DNA COMPLEXATION BY CATIONIC PULLULAN POSSESSING THERMO-SENSITIVE UNITS

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Cationic pullulan (PN) with pendant thermo-responsive units were complexed with DNA to obtain a potential thermo-sensitive gene carrier. Semitelechelic oligomers of N-isopropylacrylamide/N,N-dimethylacrylamide possessing –COOH end groups and a lower critical solution temperature of about 36 ^{o}C at pH = 7.4 (50 mM phosphate buffer) were prepared by free radical polymerization in the presence of mercaptopropionic acid as the chain transfer agent. Then, the functionalized pullulan with cationic groups was coupled with thermo-responsive oligomers by reaction between the –COOH end groups of oligomers and the remaining–OH groups of PN. Lastly, the thermo-responsive cationic pullulan chains were complexed with DNA at different charge (+/-) ratios. The size of the complexes ranges between 160 and 510 nm depending on the polymer/DNA charge ratio and temperature. The complexes obtained at a charge (+/-) ratio of 5/1 showing a diameter < 200 nm, were stable in the presence of bovine serum albumin, and were unaffected by nuclease degradation.

(Received April 14, 2011; Accepted May 2 , 2011)

Keywords: Pullulan, poly(N-isopropylacrylamide) copolymers, Smart polymers, DNA complexes, Potential gene carrier

Abbreviations: AIBN, N,N'-azobisisobutironitrile; BSA, bovine serum albumin; CDI, N,N'-carbonyldiimidazole; DFT, defibrotide, single stranded polydesoxyribonucleotide; DMAAm, N,N-dimethylacrylamide; DMF, N,N'-dimethylformamide; DMSO, dimethylsulfoxide; DMAPA, 3-dimethylamino-1-propylamine; DS, degree of substitution; FCS, fetal calf serum; LCST, lower critical solution temperature; MPA, mercaptopropionic acid; NIPAAm, N-isopropylacrylamide; OIn, poly(NIPAAm-co-DMAAm) with COOH end-group, PNIPAAm, poly(N-isopropylacrylamide); poly(NIPAAm-co-DMAAm), poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide); POIn, cationic pullulan with thermosensitive units; PN, cationic pullulan.

1. Introduction

Gene therapy may take advantage by designing sophisticated gene vectors. A number of vectors for gene delivery have been synthesized; however they are unsuitable in terms of efficiency, selectivity, and safety [1]. Thus, although non-viral vectors, such as cationic lipids and

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cationic polymers, possess advantages over viral vectors, they display lower transfection efficiency than adenovirus vectors which show the highest transfection efficiency [1,2]. Polymeric gene carriers may have some advantages over lipid systems: (*i*) a relatively smaller size and narrow distribution, (*ii*) a higher stability against nucleases, and (*iii*) an easier control of the hydrophilicity/hydrophobicity by copolymerization [3].

Cationic lipid and polymeric carrier systems have a common dilemma in that they must fulfil the following requirements: (*i*) cell uptake and prevention of DNA degradation; and (*ii*) intracellular DNA release for transcription by RNA polymerase [4].

To investigate carrier system-DNA formation and dissociation, we focused our attention on the synthesis and characterization of a thermo-responsive cationic carrier whose DNA complexating abilities are modulated by temperature.

Cationic natural polysaccharides as gene delivery carriers (*i.e.*, cationized dextran [5] including DEAE-dextran [6], schizophyllan [7], and chitosan [8]) have several attractive advantages over synthetic polymer-based gene carriers. Indeed, since polysaccharides are derived from natural sources, they are expected to be non-toxic, biocompatible, and biodegradable. Moreover, they are as flexible as synthetic polymers in terms of modification for improved gene transfer and possibly cell internalization by sugar recognition receptor(s) present on the cell surface [9].

Pullulan, a water-soluble polysaccharide with a repeated unit of maltotriose condensed through the α-1,6 linkage, is know for its non-toxic, non-immunogenic, non-mutagenic, and non-carcinogenic properties, moreover it has been widely explored for various biomedical applications, including tissue engineering as well as targeted drug and gene delivery [10]. In addition, it has been established that pullulan exhibits extremely high flexibility in aqueous solutions [11] and inherent liver affinity [12]. Lastly, the hemocompatibility and efficiency of quaternary ammonium group-introduced PN has been reported as a gene delivery vector [13].

