Dense cross-linked interpolyelectrolyte complexes modified by targeting peptide as pH-responsive nucleic acids delivery system

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Summary

Development of drug delivery systems which could exploit difference in pH between bloodstream and endosomes is a promising direction for solving the problems of the efficiency of drugs delivery and it's release inside the cell. The nanoparticles based on interpolyelectrolite complexes of poly-l-lysine with heparin capable of encapsulation of genetic constructions, i.e. model oligonucleotide, DNA, and siRNA, cross-linked with pH-sensitive ketal linker were obtained. The particles were modified with RGD-targeting peptide with the aim to facilitate cellular uptake. The cytotoxicity of particles was studied. The cellular uptake of Cy5 labeled particles was investigated by flow cytometry method. The efficacy of cross-linked particles as siRNA delivery system was evaluated.

1. Introduction

Application of genetic constructions as novel generation of medicines in nearest future will enable the effective treatment of different genetic disorders as well as curing of serious social diseases, such as cancer and diabetes. The prospective therapeutic medicines can be developed as based on small interfering RNAs (siRNAs) could be used to effectively "switch of" the expression of undesired genes, while plasmid DNAs (pDNAs) are useful for induction ("switch on") of protein expression.

The instability of nucleic acids (NAs) in the physiological environment as well as their toxic effects on healthy cells hinder the application of genetic drugs. Thus, the development of the delivery systems, which protect NAs and enhance the efficiency of their intracellular internalization into the target cells, is obviously necessary. It should be also noted, that the milestone of the gene delivery systems is the possibility of their systemic in vivo administration, which should make them commercially applicable.

The prospective strategy in design of NA delivery systems is the development of the stimuli-responsive delivery systems which are able to release the therapeutic cargo by a certain physical, chemical or physiological trigger [1]. For example, delivery systems could exploit the difference in pH level between extracellular and intracellular compartments. Such pH-responsive delivery systems are able to retain therapeutic NAs in the bloodstream at pH about 7.4 and release the drug after cell penetration at a characteristic endosomal pH 5.0-6.5 [2, 3].

In this study, we developed and tested pH-responsive nanogels for NAs delivery based on interpolyelectrolyte complexes of poly-L-lysine and heparin, which are cross-linked with a pH-sensitive ketal linker. Polymers and linker were modified with methacrylic groups, which can be cross-linked via thiol-ene click-reaction. Poly-L-lysine is aimed to condense NA due to electrostatic interaction between it's protonated NH_2-groups and PO_4-groups of NA in order to preserve it against degradation and provide compact structure for intracellular penetration. The

competitive polyanion heparin included is aimed to weaken the electrostatic interaction and provide effective intracellular release. The cross-linking with a ketal linker in turn reduces the molecular mobility of the heparin and stabilizes the particle structure in the blood and extracellular matrix. After the penetration of the particles into the cells, the pH-sensitive linker should degrade at endosomal pH, therefore unbound macromolecular components become more mobile and start to release NA due to competitive action of heparin.

The particles were modified with RGD-targeting peptide. This peptide is known to specifically bind with integrin $a_v b_3$ receptors and facilitate receptor-mediated endocytosis [4]. The modified with RGD-targeting peptide particles were investigated with the aim to test if the peptide increase the efficacy of particles intracellular uptake.

2. Experimental section

2.1 Synthesis and modification of polymers and pH-sensitive linker

2.1.1 Synthesis of poly-L-lysine

The synthesis of poly-l-lysine (pLys) was performed via n-hexylamine initiated ring-opening polymerization of corresponding N-Carboxyanhydride in anhydrous ethylacetate as described elsewhere [5]. The reaction was carried out at 25 °C 48 h. The product was precipitated with an excess of diethyl ether, then the precipitate was filtrated, washed with diethyl ether and dried. The product was cotton-like white polymer.

