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Kinetic studies of the interaction between DNA and polycations based on polyasparthylhydrazide

M.L. Turco Liveri [b], E.F. Craparo [a], M. Licciardi [a], C. Sbriziolo [b], G. Cavallaro [a,*]

[a] *Dipartimento di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy*

[b] *Dipartimento di Chimica Fisica "F. Accascina", Università degli Studi di Palermo, Viale delle Scienze – Parco d'Orleans II, Ed. 17, 90128 Palermo, Italy*

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Abstract

In the present paper, a systematic kinetic study on the interaction between interpolyelectrolytes such as positive-charged polymers and DNA was carried out. In particular, a qualitative–quantitative kinetic investigation on the interaction between copolymers of the α,β-poly(aspartylhydrazide) and DNA calf thymus filaments was performed. This study gives a new model starting from a well known "pseudo-phase model", and permits to give a qualitative explanation about the trends of experimentally observed kinetic constants by varying the concentration of one of the two poly-electrolytes. Moreover, this study permits to verify the dependence of the binding constants $K_{PAHy–CPTA}$ and K_{DNA} from the cationic copolymer (PAHy–CPTA) concentration and from the DNA concentration, respectively, during the formation process of polyplexes, by the kinetic analysis. It was also possible to know the kinetic constants of the complex formation by the proposed kinetic model, both in the aqueous pseudo-phase than in the non-aqueous pseudo-phase formed by the component in excess. From the parameters (*a*) and (*b*), information on the kinetic nature of the interaction between these electrolytes were obtained (cooperative and anti-cooperative bond).

Keywords: Kinetic study; DNA/polycation interaction; Polyasparthylhydrazide copolymers

1. Introduction

The formation of complexes between DNA and polycations, usually called polyplexes, have been widely studied from the pharmaceutical and biological point of view due to their potential for introduction of functional genes into specific cells and their application in gene therapy [1–3]. Nevertheless, the design of polycationic systems potentially is able to act as gene delivery carrier, cannot overlook the importance of a complete physico-chemical characterization of polyplexes, including the study of the kinetic of DNA–polycation complex formation. The kinetic analysis not only can give important information on the formation rate of the polyplex but also on the forces governing the DNA–polycation interaction and complex stabilization and, in addition, it can enable a correlation between these peculiarities and the structural properties of polymeric materials, their condensation properties with DNA and the resulting biological performance of the complex. From the technological point of view, these informations allow us to evaluate the more proper formulation kind and the physical stability of the polyplexes to the dilution or during time.

For all these reasons in this paper a systematic kinetic investigation of the interaction between DNA calf thymus, chosen as genetic material model and biocompatible polycations based on polyasparthylhydrazide have been performed.

These polycations have been chosen because the basic polymer, the α,β-polyaspartylhydrazide (PAHy), is a water soluble, non-toxic, non-antigenic and non-immunogenic polymer, obtained from polysuccinimide by reaction with hydrazine [4,5]. Besides these favorable pharmacological properties, this polymer shows a good chemical reactivity due to the presence of hydrazinic groups (one for each repeating unit). This allows simple insertion of molecules including drugs or positively charged groups into its structure [5–7], so that PAHy-based polycations with easily modulable positive charge amounts have been prepared by the reaction of PAHy with carboxypropyltrimethylammonium chloride (CPTA) (PAHy–CPTA) to obtain proper molecular weights of the resulting polycations

* Corresponding author. Tel.: +39 091 623 6131; fax: +39 091 623 6150.
E-mail address: gennacav@unipa.it (G. Cavallaro).

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[7]. Preliminary physico-chemical charaterization studies on polyplexes obtained with DNA (from calf thymus) evidenced the notable properties of these polycations as gene delivery systems [7]. In addition, PAHy–CPTA has an excellent toxicity profile both *in vitro* and *in vivo* being almost non-toxic even at elevated polymer doses and apart from the positive charge amount [8]. Polyplexes with pDNA are efficiently internalized by cells and lead to considerable levels of transgene expression. These properties make PAHy–CPTA a promising candidate for systemic gene delivery *in vivo* [8].