Remarkably, recent investigations on thermo-responsive polymers for gene delivery applications have attempted to regulate the degree of DNA condensation via temperature cues. This class of polymers undergoes a reversible coil-to-globule phase transition in response to changes of temperature. Poly(N-isopropylacrylamide) (PNIPAAm) exhibits a lower critical solution temperature (LCST) at 32 °C [14] and has been applied extensively to obtain thermo-responsive hydrogels [15], bioconjugates [16], chromatographic systems [17], and thermo-responsive polymeric micelles as drug carriers [18]. Below this LCST, PNIPAAm is water soluble and hydrophilic, existing in an extended chain form. Above the LCST, PNIPAAm undergoes a reversible phase transition to an insoluble and hydrophobic aggregate [14]. Oligomers with reactive end groups allow facile synthesis not only of block and graft copolymers but also of oligomer-modified biomolecules. If biomolecules, such as enzymes, antigens, antibodies, and nucleotides, could be modified while retaining their bioactivity, new macromolecular bioconjugates with an array of potential applications would become available [19]. In this context, semitelechelic oligomers could not display unspecific aggregation with biomolecules because they possess one reactive group per oligomer only and it is expected to import high temperature sensitivity due to the inherently high mobile nature of the oligomer free-end groups [20]. As the phase transition of PNIPAAm corresponds to the stability of hydrophobic groups present in the polymer chain in aqueous media, oligomers copolymerized with another hydrophobic or hydrophilic comonomers show different LCSTs compared to homogenous PNIPAAm [21]. Moreover, polysaccharide coupled with semitelechelic oligomers of different LCSTs should display different LCSTs reflecting the respective conjugated oligomers [22]. Moreover, it has been reported the possibility to improve the transfection efficiency in a temperature-dependent mode by NIPAAm-based vectors [22,23].

The extent to which thermo-responsive polymers can enhance transfection depends on optimizing several important parameters including (*i*) the time and temperature protocol for effective intracellular release, (*ii*) the balance of functionalities to complement the thermo-responsive properties (*e.g.*, cationic character, hydrophobicity, and endosomal escape capabilities), and (*iii*) the LCST range suitable for in vivo applications. The use of such polymers for gene delivery applications represents a growing area of research with a potential that remains to be seen [24].

From these perspectives, here we report the synthesis and characterization of a water-soluble polymer with thermo-responsive properties obtained by conjugation of cationic pullulan with a semitelechelic oligomer containing carboxyl end groups, based on poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide) (poly(NIPAAm-co-DMAAm)). Then, the cationic thermo-responsive cationic pullulan was complexed with DNA, the effect of bovine serum albumin (BSA) on the complex stability was investigated, and the resistance against nuclease degradation was examined.

2. Experimental

2.1. Materials

Pullulan (Mw = 200,000 g/mol) was purchased from Hayashibara Lab. Ltd. (Okoyama, Japan). N-isopropylacrylamide (NIPAAm; from Sigma-Aldrich Chemie Gmbh, Germany) was recrystallized from hexane. N,N-Dimethylacrylamide (DMAAm; from Sigma-Aldrich) was purified by vacuum distillation at 81°C/ 20 mmHg. N,N'-Azobisisobutironitrile (AIBN; from Fluka, Buchs, Switzerland) was recrystallized from methanol. Bovine serum albumin (BSA) and Fetal calf serum (FCS) were obtained from Sigma-Aldrich Chemie Gmbh, Germany. Dimethylsulfoxide (DMSO), N,N'-dimethylformamide (DMF), mercaptopropionic acid (MPA), 3-dimethylamino-1-propylamine (DMAPA), N,N'-dimethylaminopyridine (DMAP), N,N'-carbonyldiimidazole (CDI), and all the other chemicals were purchased from Fluka. DMSO and DMF were distilled under vacuum. All chemicals were used without purification unless stated. The single stranded polydesoxyribonucleotide (DFT) was obtained from Crinos Industria (Como, Italy).

2.2. Synthesis

2.2.1. Synthesis of poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)

Synthesis of linear poly(NIPAAm-co-DMAAm) was carried out by free radical copolymerization in 1,4-dioxane using AIBN as the initiator. Typically, 1.13 g NIPAAm (10 mmol), 0.198 g DMAAm (2 mmol), and 0.010 g AIBN (0.06 mmol) were solubilized in 10 ml 1,4-dioxane. Dried nitrogen was bubbled through the solution for 30 min prior to polymerization. The reaction mixture was let to react at 70 °C for 10 hours. Then, the polymer was precipitated into diethyl ether, and dried under vacuum. Finally, the copolymer was solubilized in distilled water, dialyzed for 5 days at 20 °C, and recovered by freeze-drying.

2.2.2. Synthesis of semitelechelic poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide) with a carboxyl end-group

Poly(NIPAAm-co-DMAAm) with one reactive carboxyl end-group **(OIn)** was synthesized by chain transfer free-radical copolymerization of NIPAAm and DMAAm, in dioxane, using AIBN and MPA as the initiator and the chain transfer reagent, respectively. The initial solution was purged with nitrogen to remove oxygen. The reaction was performed in a glass reactor at 70±1 ^{o}C for 15 h. The experimental conditions used to prepare the co-oligomers are summarized in Table 1. Oligomers were recovered by repeated precipitation from dioxane into diethyl ether, followed by drying in vacuum.

2.2.3. Preparation of cationic pullulan

2.42 grams (14.9 mmol) of pullulan were dissolved in 25 ml DMSO, followed by the addition of N,N'-carbonyldiimidazole (7.48 mmol), under stirring. After reaction at 25±2 ^{o}C for 6 hours, 7 ml (55.9 mmol) of 3-dimethylamino-1-propylamine were added and the reaction

continued for 48 hours under stirring. The mixture was poured into acetone, and the precipitate was washed 3 times with 30 ml methanol. Finally, the precipitate was solubilized in distilled water, dialyzed for 5 days at 20 ^{o}C, and pure cationic pullulan **(PN)** was recovered by freeze-drying.