2.1.2 Modification of poly-L-lysine

Modification with methacryloyl chloride: poly-L-Lys (33 mg, 0.257mmol of NH_2-groups) was suspended in mixture of distilled water (400µL) with acetone (200µL) and under magnetic stirring at room temperature. Then, the hydroxide aqueous solution (11 mg, 0.196 mmol of KOH in 100µL of distilled water) was added under magnetic stirring and cooling with an ice bath. Then, methacryloyl chloride in the corresponding amount (8mg, 0.077 mmol - desired modification of 30% NH_2-groups, 11mg, 0.103 mmol - 40%, 16mg, 0.154 0.077 mmol - 60%) was added and potassium hydroxide aqueous solution (11 mg, 0.196 mmol of KOH in 100µL of distilled water) was added for four times every 5 min, afterwards the reaction was left for 12 h. After that, the precipitate obtained was purified via dialysis (Spectra/Pore® dialysis bags, MWCO 1kD) against distilled water for 36h followed by freeze-drying for 1 day.

Transferring of methacrylated poly-L-lysine into water solution: methacrylated poly-L-Lys was dissolved in mixture of Dimethyl Sulfoxid (DMSO) and Trifluoroacetic acid (TFA) (1mg/2ml, DMSO:TFA=50:50) with ultrasonic dispersion. Then, the solution was transferred to dialysis tubes (MWCO 1000) and the solvent was replaced by distilled water via dialysis for 48h.

Modification with targeting RGD-peptide: Maleimide-PEG_{12}-succinimidyl ester Reagent (MI-PEG_{24}-NHS) solution (11mg/200µL dry DMSO) was added to the solution of poly-L-Lys (1.1mg/2ml PBS) or methacrylated poly-L-Lys (0.5mg/2ml PBS) in the equivalent amount to NH_2-groups of the corresponding polymer. The reaction mixture was left in a shaker at room temperature for 30 min. Afterwards, the unbound Reagent was removed by ultra-filtration (Vivaspin filter tubes, MWCO 3kD). Then, to the (poly-L-Lys)-NHS obtained the RGD-SH peptide was added in 10% amount in relation to NH_2-groups and the reaction mixture was left in a shaker at room temperature for 30 min. The product was purified via ultra-filtration (Vivaspin filter tubes, MWCO 3kD).

2.1.2 Modification of heparin

Modification with 2-Aminoethyl methacrylate:

At the first stage, 2-aminoethyl methacrylate hydrochloride (AEMA•HCl) was transferred to free amine. AEMA•HCl (300 mg) was dissolved in CH_2Cl_2 (10 ml) and then, the diisopropylethylamine (450 µl) was added dropwise with stirring. The reaction mixture was vigorously stirred for 2 hours at room temperature. The salt precipitate was separated using Schott filter and discarded. The filtrate containing AEMA was concentrated with rotary evaporator and then, diethyl ether was added to precipitate unreacted AEMA•HCl. After the salt precipitated was filtered out and the diethyl ether was removed by rotary evaporation, AEMA was obtained as clear yellowish liquid.

At the second stage, the heparins carboxylic groups were activated. Heparin (150mg, 0.218mmol) was dissolved in MES buffer solution (65 ml, pH 5.6). Then, N-hydroxysuccinimide (NHS) (19 mg, 0.164mmol) dissolved in MES buffer solution (5 ml, pH 5.6) was added with stirring to the cooled solution (ice bath). After 5 min, EDC (25 mg, 0.164mmol) dissolved in MES buffer solution (5 ml, pH 5.6) was added to the reaction mixture and it was stirred at 0°C for an hour. The amounts of NHS and EDC were taken from the calculation of the activation of 30% COOH-groups of the heparin. Activated heparin was purified from the components of the reaction mixture and concentrated via ultra-centrifugation (Vivaspin filter tubes, MWCO 5kD).

Then, the AEMA solution in borate buffer (pH 8.4) was prepared and activated heparin was added into cooled to 3°C solution with stirring. The reaction mixture was stirring in 3°C (fridge) for 2 hours and then left in the room temperature with stirring overnight. The methacrylated heparin was purified via dialysis (Spectra/Pore® dialysis bags, MWCO 5kD) for 36 hours followed by freeze-drying for 2 days.