Based on the excellent biopharmaceutical and toxicological properties of these copolymers, four polycations differing in positive charge amount, have been considered in the present study and kinetic constant related to the complex formation, both in water and in buffer solution at pH 7.4 at 37 °C have been determined. Kinetic studies have been performed as a function of PAHy–CPTA concentration keeping constant DNA concentration and vice versa for all copolymers. The probable formation mechanism of polyplex has been also discussed. The quantitative analysis of the kinetic data enabled us to obtain the binding constant of the PAHy–CPTA to DNA and vice versa.

2. Material and methods

2.1. Chemicals

D,L-Aspartic acid, hydrazine hydrate, *N*,*N*′-dimethylformamide (DMF), 3-(carboxypropyl)trimethyl-ammonium chloride (CPTA·Cl), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), were obtained from Fluka (Switzerland). DNA (calf thymus sodium salt) was purchased from Sigma–Aldrich (Italy). Water was obtained by a deionization instrument Culligan Pharma System 20.

The polysuccinimide (PSI) was obtained from a thermal polycondensation of D,L-Aspartic acid, at 180 °C in the presence of H_3PO_4 [9].

2.2. Instruments

Lyophilisation was performed by using a freeze-dried Hetossic HETO lab equipment (Birkerod, Denmark).

^{1}H NMR spectra were obtained with a Bruker AC-250 instrument operating at 250.13 MHz.

The molecular characterization of PAHy and PAHy–CPTA copolymers was performed using a size exclusion chromatography (SEC) system consisting of Water 600 Pump with a Water 2410 Refractive Index Detector equipped with two Ultrahydrogel columns from Water (1000 and 250 Å). The following elution conditions were used: flux, 0.8 ml/min (PBS pH 7.8 with 0.1 N $NaNO_3$); temperature 25.0 ± 0.1 °C.

UV spectra were obtained on a Beckman DU-640 instrument.

The kinetic analyses were carried out by a Stopped Flow Hi-Tech Scientific SF-61 instrument with a conductometric and spectrophotometric detection. Stopped Flow instrument contains two syringes, which were loaded with the solutions and a mixing cell. Solutions of PAHy–CPTA and DNA were in the mixing cells thanks to a pressure given by a piston. In the mixing cell there were two electrodes (for the conductometric measurements) or an optic cell (for the spectrophotometric measurements). The flow in the mixing cell was stopped by a "stopping syringe".

For all systems studied, the polyplexes formation was found to be first order with respect to both PAHy–CPTA and DNA.

The rate constant values, (k), with a reproducibility within ±5%, were obtained with the method of least squares by using the Origin 7.0 program.

Both the Stopped Flow instrument and the spectrophotometer are connected with a cryothermostat in order to control and maintain the temperature at 37.0 ± 0.1 °C.

2.3. Synthesis of α,β-polyspartylhydrazide (PAHy)

α,β-Polyspartylhydrazide (PAHy) was prepared via PSI by reaction with hydrazine hydrate in DMF solution. PAHy was isolated by filtration and purified as reported elsewhere [4,5]. Analytical and spectral data of PAHy were in agreement with Refs. [4,5]. PAHy weight–average molecular weight determined by SEC was 20 kDa ($M_w/M_n = 1.50$).