The content of cationic amine groups, determined from the degree of substitution (DS), was calculated according to Equation 1:

$$EC = \frac{DS \times 1000}{M_{PN}} \text{ (meq/g)} \tag{1}$$

where EC is the exchange capacity, M_{PN} is the molecular weight of the cationic glucopyranosic unit. DS is the substitution degree.

The DS value with secondary amine groups present in the glucopyranosic unit of PN was calculated from the total nitrogen content according to Equation 2:

$$DS = \frac{162 \times N\%}{28 \times 100 - 128.18 \times N\%} \tag{2}$$

where N is the total nitrogen content (wt. %) and 128.18 is the molecular mass of aminopropyl-3-dimethylamine. M_{PN} is 174.17 for PN with a DS value calculated using equation 2.

2.2.4. Synthesis of cationic pullulan grafted with semitelechelic poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)

Cationic pullulan grafted with poly(NIPAAm-co-DMAAm) **(POIn)** was prepared by coupling reaction between −OH groups of PN and −COOH groups of OIn with molecular weight of 4500 g/mol, using CDI as a condensation agent. Typically, 1.3 equivalents of CDI were added to 0.5 g of OIn dissolved in 4 mL DMSO. After the activation of the carboxyl groups at room temperature for 6 hours, the appropriate amounts of PN and DMAP dissolved in 6 ml DMSO were added and the reaction continued at 60 ^{o}C for 48 hours. The reaction mixture was precipitated into acetone and recovered by filtration. Finally, the product dissolved in water was dialyzed against water for 5 days (Mw cut off = 10,000-12,000; from Medi Cell International, London, England) to remove low molecular weight compounds and unreacted oligomers, and subsequently it was lyophilized. The [COOH]/CDI/DMAP molar ratio was 11/1.3/0.7.

2.2.5. Preparation of polymer/DNA complexes

For calculation of the charge ratios, an average mass per charge of 324 Da was used for DNA. The PN/DNA and POIn/DNA charge (+/-) ratio was expressed as the ratio between moles of the amino groups of PN and POIn and moles of the phosphate groups of DNA. Complexes were allowed to self-assemble in standard phosphate buffer (PB, 50 mM, pH = 7.4) by mixing DNA (20 mg/ml) with the appropriate polymer solution at the desired charge ratio; the DNA concentration was adjusted to 10 mg/ml with PB and left standing at room temperature for 24 h before use. All the polymer/DNA complexes were mixed by vortexing for 5 s immediately prior to use. Complex formation was confirmed by electrophoresis on a 0.8 % (w/v) agarose gel with Tris-acetate running buffer at 60 V for 40 min. Each sample was loaded in a well for electrophoretic separation and DNA was visualized with ethidium bromide.

2.3. Methods

2.3.1. Characterization of the semitelechelic oligomers and polymers

^{1}H-NMR spectra of the polymers were recorded using a Bruker Avance DRX 400 NMR spectrophotometer operating at 400 MHz frequency in deuterated solvents ($CDCl_3$ and D_2O).

The molecular weight of semitelechelic oligomers was determined by end-group titration with 0.01 N NaOH and phenolphthalein at 20 ^{o}C. The molecular weight was calculated according to Equation 3:

$$\text{Mn (g/mol)} = \frac{1}{\text{CS}} \times 1000 \quad (3)$$

where CS is the exchange capacity (meq/g).

2.3.2. LCST determination

Optical absorbance of oligomer and polymer solutions (1 %, w/v) at various temperatures was carried out by monitoring absorbance at 450 nm using a UV-Vis spectrophotometer coupled with a temperature controller.

The polymer solution was prepared in PB. The heating rate was 0.2 ^{o}C every 10 min. The cloud point (CP) is the temperature at the inflexion point in the normalized absorbance versus temperature curve.

2.3.3. Dynamic light scattering measurements

The particle size distribution was determined by using a dynamic light scattering (DLS) technique (Zetasizer model Nano ZS (Malvern Instruments, Malvern, England)) equipped with a red laser 633 nm (He/Ne). Complexes were prepared as previously described, by vortexing for 5 s followed by standing for 20 min, at 23 or 40 ^{o}C. The solutions were filtered (0.4 μm cut off) before any measurement. Each sample was analyzed in triplicate, and the reported data represent the mean values.

2.3.4. Stability of polymer/DNA complexes in the presence of bovine serum albumin

Dispersive stability of PN/DNA and POIn/DNA complexes in the presence of BSA was evaluated by turbidity measurement at 340 nm using a UV-Vis spectrophotometer (PerkinElmer, Norwalk, CT, USA). The polymer/DNA complexes were dispersed in PB. The turbidity of POIn/DNA and PN/DNA (control) complexes at various charge ratios was measured 24 h after incubation at 23 ^{o}C (under the LCST of polymers).