Labeling with Cy5-dye: For the fluorescent labeling Cy5-NH_2 dye was used. Before conjugation, heparin's COOH-groups were activated. NHS (0.4eq to COOH-groups) and EDC (0.4eq to COOH-groups) dissolved in PBS were added to the heparin's solution in PBS (2.5 mg/1ml) and the reaction mixture was stirring at 0°C for 1 hour. Afterwards, the Cy5-NH_2 solution (0.01eq, 2.2mg/1ml, dry DMSO) was added to the activated heparin and the reaction was left in the dark with stirring at room temperature for 24hours. The product was purified via ultra-centrifugation (Vivaspin filter tubes, MWCO 10kD).

2.2.3 Modification of pH-sensitive linker

Modification with methacryloyl chloride:

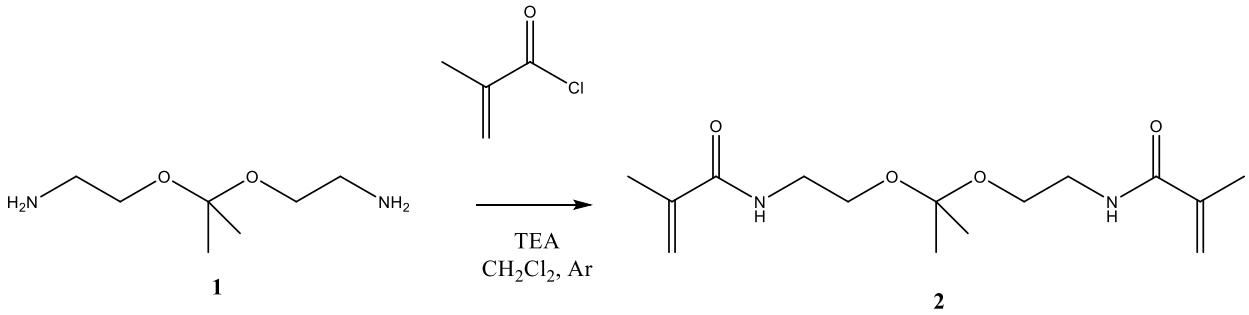

Figure 1. Brief scheme of pH-sensitive linker methacrylation

The pH-sensitive aminoketal linker **1** (**Figure 1**) was synthesized by our colleagues at the Organic Chemistry Department (Institute of Chemistry, SPbU). With the aim of double bonds introducing **1** (0.500g, 9 mmol) was dissolved in THF (15mL) and triethylamine (1.8g, 18 mmol) was added. After purging the reaction mixture with argon for 10 minutes, methacryloyl chloride (1.9g, 18mmol) in THF (5mL) was added dropwise with syringe through a septum and the reaction was stirring at 0°C (ice bath) for 60 minutes. Afterwards, the reaction mixture was left stirring at room temperature overnight. The reaction was carried out in the presence of triethylamine excess, that bind the chloride anions, resulting triethylamine hydrochloride was removed by filtration. The solvent was removed by rotary evaporation. The residue was dissolved in ethyl acetate (100 mL) and washed with 10% (w/v) K_2CO_3 solution (70 mL × 3). The organic phase was combined and dried over anhydrous Na_2SO_4. The solution was filtered and concentrated by rotary evaporation. The crude oily product was purified with column chromatography (silica gel, ethyl acetate with addition of TEA 15%). As a result, methacrylated ketal linker **2** (**Figure 1**) was obtained as yellow oil (Yield: 0.328g, 36%).