2.4. Synthesis of PAHy–CPTA copolymers

A proper amount of carboxypropyltrimethyl ammonium chloride (CPTA·Cl) was added to a 20 mL solution of PAHy 1% (w/v). The solution pH was increased to 4.75 with NaOH 0.1 N, then the suitable amount of EDC was added under mixing. The moles of CPTA·Cl and EDC added for each copolymers were expressed, respectively as X and Y ratios: where X was defined as ratio of moles of CPTA·Cl and moles of PAHy repeating units and Y as ratio of moles EDC and moles of CPTA·Cl. Three different reactions were performed maintaining constant X value equal to 1 and changing Y value (Y = 0.25, 0.5, 1.0 or 1.5). The pH mixture was adjusted to 4.75 with HCl 0.1 N and the reaction was kept for 2 h at 25.0 ± 0.1 °C. After this time the reaction was stopped increasing the pH value to 7 with NaOH 0.1 N and the solution was exhaustively dialyzed using Visking Dialysis Tubing with molecular weight cut-off of 12,000–14,000, then lyophilized. All PAHy–CPTA copolymers were obtained with a yield range between 60 and 96% (w/w) based on starting amount of PAHy (see Table 1). PAHy–CPTA copolymers were characterized by FT-IR, ^{1}H NMR and SEC analyses and data were in agreement with attributed structures (see Section 3).

2.5. Kinetic studies

Solutions of PAHy–PTA copolymers at different derivatization degree (mol.%: 15, 23, 48 and 60%) and of DNA calf thymus sodium salt were prepared both in twice-distilled water or in phosphate buffer at pH 7.4 [$Na_2HPO_4 \cdot 12H_2O$ and KH_2PO_4] by weighting the desired amount. The measurements were carried out on fresh prepared solutions.

Spectrophotometric UV–vis as a function of time were recorded on aqueous solutions of DNA, PAHy–CPTA and on the DNA/PAHy–CPTA complex. The complex DNA/PAHy–CPTA showed a maximum of absorbance at 270 nm. For the kinetic

Table 1
Molecular characterization parameters of PAHy–CPTA copolymers

PAHy–CPTA	Y	M_w (kDa)	PI	DD% (mol/mol)	Yield (%PAHy)
	0.25	13.8 ± 1.1	1.3 ± 0.1	15 ± 0.8	60 ± 3
	0.50	11.1 ± 0.9	1.3 ± 0.1	23 ± 1.1	79 ± 4
	1.00	9.8 ± 0.8	1.3 ± 0.1	48 ± 2.4	92 ± 5
	1.50	9.9 ± 0.8	1.3 ± 0.1	60 ± 3	96 ± 5
PAHy	/	20.0 ± 1.4	1.5 ± 0.1		

runs, the two solutions were placed inside of each syringe of the Stopped Flow instrument and then introduced inside the mixing cell, which is equipped with two measurement systems, for the conductance (conductometric detection) or the absorbance (spectrophotometric detection) at 270 nm, so the variation of the property as a function of the time has been followed.

For this study, (1) PAHy–CPTA solutions at constant concentration (4.2×10^{-7} mol L^{-1} for PAHy–CPTA$_{60\%}$, 4.6×10^{-7} mol L^{-1} for PAHy–CPTA$_{48\%}$, 5.8×10^{-7} mol L^{-1} for PAHy–CPTA$_{23\%}$ and 6.4×10^{-7} mol L^{-1} for PAHy–CPTA$_{15\%}$) and progressively rising of DNA (from 8.2×10^{-12} to 4.1×10^{-6}), and (2) DNA solutions at constant concentration (1.64×10^{-7} mol L^{-1}) and progressively rising of PAHy–CPTA (from 5.8×10^{-10} to 1.6×10^{-5} mol L^{-1}) were used.

We suppose that when the polycation interacts with the DNA by electrostatic interactions [10], the counter-ions linked to the DNA negative and PAHy–CPTA positive charges, are released in accordance with the theory of Manning [11,12]. Accordingly, the driving force of the complexation process is due to the high entropic contribution, which is given from the release of the same counter-ions [11,12], which allows to exploit the conductometric detection for the systems in aqueous solutions.

For the studies in phosphate buffer at pH 7.4, it was not possible to use the conductometric detection for obtaining information about the complexation process, due to the high salt concentration of the buffer.