2.3.5. Thermal stability of polymer/DNA complexes

The thermal stability of polymer/DNA complexes was determined as follows. Complexes at the desired charge ratio were incubated under (23 ^{o}C) and upper (40 ^{o}C) the LCST of the polymers in a thermostatic bath, in the presence of 10 wt % FCS. At different time intervals, between 0 and 240 min, samples were withdrawn and stored at -20 ^{o}C until the electrophoretic analysis was performed. Electrophoresis was performed on a 0.8% (w/v) agarose gels containing 0.5 mg/ml ethidium bromide for 40 min at 60 V constant current.

3. Results and discussion

3.1. Synthesis of NIPAAm-based copolymers and oligomers

The biomedical and biological applications of poly(NIPAAm) are possible for copolymers exhibiting a sharp phase transition around the physiological pH and temperature. As previously reported [14], PNIPAAm shows a LCST value of about 32 ^{o}C in aqueous solution. Moreover, in simulated physiological conditions (PB at pH = 7.4) the LCST decreases to a lower value [25]. In order to increase the LCST to body temperature, a series of copolymers of NIPAAm and DMAAm, obtained at different molar ratios of the comonomers in the initial reaction mixture, were tested (Table 1).

DMAAm was used as a hydrophilic comonomer that possibly increases the LCST of the copolymer [26]. As shown in Table 1, the LCST increases toward higher temperatures as the content of DMAAm increases in the copolymer. The copolymer with a comonomer molar ratio in

the feed of 10/4 (Sample In 3) has a LCST of about 36.5 °C in PB. Therefore, these experimental conditions were used for the preparation of semitelechelic oligomers.

Semitelechelic oligomers of NIPAAm with DMAAm possessing −COOH free end groups were synthesized by telomerization using MPA as the telogen at 70 °C in 1,4-dioxane [19]. The composition of OIn was determined from the analysis of ^{1}H-NMR spectra in deuterated chloroform solutions (not shown). The molar fraction of DMAAm in OIn was calculated from the area of the triplet centered at 2.79 ppm, attributed to the methyl protons of the N-substituted methyl groups, and the area of the singlet at 4.01 ppm, attributed to the resonance of the C-2 proton of the isopropyl groups. ^{1}H-NMR analysis confirms copolymer formation (see Table 1).

Table 1. Experimental conditions for copolymer synthesis [a].

Code sample	NIPAAm/ DMAAm (molar ratio)	Content of DMAAm (% mol ratio) in the		LCST (°C)[b]		
		Feed	Copolymer [c]	water	pH = 1.2	pH = 7.4
In 1	10/2	16.66	15.2	36.5	35.7	34
In 2	10/3	23.07	21.5	36.8	36.2	35.4-35.6
In 3	10/4	28.6	25.0	40.8	40.2	36.2-36.6
In 4	10/5	33.33	30.9	-	-	42-44
In 5	10/6	37.5	35.24	46.5	45-45.5	42-44

[a] The values are the mean of two independent experiments. The reaction was carried out in 1,4-dioxane for 15 hours, at 70 ± 1°C.
[b] Determined by UV-Vis spectrophotometry.
[c] Determined by ^{1}H NMR spectroscopy.

As expected, the molecular weight of oligomers decreases as the amount of the chain transfer agent increases (Table 2). All data were consistent with one OIn molecule averaging one −COOH free end group per polymer chain. The oligomer with molecular weight of 4500 g/mol (OIn 4) was chosen to be used in this study because of its moderate molecular weight.

Table 2. Preparation and characterization of semitelechelic oligomers.

Code sample	[S]/[M] [a]	Mn [b] (g/mol)
OIn 1	0.01	12000
OIn 2	0.02	10000
OIn 3	0.03	6500
OIn 4	0.04	4500
OIn 5	0.05	3200
OIn 6	0.07	1900
OIn 7	1	1400

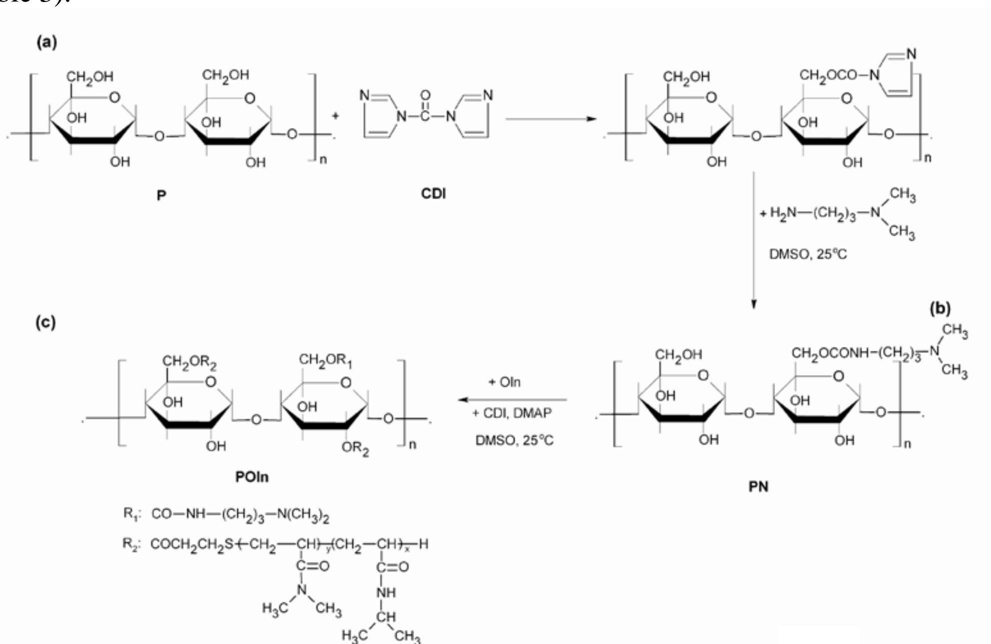
[a] [S]/[M] represents the molar ratio between the chain-transfer agent MPA (S) and the monomers (M).
[b] From the analysis of end-groups.