2.2 Particles preparation with oligonucleotide, DNA and siRNA encapsulation

Cross-linked hydrogel nanoparticles were obtained by ionotropic gelation due to the electrostatic interaction of protonated amino groups of pLys (or methacrylated pLys - further interchangeable) with anionic groups of nucleic acid (NA) and heparin, followed by cross-linking of pLys and heparin by photopolymerization. On the first stage of particle preparation, an aqueous solution of NA (i.e., model oligonucleotide/ siRNA/ DNA) was added into a PBS solution (nuclease-free water solution in case of siRNA) of pLys (1mg/2mL) and left on the left on the shaker at 21°C for 1 hour. Then, a heparin solution in PBS (nuclease-free water solution in case of siRNA) (2mg/1mL) was added to the solution of interpolyelectrolite complexes formed between pLys and NA and the mixture was stirred for 1 minute (Vortex, Thermo Fischer Scientific). Afterwards, the 10wt% of methacrylated pH-sensitive linker, 1.5wt% of dithiothreitol (Sigma,) and 0.05wt% of photoinitiator (Irgacure-2959) were added into particles solution and the solution was transferred into a well of 240well-plate provided with magnetic stirrer and subjected to UV (Bio-Link365, 365 nm, 1.4 J/cm^2) for 25 minutes followed by stirring at room temperature for 2 hours. Unreacted components were removed from the particles solution via ultra-centrifugation (Vivaspin filter tubes, MWCO 10kD) and particles were washed with PBS (nuclease-free water solution in case of siRNA) 5 times.

Size and ζ-potential: hydrodynamic diameter, polydispersity index (PDI), and ζ-potential were determined by dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively, by using Litesizer500 (Anton Paar).

2.3 Cell culture experiments

2.3.1 Cytotoxicity

HEK 293 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (P/S) culture medium. 8×10^3 cells per well were seeded in a 96-well plate in culture medium (100 μL/well). After 24 h, the medium was replaced with the culture medium containing particles of different concentration. The viability was determined after 48 h incubation via application of CellTiter-Blue (CTB-assay).

2.3.2 Cellular uptake

For evaluation of IPECs cellular internalization dynamics, flow cytometric (FCM) analysis was used. 1×10^5 of HEK 293 cells in 600μL of medium were seeded in each well of 24-well plates. After 24 h cultivation medium was removed and 300 μL of medium containing labeled cross-linked particles (50 μg/mL) was added. The exposure times were 0, 5, 30, 60, 120, 240, 360 min and 24 hours. At each time point, the cells were washed with warm PBS, detached by trypsin solution and centrifuged at 300 g for 5 min. Then the cells were washed with PBS and resuspended in 200μL of PBS. The fluorescence signals were measured via flow cytometry (BD Accuri™ C6 Plus Flow Cytometer). At least 10,000 events per sample were analyzed. Only viable cells were taken for the analysis. Particles cellular internalization is presented as mean fluorescence intensity of viable cells. Data are presented as mean ± SD (n = 3).

2.3.3 GFP Gene silencing

NIH 3T3 GFP+ mouse fibroblast cells were used to test the efficiency of GFP expression knockdown by siRNA delivered by developed cross-linked nanoparticles. For this, 3×10^3 cells in 100 μL of medium were seeded per each well of a 96-well plate. After 24 h, the medium was

removed and 200 μL of siRNA loaded cross-linked particles solution in culture medium, containing 0.05nmol of encapsulated siRNA was added to each well. The silencing of GFP gene was being monitored with Incucyte S3 Live-Cell Analysis Systems during 72 hours.

3. Results and Discussion

3.1 Particles formation

Poly-L-lysine modified with 30% 40% and 60% of methacrylic groups (pLysMA(30), pLysMA(40), pLysMA(60) respectively) was prepared as described in experimental section. To our surprise pLysMA(30) turned out to be extremely low soluble and could not be exploit for the particles formation. The polymers pLysMA(30), pLysMA(40) demonstrated law solubility and required optimization of the conditions for polymer transferring into solution with the aim of further binding with NA. The optimized procedure is described in detail in experimental section (*2.2.1)*.

Based on the fact, that both polymers showed comparable low solubility, for further investigation pLysMA(30) was chosen, as the higher number of NH_3^+ groups in the counterionic polymer seems to facilitate more effective NA binding. Also, in order to investigate the effect of counterion polymer on particles' efficacy non-modified poly-L-lysine (pLys) was used as a proven efficient counterionic polymer for the NA binding.