3. Results and discussion

3.1. Synthesis and characterization of the polycations

The use of complexes between polycations and DNA represent a very promising approach as non-viral release systems in the field of gene therapy. The success of polycation use in gene therapy is mainly based on the optimization of two peculiar aspects such as the chemical and biological properties of these materials. The copolymer synthesis must be fast, reproducible and the amount of positive charge on the polymeric backbone easily modulated. The obtained polycations have to be biocompatible, able to complex DNA moieties at very low polymer/DNA ratios and not possess any particular tropism towards organs such as for example liver.

In a previous paper, the synthesis and characterization of novel polycations based on α,β-polyaspartylhydrazide (PAHy) have been described.

PAHy is a non-toxic, non-antigenic, non-teratogenic polymer, already proposed as a plasma expander [4], carrier for macromolecular conjugates [4] and starting polymer for preparing hydrogels [13]. Moreover, it is a water-soluble polymer with a protein like structure carrying in side chains reactive hydrazide groups which are able to react with carboxylic groups properly activated by a carbodiimide [7]. For this reason the 3-carboxypropyltrimethylammonium chloride (CPTA·Cl) was chosen as reagent for obtaining polycationic derivatives of PAHy (see Fig. 1).

With the aim at activating the carboxylic groups of CPTA·Cl and make easier the formation of bonds with the hydrazide groups of PAHy, the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was chosen as activating agent; the reaction was carried out in aqueous solution and pH value kept between 4 and 5.

Under these conditions the hydrazide groups are enough nucleophilic to be able to react with the activated carboxylic groups, bearing to the formation of new amide linkage and uridic derivatives as secondary products. However, the reaction at pH 4.75 is fast enough that an increase in the pH value of the reaction mixture is detected for about 30 min after the EDC addiction and the reaction seems to be complete after about 1 h. The reaction mixture was maintained at 25.0 ± 0.1 °C for 2 h in any case and subsequently, after pH neutralization, subjected to an exhaustive dialysis by using Visking Tubing Dialysis 18/32″ with a molecular weight cut-off of 12,000–14,000. After dialysis, the solution was freeze-dried. The reaction yield ranged between the 60 and 90 wt.% based on the starting PAHy. By the modification of the used amount of EDC (expressed as Y = moles of EDC/moles of CPTA), four different PAHy–CPTA copolymers were synthesized with a different derivatization degree. The obtained products were characterized by FT-IR and ^{1}H NMR analyses.

The FT-IR spectra of copolymers (done in KBr tablets) showed a broad band centered at 3300 cm^{-1} (attributable to the stretching of –NH, –NH$_2$ groups) and two bands centered at 1655 and 1540 cm^{-1} (attributable respectively to the amide I and II) with a shoulder at 1495 cm^{-1}, attributable to the bending of –CH$_3$.

The ^{1}H NMR spectrum (in D$_2$O) (Fig. 2) showed the following peaks: δ 2.2 [–CH$_2$*CH$_2$*CH$_2$N(CH$_3$)$_3$]; δ 2.5 [–CH$_2$CH$_2$*CH$_2$*N(CH$_3$)$_3$]; δ 2.9 [–CH–*CH$_2$*–CO–NH–]; δ 3.2 [–CH$_2$CH$_2$CH$_2$N(*CH$_3$*)$_3$]; δ 3.4 [–*CH$_2$*CH$_2$CH$_2$N(CH$_3$)$_3$]; δ 4.75 [–*CH*–CH$_2$–CO–NH–].