3.2. Synthesis of cationic pullulan with thermo-sensitive units

Firstly, pullulan was derivatized with 0.545 meq/g (7.04 wt. %) of secondary amine groups by the reaction with 3-dimethylamino-1-propylamine in DMSO, in the presence of CDI as the condensating agent (Scheme 1a,b).

Secondly, cationic pullulan with thermo-sensitive units (POIn) were obtained by the coupling reaction of PN with OIn 4 in the presence of CDI as the condensating agent and DMAP as the catalyst (see Scheme 1c). The reaction parameters and the results of the graft copolymers are summarized in Table 3. The weight content of grafted units was calculated both from sulfur elemental analysis and from the ^{1}H-NMR peak integration of methyl protons of NIPAAm in

poly(NIPAAm-co-DMAAm) and of the anomeric proton from the glucose unit of PN (see Figure 1 and Table 3).



Scheme 1. Synthesis of cationic pullulan grafted with oligo(NIPAAm-co-DMAAm) (POIn).

The ^{1}H-NMR spectrum of P_3OIn shown in Figure 1 evidences the presence of grafted thermo-sensitive units. Indeed, the peaks *a*, *b*, *c*, *d*, and *e* belong to NIPAAm and DMAAm units of OIn 4, that are linked on the main chain of cationic pullulan.

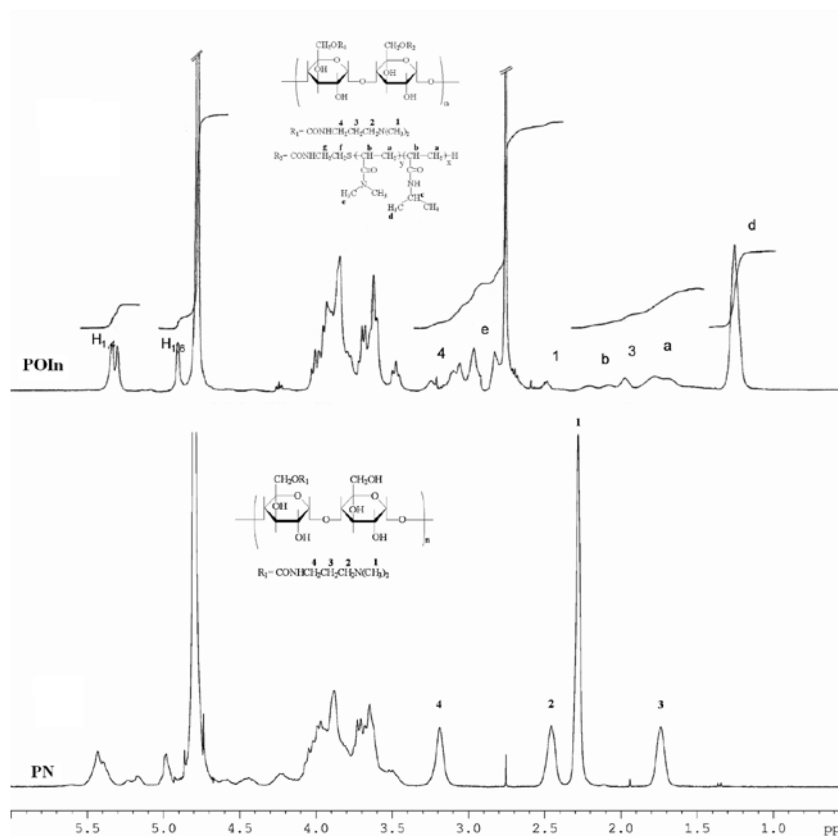


Figure 1. ^{1}H NMR speactra of PN and P_3OIn in D_2O.

As expected, the amount of grafted thermo-responsive units increases on increasing the amount of semitelechelic oligomers in the feed (see Table 3). However, further increase of the oligomers in the feed was not possible due to their limited solubility.

Table 3. Reaction parameters for synthesis of cationic pullulan with thermo-sensitive units

Code Sample	OIn (Mn)	−COOH/UG [a] (molar ratio)	OIn content (wt %) calculated		LCST (°C)
			From sulfur analysis	From ^{1}H-NMR analysis	
P_1OIn	OIn 4	0.039	28.34	27.63	36.2 - 36.6
P_2OIn	(4500)	0.088	39.607	38.72	36.2 - 36.6
P_3OIn		0.16	45.51	47.47	36.2 - 36.6

[a] UG = glucopyranosic unit

3.3. LCST behavior

The phase transition behavior of NIPAAm-based polymers is often studied in pure water or under conditions which may not be fully relevant for biomedical applications [25]. The analysis of the phase transition behavior under conditions more relevant for the intended use of the polymers is necessary to translate any promising results into real settings. The phase transition of the grafted copolymers POIn was therefore measured by observing the change in optical transparency in PB at pH = 7.4 and compared with that of semitelechelic OIn (Figure 2).