The cross-linked particles obtained with pLysMA(30) and pLys and loaded with model oligonucleotide, DNA and siRNA were investigated using dynamic light scattering (DLS) and electrophoretic light scattering (ELS). All particles demonstrated hydrodynamic diameter smaller 700nm and negative ζ-potential in the range from -28.4±0.5 mV to -41±1 mV that is suitable for cellular uptake according to the previously published data [6].

3.2 Cytotoxicity of cross-linked nanoparticles

The *in-vitro* cytotoxicity of cross-linked particles at concentrations from 16 to 500 μg/mL was studied for HEK-293 (human embryonic kidney) cell line. Results of CTB-assay for HEK-293 cells incubated with particles loaded with model oligonucleotide ([oligonucleotide-PO_4^-]:[pLysMA(30)-NH_3^+]:[Hep-SO_3^-]) = 1:2:6) for 48 h presented in **Figure 2**. It can be seen that particles do not demonstrate toxicity for HEK-293 cell line at all concentrations.

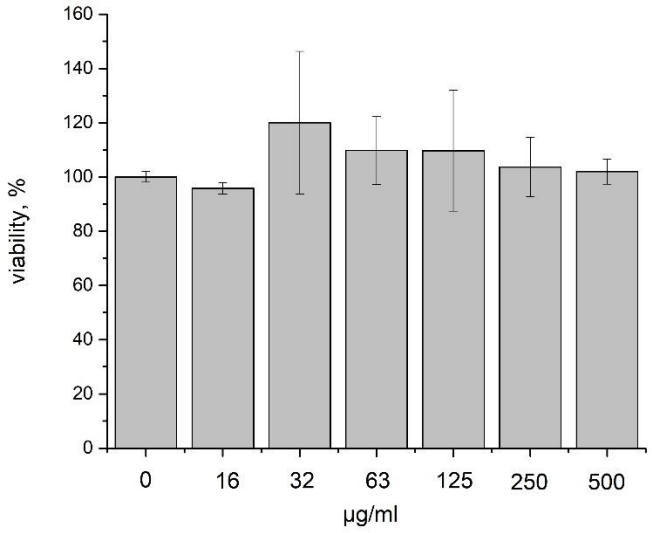

Figure 2. CTB test: viability of HEK 293 incubated with different concentrations of cross-linked particles loaded with model oligonucleotide.

3.2 Cellular uptake

For cellular uptake study oligonucleotide loaded nanoparticles with heparin covalently labeled with Cy5 dye (1 mol% of Hep-COOH groups) were formed. Cellular uptake of nanoparticles was studied using HEK-293 cell line, the time of exposure was 2 and 4 and 24 hours. To evaluate efficacy of cellular uptake flow cytometry was applied. The fluorescent intensity calculated according to the formula:

$$Fluorescence\ intensity = Sample\ fluorescence - Control\ Sample\ flourescence$$

Fluorescence intensity for cells incubated with particles containing pLysMA(30) ([oligonucleotide-PO_4^-]:[pLysMA(40)-NH_3^+]:[Hep-SO_3^-])=1:1:3] and pLys ([oligonucleotide-PO_4^-]:[pLysMA(40)-NH_3^+]:[Hep-SO_3^-] = 1:2:6) are shown in the **Table 1.**

Table 1. Fluorescence intensity of viable cells (mean ± SD (n = 3))

	2h	*4h*	*24h*
pLys	(140 ± 2)*10	(200 ± 10)*10	(70 ± 8)*10
pLysMA(30)	(6 ± 1)*10	(7 ± 4)*10	-

The data obtained demonstrate effective cellular uptake of particles containing pLys during first 4 hours. The diminishing of fluorescent signal after 24 hours of incubation cells with pLys containing particles can be ascribed to the intracellular decay of delivery particles with the loss of fluorescent label.