The derivatization degree (%), expressed as percentage ratio between the moles residues bearing ammonic groups and

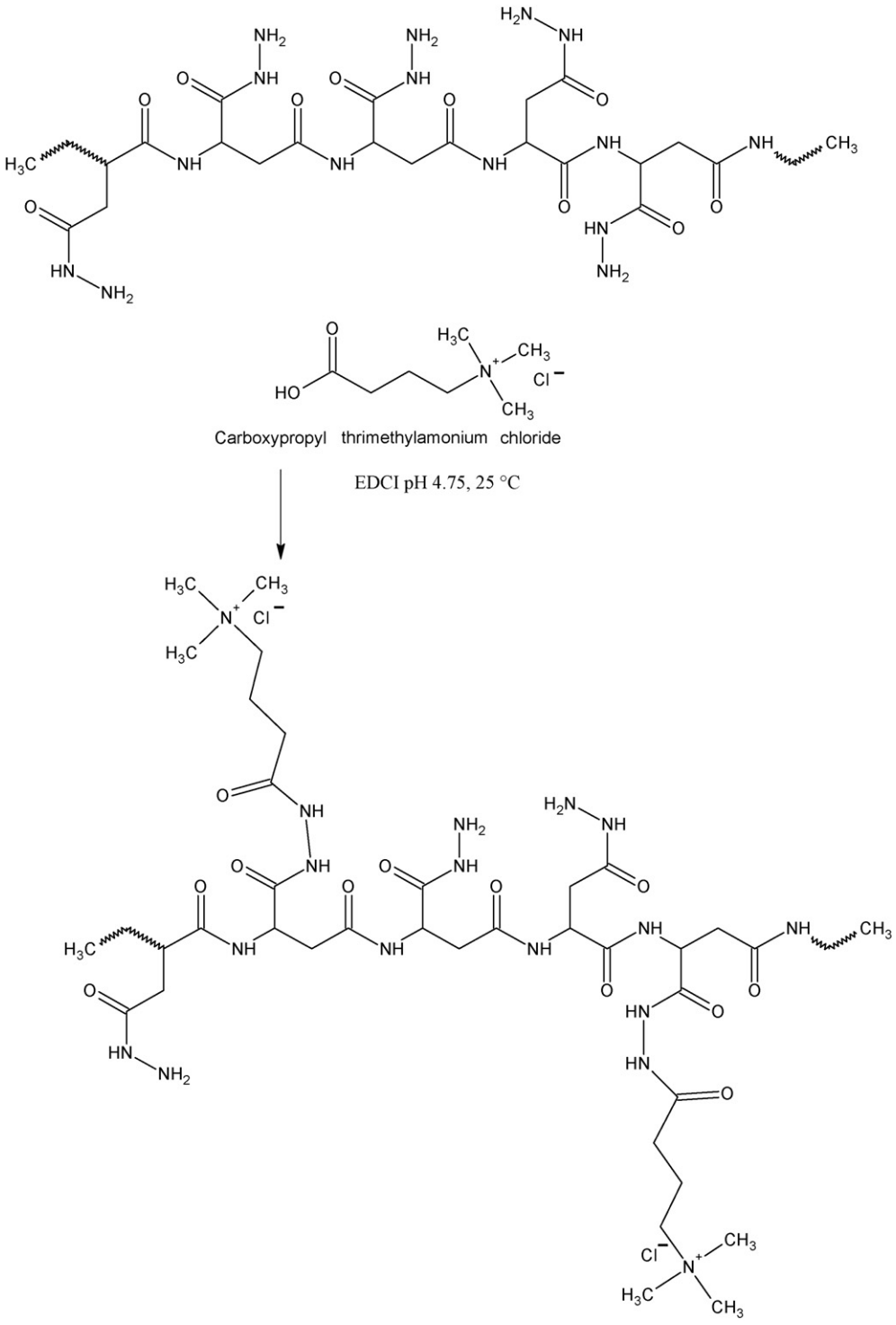


Fig. 1. Scheme of reaction of PAHy–CPTA copolymers.

the moles of repeating units of PAHy, was calculated from the ^{1}H NMR spectrum by the average of the ratios of the integrals of each peak attributable to the CPTA·Cl residues linked to PAHy (δ 2.2, 2.5, 3.2 and 3.4) with that of PAHy (δ 2.9).