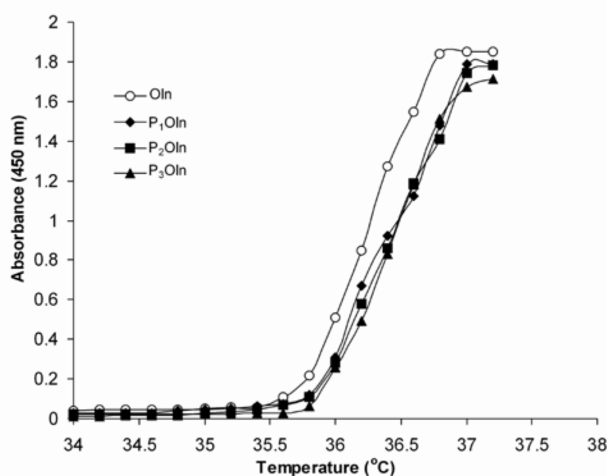


Figure 2. LCST profile of OIn and POIn in phosphate buffer at pH = 7.4 (determined by cloud point technique at 450 nm). The polymer concentration was 1 % (w/v). The continuous lines are "hand-drawn" lines.

As shown in Table 3, the graft copolymers exhibit LCST values close to that of the pure oligomer. Moreover, the sharpness of the phase transition seems to be not altered and lasts in the range of no more than two Celsius degrees (Figure 2). In fact, the comonomer sequences in copolymer are unaffected by the coupling reaction. This is in agreement with literature [27], and suggests that the phase transition temperature of the grafts is not affected by coupling to the polymer backbone. Hydrophilic polymers containing poly(NIPAAm) grafts often exhibit an LCST similar to that of poly(NIPAAm) because the cooperative domains in poly(NIPAAm) grafts that undergo phase transition are not significantly perturbed by the other components [28].

3.4. Binding of DNA to polymers

In order to evaluate the capability of cationic pullulan and cationic thermo-responsive pullulan to complex DNA molecules by electrostatic interactions, a series of experiments was performed using defibrotide (DFT) as a model compound.

PN is a water soluble polymer with tertiary amine side groups which have a pK_a value of about 9.3 [29], therefore, all side groups are protonated under physiological conditions. The ability of PN and POIn to interact with DNA was evaluated by gel electrophoresis and DLS experiments.

DNA binding to PN and POIn copolymers was investigated by agarose gel electrophoresis. Figure 3 shows the ability of cationic pullulan (Figure 3a) and cationic pullulan with thermo-responsive units (P_3OIn, Figure 3b) polymers to bind DNA. Both polymers are able to efficiently load DNA. Migration of DNA was retarded on increasing the charge (+/-) ratio, leveling off at the charge (+/-) ratio higher than a certain value. PN showed DNA complexation with increasing polymer charge (+/-) ratio from 1 to 4. DNA was completely retained by the P_3OIn over a charge (+/-) ratio of 3:1 (Figure 3b). The retardation could reflect the increase of the molecular weight of DNA upon complexation with the cationic polymer bringing thermo-sensitive units.

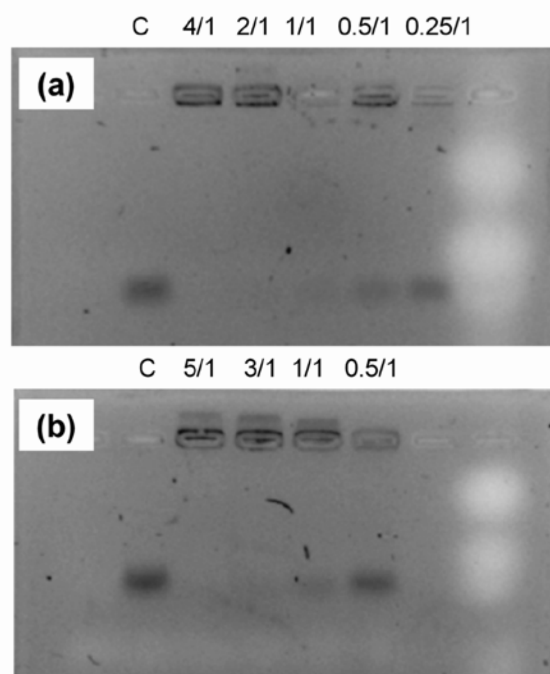


Fig. 3. Gel electrophoresis of PN/DNA (a) and P_3OIn/DNA (b) complexes. The experiments were performed at T = 23±1 °C at different (+/-) charge ratios, DNA was 10 μg/ml. C, control.

To investigate the effect of temperature on the size of polymer/DNA complexes, DLS measurements were performed at 23 °C (below the LCST) and 40 °C (above the LCST) (see Figure 4).