To study the cellular uptake of particles containing pLysMA(30) a different procedure with Propidium Iodide application was suggested. Propidium Iodide (PI) is a red-fluorescent counterstain (λ_{ex}/ λ_{em} = 535/617) which binds to DNA by intercalating between the bases and is unable to penetrate the membrane of living cells.

For the usage of PI as a fluorescent agent the particles loaded with DNA was obtained. To improve the internalization of particles, the starting pLysMA(30) was modified with 10% of targeting RGD-peptide (the procedure described in *2.2.1*). On the stage of pLysMA(30)/DNA particles formation PI was added to stain DNA (0.5μL PI/1μg DNA). Before the cross-linking of particles with heparin, unbound PI was removed by ultra-filtration (MWCO 5kD), followed by washing with water for 4 times. As double-strand DNA has higher charge density than oligonucleotide, the higher ratio of pLysMA(30) was applied: [DNA-PO_4^-]:[pLysMA(30)-NH_3^+]:[Hep-SO_3^-] = 1:10:6). The fluorescent image of the particles was obtained with Cytation5 (Texas Red filter, 593 nm) (**Figure 3**).

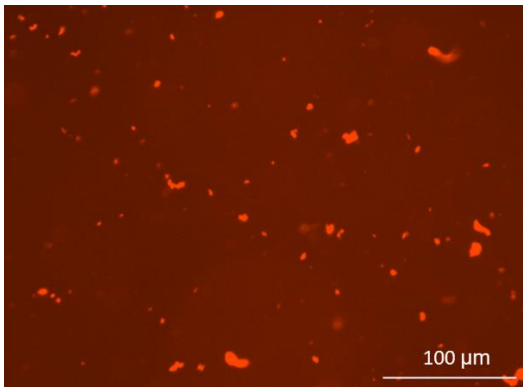

Figure 3. Image of RGD-modified cross-linked particles containing DNA stained with PI (Cytation5, Texas Red Filter) ([DNA-PO_4^-]:[pLysMA(40)-NH_3^+]:[Hep-SO_3^-] = 1:10:6)

Cellular uptake of particles was investigated via flow cytometry (**Figure 4**). The possibility of dead cells fluorescent staining was excluded due to the cutting off the population of dead cells by size and shape.

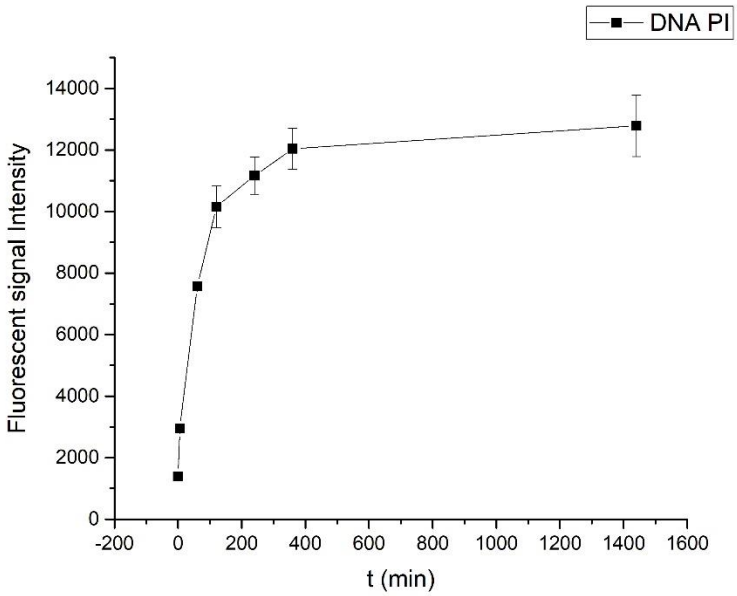

Figure 4. Fluorescence signal intensity after incubation of HEK 293 cells with cross-linked particles obtained via flow-cytometry.