The four obtained PAHy–CTPA copolymers were characterized from the molecular point of view by size exclusion chromatography (SEC), with the aim to point out eventual effects on the obtained molecular weight and on the polydispersity index (PI) of the copolymers due to the experimental conditions of reaction (see Table 1).

As it can be seen from Table 1, the derivatization degree becomes higher, increasing EDC amount, while no increase in the derivatization degree was observed by prolonging the reaction time (data not shown). This fact indicates that the reaction is already complete after 2 h of reaction.

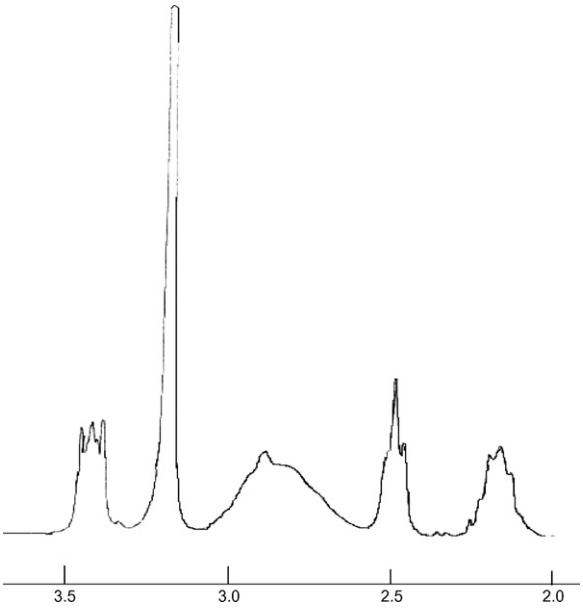


Fig. 2. Typical ^{1}H NMR spectrum of a PAHy–CPTA copolymer.

As it shown in Table 1, only a limited reduction of the copolymer molecular weight is observed as regards to the starting molecular weight of PAHy, indicating that during the derivatization reaction moderated degradation processes occurred. On the other hand, no differences were observed in the molecular weight of obtained copolymers as the Y value increased (Y = moles of EDC/moles of CPTA) from 0.5 to 1.5. Moreover, an exclusion effect of the dialysis membrane could be supposed by considering both the low polydispersity index values of all the obtained copolymers and the molecular weight cut-off of 12,000–14,000 of the dialysis membrane.

By the chlorine determination carried out on the PAHy–CPTA copolymers, the presence of about 85% of chloride ions in comparison with estimated value (based on the derivatization degree in ammonium groups) was evidenced.

Taking into account the above-described characteristics, PAHy–CPTA copolymers seem to be good tools as DNA vectors inside of the living organism.

The transfecting ability of any DNA vector depends on several factors. The fundamental requirement for obtaining the polymeric interaction with DNA, is the presence of positive charges on the polymeric backbone, which in the case of PAHy–CPTA copolymers are the ammonic quaternary groups of the CPTA·Cl linked to PAHy by amidic bonds. In fact, is well known from Ref. [10] that the interactions between polycations and DNA are electrostatic, i.e. between phosphate groups of the DNA double helix and the positive-charged groups of the polycation. This interaction is promoted by the entropic contribution due to the release of counter ions at low molecular weight, condensed both on the DNA and polycation [11,12]. The counter ion release and the associated favorable entropic contribution are the driving force for the formation of the polyplex. The equilibrium of the complex formation at the same charge ratio, depends on three factors [14]:

- The effect of the polycation charge density on the condensed counter ion release.
- The effect of DNA charge density on the condensed counter ion release.
- The effect of polycation charge density on the release of counter ion condensed on DNA.

It is well known [10] that this interaction is like "cooperative" and depends on the polycation concentration. As polycation concentration increases, the negative charges of DNA are neutralized, with the formation of polyplexes with spherical shape due to the formation of hydrophobic sites [15]. Moreover to have *in vitro* transfections the two main conditions required are size not larger than 100 nm and as much spherical shape of the polyplex [16].