The size of the PN/DNA particles decreases on increasing the charge (+/-) ratio at 20 °C, reaching the minimum value at about 100 nm at the charge (+/-) ratio of 5 (Figure 4a). As expected, control PN/DNA complexes show only small changes in their size at 40 °C, the smallest diameter being 98 nm at the charge (+/-) ratio of 5. This observation is in agreement with the previous finding [27], suggesting that on increasing temperature only a small increase of the molecular weight and size of the control cationic polymers/DNA complexes occurs.

The size of POIn/DNA particles showed a similar trend as a function of the charge (+/-) ratio. At 23 °C, DLS experiments showed double peaks for all the POIn/DNA particles indicating a large polidispersity of 1.3-1.6. The presence of double peaks indicates a different aggregation degree of particles. Only the P_3OIn/DNA complex showed an almost uniform aggregation state with a single peak (*i.e.*, 5:1 of the charge (+/-) ratio). At a smaller charge (+/-) ratio, POIn/DNA complexes formed large particles, possibly reflecting their aggregation. On increasing the charge ratio, the size of POIn/DNA complexes increases and reaches a maximum of 511 nm at 2:1. The further increase of the charge (+/-) ratio up to 5:1 induces the decrease of the size of complexes

(about 236 nm); this could reflect the fall down of complexes as the consequence of strong electrostatic interactions.

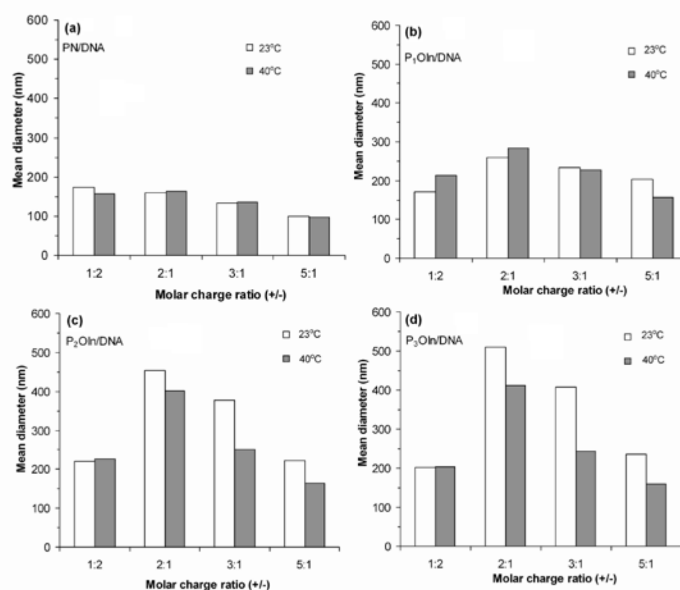


Fig. 4. Particle sizes of polymer/DNA complexes at different (+/-) charge ratios.

The size of the complexes increased with raising the thermo-responsive unit content at 23 °C. Similar trends were observed for DNA complexes of graft copolymers of PNIPAM on poly (L-lysine) [28] and for those which contain poly[*N*-(2-hydroxypropyl)methacrylamide] instead of PNIPAM as the hydrophilic non-ionic block [30].

Temperature increase above the phase transition of OIn induced changes in the size of particles. As expected, the temperature-induced changes were more evident in samples with a higher content of the thermo-responsive component (Figure 4c). Indeed, at the (+/-) charge ratio of 2:1, the hydrophobic chains are prone to self-aggregate leading to the enlargement of the diameter of particles. On the other hand, at the (+/-) charge ratio of 5:1, complex aggregation decreases, and instead, the collapse of PNIPAAm chains in combination with electrostatic interactions facilitates the formation of dense complexes, characterized by a smaller size of particles. These two effects produce particles with different size above/below the LCST and at different (+/-) charge ratio values. The same particle size (~160 nm) for P_2OIn/DNA and P_3OIn/DNA at the (+/-) charge ratio of 5:1 indicates the two opposite contributions are equivalent.

3.5. Stability measurement of the polymer/DNA complexes

Stability measurements were conducted accounting for P_3OIn/DNA complexes prepared at the 5:1 molar ratio.

The turbidity of P_3OIn/DNA and PN/DNA complexes, at various charge ratios, was measured 24 h after incubation with the representative plasma protein BSA. Since BSA is the dominating component of the blood plasma with a net negative charge (concentration as high as 50 mg/ml), it could promote the interaction with oppositely charged complexes after intravenous administration. The results shown in Figure 5 indicate that P_3OIn/DNA complexes showed constant or slightly decreasing turbidity without precipitation, this indicates the enhanced stability of the complex and suggests that PNIPAm lateral units at the surface of the complexes prevent the BSA binding. At 23 °C, the POIn/DNA particles can prevent the adsorption of BSA because of the hydrophilic poly (NIPAAm-co-DMAAm) chains present on the particle surface. Obviously, in the presence of a higher content of thermo-sensitive lateral units an improved protective effect against interaction with BSA could occur.

