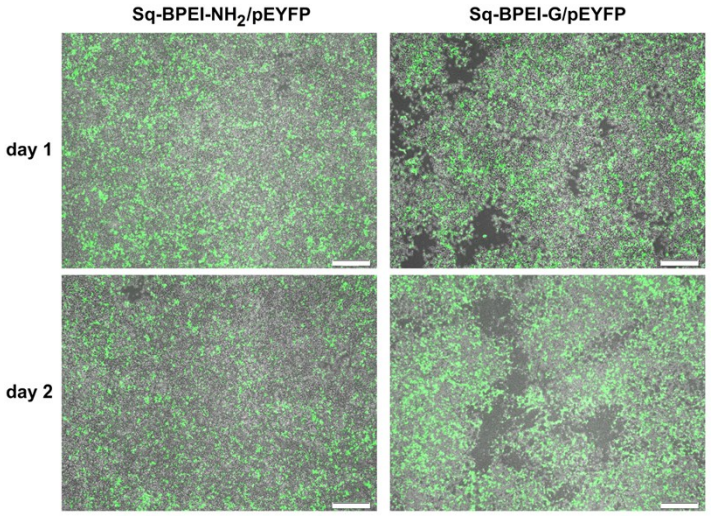
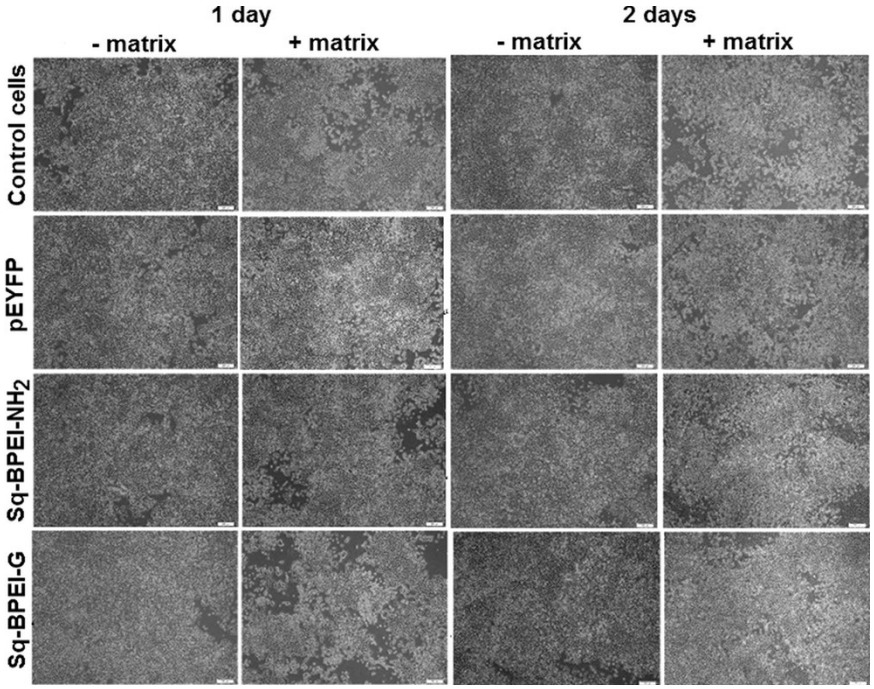


Figure S5. Fluorescence microscopy images for *in vitro* transfection activity on HEK 293T cells of the polyplexes Sq-BPEI-NH_2/pEYFP and Sq-BPEI-G/pEYFP, for N/P ratios of 20 and 15, respectively. Scale bar 500 µm.



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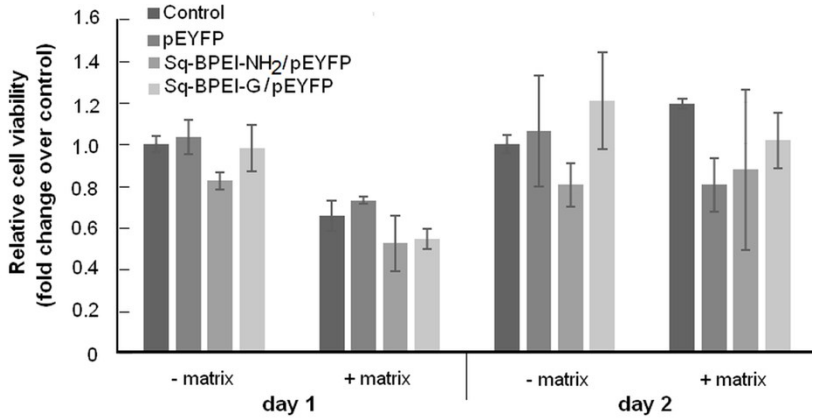

b

Figure S6. Cytocompatibility comparative data. (a) Phase contrast images obtained for HEK 293T cells incubated in the presence of polyplexes formed by conjugates Sq-BPEI-NH_2 and Sq-BPEI-G with pEYFP, free or embedded into matrices, as compared to control samples (cells alone or exposed to free plasmid, in the absence or presence of matrix). Scale bar 200 μm; (b) Viability of HEK 293T cells in similar conditions.