According to the flow-cytometry study results it can be concluded that the particles are effectively captured by the cells during first 2 hours of incubation and the internalization process reaches the plateau within 6 hours.

3.3 GFP Gene silencing

With the aim to estimate the possibility of using the particles obtained for gene therapy, the particles loaded with siRNA which could knockdown the expression of green fluorescent protein (GFP) was prepared. Particles containing non-modified pLys and pLys modified with targeting RGD-peptide ([siRNA-PO_4^-]:[pLys-NH_3^+]:[Hep-SO_3^-] = 1:1:3) was tested on NIH-3T3 GFP+ cells. The silencing of GFP gene was monitored with Incucyte S3 Live-Cell Analysis Systems (**Figure 5**).

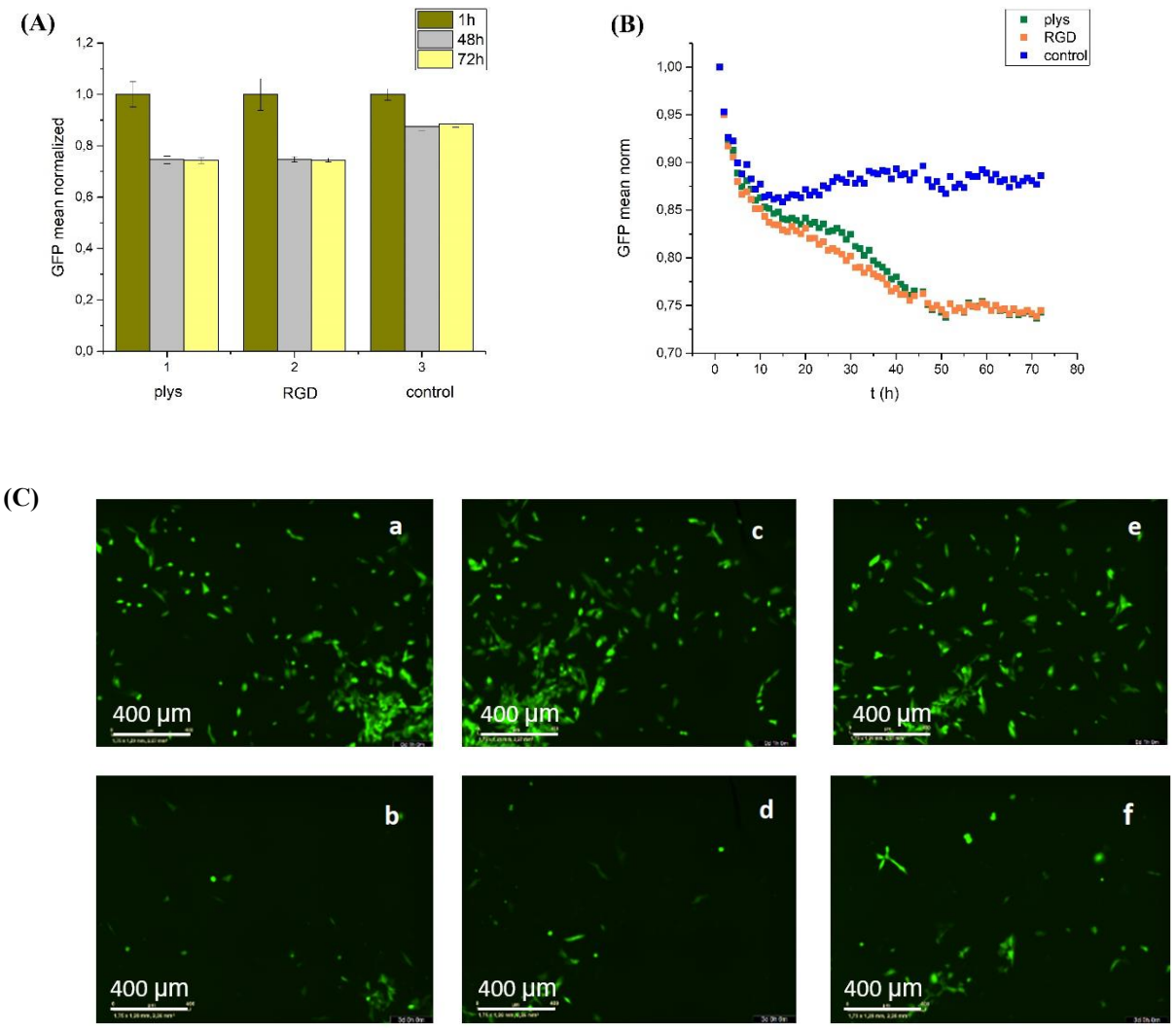

Figure 5. (**A**) Comparison of fluorescence decreases after 1 hours, 48 hours and 72 hours of and intact cells incubation as a control (control) and cells incubation with particles containing non-modified pLys (plys), RGD-peptide modified pLys (RGD). (**B**) The time-fluorescence curve for the period from 1 hour to 72 hours. (**C**) The images from Green fluorescent channel for **a** - cells incubated with particles containing non-modified pLys (plys) for 1h, **b** - cells incubated with plys for 72h, **c** – cells incubated with particles containing RGD-peptide modified pLys (RGD) for 1h, **d** - cells incubated with RGD for 72h, **e** – intact cells incubated for 1h, **f** - intact cells incubated for 72h

The cells demonstrated (25±1)% decrease in fluorescence after 72h of incubation with siRNA loaded particles. While the final decrease of fluorescence turned to be the same for particles with pLys and with targeting RGD-peptide, one can observe that the fluorescent decrease pattern for RGD-modified particles is more rapid in the period from 10 hours to 35 hours of incubation (**Figure 4(B)**). Such pattern might be ascribed to the faster cellular internalization facilitated by efficient RGD interaction with the cell membrane.