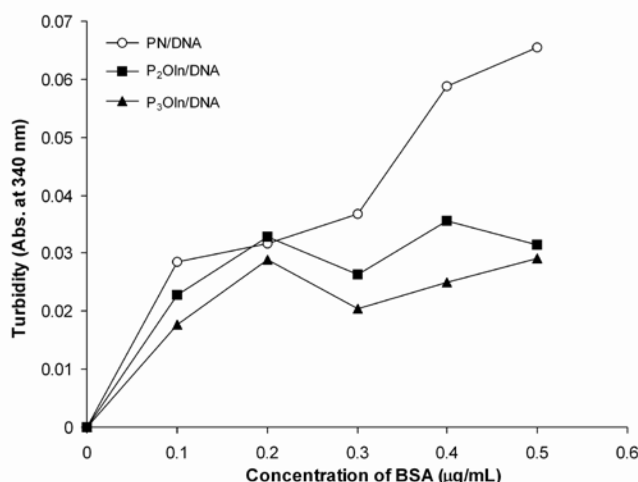


Fig. 5. Turbidity of PN/DNA and POIn/DNA complexes (5/1) after incubation for 24h with BSA. The experiments were performed in PB, at 23±1 °C (n = 3). The continuous lines are "hand-drawn" lines.

3.6. Resistance of polymer/DNA complexes against nucleases degradation

The protective effect of POIn and PN (as the control) on the stability of DNA in the complexes was studied by following nuclease-catalyzed DNA degradation. As source of nucleases it was used FCS since it is routinely employed in cell culture experiments.

The P_3OIn/DNA and PN/DNA complexes at the 5:1 charge ratio were incubated under (23 °C) and upper (40 °C) the LCST of polymers in the presence of 10 % (w/v) FCS. At different time intervals, from 0 to 240 min, samples were withdrawn and stored at –20 °C until electrophoretic analysis was performed. Electrophoresis was performed on 0.8 % agarose gels containing 0.5 mg/ml ethidium bromide for 40 min at 60 mV constant current (see Figure 6).

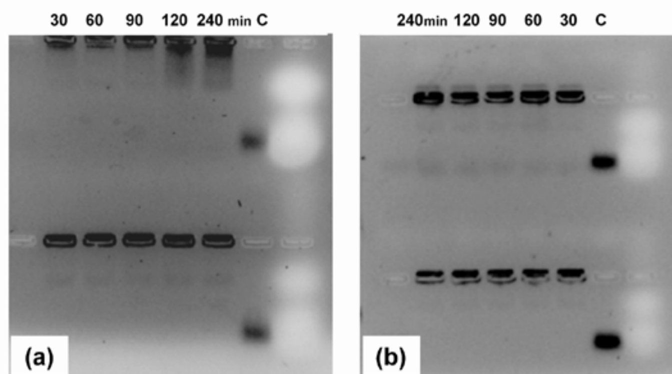


Fig. 6. Gel electrophoresis for PN/DNA (a) and P_3OIn/DNA (b) complexes after incubation with 10 % FCS (w/w) at 23 °C (upper lanes) and 40 °C (under lanes). C, control. DNA was 10 µg/ml.

Data shown in Figure 6 indicate that after incubation with 10 % (w/v) FCS at 23 °C, the PN/DNA complex undergoes complete degradation within 240 min, whereas a small fraction of DNA is retained in the P_3OIn/DNA complex after the same treatment. Again, this phenomenon can be explained by the protecting effect of the thermo-sensitive poly (NIPAAm-co-DMAAm) lateral chains. At 23 °C, the thermo-sensitive chains residing on the particle surfaces are hydrophilic alike PEG, so the POIn/DNA particles can repel the serum proteins. Notably, both PN/DNA and P_3OIn/DNA complexes resist to FCS-induced degradation at 40 °C, reflecting the stability of the polymer/DNA complex (Figure 6B), under lane). However, POIn binds DNA in a

more tight fashion than PN, because of the presence of additional hydrophobic interactions between DNA and thermo-responsive oligomers above the LCST. The collapsed chains tightly cover the surface of complex, protecting DNA from exposure.

4. Conclusions

Here, the characterization of the polyelectrolyte complexes of DNA and thermo-responsive grafted cationic pullulan are reported. The presence of PNIPAm copolymers in the complex structure provides a temperature responsiveness that is manifested by significant differences of physicochemical properties below and above the phase transition temperature of PNIPAm. The complexes obtained at (+/-) charge ratio of 5:1 were less than 200 nm in size and were stable in the presence of BSA and FCS.

This study demonstrates the potential use of temperature-responsive polymers in gene delivery and expands their wide use in biomedical applications. The changes of complex physico-chemical properties can be useful for modulation of biological activities. As such, these complexes can represent a platform for the development of “smart” gene delivery vectors capable of changing their properties in a specific stimulus-dependent manner and can contribute to the improvement of synthetic gene delivery vectors.

Aknowledgements

This work was supported by CNCSIS –UEFISCSU, project number 644/19.01.2009, PNII – IDEI code 989/2008. M. C. acknowledges the financial support of European Social Fund – ”Cristofor I. Simionescu” Postdoctoral Fellowship Program (ID POSDRU/89/1.5/S/55216), Sectoral Operational Programme Human Resources Development 2007 – 2013.

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