3. Additional Tables

Table S1. Detailed assignment of bands in FTIR spectra.

Code	Bands	
Sq-CHO	2964, 2920, 2854	- ν (C-H) asim and sim in (=CH, CH_3, CH_2)
	2715,2854 (partially)	- ν (C-H) aldehyde (sat)
	1728	- ν (C=O) aldehyde (sat)
	1667	- ν (C=C)
	1446, 1382	- δ (C-H)
	1106, 990	- C-H disubstituted alkene (trans)
	898, 838	- C-H trisubstituted alkene
Sq-CH=N-EO_2-NH_2	3200-3600/peak 3363	- ν (N-H) in amine (NH_2), imine (NH)
	2957, 2925, 2869	- ν (=CH, CH_2,CH_3)
	1667.5	- ν (C=C) aliphatic
	1573	- ν (C=N) imine/ Schiff base
	1471,1378	- δ (C-H)
	1309	- ν (C-N)
	1111	- C-H disubstituted alkene, ν (C-O-C)
	811	- γ (CH)
Sq-CH=N-EO_2-NH-BAC	3200-3600, peak 3250	- ν (N-H) imine/ Schiff base and amide (sec amide, NH associated)
	3066	- ν (C-H) in (=CH_2 vinyl)
	2958, 2923, 2855	- ν (=CH, CH_3, CH_2)
	1652	- ν (C=O) amide I (sat), ν (C=C) aliph
	1620	- ν (C=C) vinyl
	1553	- ν (C=N) imine, ν (C-N), NH def/amide II
	1445,1386	- δ (C-H) and CH_2-S def
	1311	- CH_2-S- wag
	1252	- (C-N amide) amide III
	1124	- ν anti-sim (C-O)
	1103	- ν (C-O), C-H disubstituted alkene (trans)
	1071	- ν (C-S)
	989	- C-H disubstituted alkene (trans)
	962	- δ (H-C-S) bend
	811	- γ (CH)
	696	- ν (C-S)
Sq-BPEI-NH_2	3600-3200, peak 3434	- ν (N-H) NH2, NH amide and imine
	2958, 2920, 2852	- ν (C-H) in (=CH, CH_3, CH_2)
	2480	- protonated form =NH_2^+
	1647 (wide)	- ν (C=O) amide I, ν (C=C) aliph, NH bend
	1463	- (CH) bending
	1125	- ν (C-O-C), C-H disubstituted alkene, ν (C-N-C) in sec amine/imine
	1082-1021	- ν (C-S), ν (C-N) in tert amine
	954	- ν (C-N)
	850-600	- NH bend, wide, multiple

Sq-BPEI-G	3600-3200, peak 3367	- ν (N-H) NH_2, NH in amide and imine
	2951, 2840	- ν (C-H) in (=CH, CH_3, CH_2)
	1651	- ν (C=O) amide I, ν (C=C) aliph, NH bend
	(sh) 1618/1651	coupled δNH/CN in plane for guanidyl group, ν (C=N) imine (Schiff base)
	1459	- (CH) bending
	1082, 1211	- ν (CN), δ (NH), ν (C-O-C), ν (C-H) alkene, ν (C-N-C) in amine (sec, tert), ν (C-S)
	954	- ν (C-N)
Sq-BPEI-FITC	3700-3200, peak 3433	- ν (N-H) NH_2, NH in amide and imine
	3050 (sh),	- ν (C-H) in =CHvinylidene and aromatic,
	2959, 2928, 2850	- CH3, CH2
	1650	- ν (C=O) amide I, ν (C=C) aliph, NH bend
	1636	- ν (C=C) arom
	1573	- superposing ν (C=C) arom, ν (C=O) in FITC with ν (C=N) imine, ν (C-N), NH def (amide II) and amide III in sec thioamide
	1463,1390	- ν (C=C) arom, ν C=O sym, δ (C-H)
	1329,1299,1211,1108	- ν (C-O)
	1171	- (C-O) phenol (bend)
	1021	- ν (C-S) + ν (C-N) in tert amine
	954	- (C-H) disubstituted alkene, ν (C-N)
	915, 852, 812, 771	- CH arom (out of plane) bend
	771	- NCS def

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