4. Conclusion

The cross-linked with pH-sensitive ketal linker particles based on interpolyelectrolite complexes of poly-L-lysine and heparin were obtained. The lack of cytotoxicity of particles was shown on the HEK 293 cells. The cellular uptake of particles obtained was confirmed with flow cytometry study. The knock-down of GFP gene expression using particles loaded with siRNA was investigated and the difference in efficacy between non-modified and modified with RGD-peptide particles was demonstrated.

5. References

[1] S. Mura, J. Nicolas, and P. Couvreur, "Stimuli-responsive nanocarriers for drug delivery," *Nat. Mater.*, vol. 12, no. 11, pp. 991–1003, Nov. 2013, doi: 10.1038/nmat3776.

[2] M. Rizwan *et al.*, "pH Sensitive Hydrogels in Drug Delivery: Brief History, Properties, Swelling, and Release Mechanism, Material Selection and Applications," *Polymers (Basel).*, vol. 9, no. 12, p. 137, Apr. 2017, doi: 10.3390/polym9040137.

[3] Y. Yang *et al.*, "One-pot synthesis of pH-responsive charge-switchable PEGylated nanoscale coordination polymers for improved cancer therapy," *Biomaterials*, vol. 156, pp. 121–133, Feb. 2018, doi: 10.1016/j.biomaterials.2017.11.038.

[4] L. Qiu *et al.*, "cRGDyK modified pH responsive nanoparticles for specific intracellular delivery of doxorubicin," *Acta Biomater.*, vol. 30, pp. 285–298, Jan. 2016, doi: 10.1016/j.actbio.2015.11.037.

[5] O. Osipova *et al.*, "Amphiphilic Polypeptides for VEGF siRNA Delivery into Retinal Epithelial Cells," *Pharmaceutics*, vol. 12, no. 1, p. 39, Jan. 2020, doi: 10.3390/pharmaceutics12010039.

[6] V. Korzhikov-Vlakh *et al.*, "Photosensitive Poly-l-lysine/Heparin Interpolyelectrolyte Complexes for Delivery of Genetic Drugs," *Polymers (Basel).*, vol. 12, no. 5, p. 1077, May 2020, doi: 10.3390/polym12051077.