More recently, thermodynamic studies were carried out in order to confirm the electrostatic and non-specific kind (90%) of the interaction between DNA and polycations. These studies lead to describe a two-step model for the process of polyplex formation: the complexation phase and the condensation phase [10,17]. The complexation phase includes the electrostatic interaction, while the condensation phase occurs because of the formation of the hydrophobic sites giving a spherical shape to the polyplex. It is also known that the effect of PEG chain introduction in the poly-lysin structure on the shape and size of polyplexes formed with DNA, that were investigated by several techniques such as the atomic force microscopy (AFM) and the scanning force microscopy (SFM). The PEG addiction seems to stabilize polyplex size because of the formation of spheroid structures with a hydrophobic core and a hydrophilic shell [18].

From that considerations, it is clear that the charge density of both polycations and DNA plays a crucial role in the polyplex formation, and the effect of the solvent have to be also considered. Moreover, to date, kinetic studies regarding polyplex formation are not known and for these reasons a systematic kinetic study on the formation of polyplexes between DNA and PAHy–CPTA copolymers was undertaken.

In particular, the kinetic of the processes (I) and (II) was studied at 37.0 °C:

$$\text{PAHy–CPTA} + \text{DNA} \rightarrow \text{PAHy–CPTA·DNA} \quad \text{(I)}$$

$$\text{DNA} + \text{PAHy–CPTA} \rightarrow \text{DNA·PAHy–CPTA} \quad \text{(II)}$$

These processes were studied both in aqueous and in buffered solution at pH 7.4. In both cases the kinetic was followed at a fixed concentration of PAHy–CPTA as a function of the DNA concentration and vice versa. It has to be underlined that when measurements were carried out in aqueous solution, a conductometric method was used, while for the measurements in buffer a spectrophotometric method was used due to the high ionic strength of the medium.

3.2. Kinetic data

As for the kinetic measurements in aqueous solution, for all cases studied, the solution electrical conductivity was found to

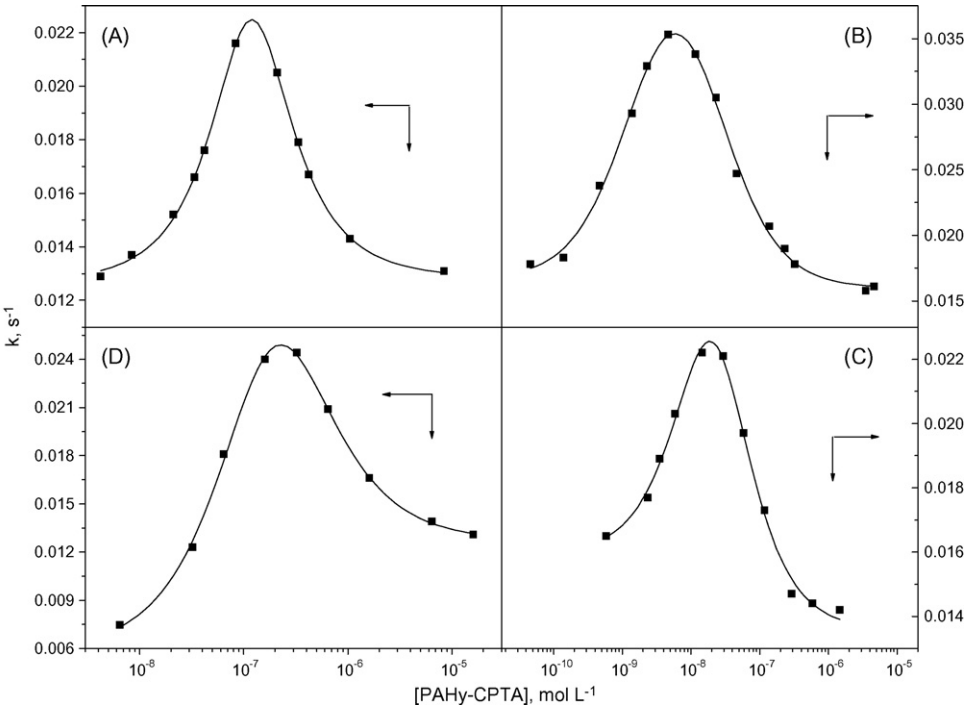


Fig. 3. Kinetic constant values for the process (I) in aqueous solution as a function of PAHy–CPTA concentration with (A) PAHy–$CPTA_{60\%}$, (B) PAHy–$CPTA_{48\%}$, (C) PAHy–$CPTA_{23\%}$, and (D) PAHy–$CPTA_{15\%}$; at a fixed [DNA] = 1.6×10^{-7} mol L^{-1}, $T = 37.0\,^{\circ}C$. (■) Experimental and (–) calculated data by means of Eq. (5).

increase with time and after a certain time it attains a constant value.

The analysis of the obtained kinetic data has permitted to obtain the rate constant values of the polyplex formation process.

In Fig. 3 the trends of the kinetic constant values obtained in aqueous solution for the process (I) as a function of PAHy–CPTA concentration are shown.

Perusal of the figure evidences that for all the PAHy–CPTA copolymers the rate constant values as function of the PAHy–CPTA concentration increase until reach a maximum value and then a subsequent decrease at higher concentration values can be noticed.

The observed trends can be reasonably explained by considering that at lower PAHy–CPTA concentration the progressive increase [PAHy–CPTA] leads to an augmentation of the rate of the polyplex formation, due to the higher probability that the polymer interacts with the DNA, while when the concentration values exceed a certain one giving the highest rate constant value, the formation process becomes slower decrease, probably because of the increased solution viscosity, that, at these concentration becomes significant.

The highest value of the rate constant is included in for concentration values ranging between 10^{-9} and 10^{-7} mol L^{-1}, with a decreasing trend when the charge density on the copolymers increases ($Y = 0.25$, $Y = 0.5$ and $Y = 1$), respectively (2.3×10^{-7} mol L^{-1}; 1.87×10^{-8} mol L^{-1}; 6.0×10^{-9} mol L^{-1}). Different trend seems to have the polycation with the higher charge density, which has a behavior similar to the polycation with the lower charge density (PAHy–$CPTA_{15\%}$) and middle between that obtained with PAHy–$CPTA_{15\%}$ and PAHy–$CPTA_{23\%}$.

The same considerations holds also for the kinetic constant values obtained at concentration values lower than 10^{-7} mol L^{-1}. In fact, a decreasing trend is obtained in the order PAHy–$CPTA_{48\%}$ > PAHy–$CPTA_{23\%}$ > PAHy–$CPTA_{15\%}$. Different trend has PAHy–$CPTA_{60\%}$, which shows, in this portion of the graphic, values of rate constant intermediate between those obtained with the PAHy–$CPTA_{23\%}$ and PAHy–$CPTA_{15\%}$. It is possible to see that for concentration values higher than that corresponding to the maximum value of the rate constant, the trend of the kinetic constants as a function of the charge density becomes more difficult to rationalize reasonably because of the coexistence of several factors which influence these values, such as the viscosity and the molar mass.

Similar trends were obtained by the kinetic study of the process (II), which was carried out at a fixed PAHy–CPTA concentration as a function of the DNA concentration (Fig. 4).

Also in this case, it is clear that as DNA concentration increases, the kinetic constant values grow, reaching a maximum value and, then, decrease. This trend analogously to that explained for process (I) is probably due to the higher probability that the phosphate groups of DNA interact with the ammonic groups of the polycation. However, when the concentration is over the value that gives the greatest value of the kinetic constant, the viscosity growth becomes determining. This way the kinetic constant value decrease can be ascribed to the enhancement in the medium viscosity. On the other hand for similar systems such as the platin–polypyridin complexes which bind DNA, it was observed that the kinetic of the interaction decreases as the viscosity grows when DNA concentration increases [19]. Another possibility is that increasing DNA concentration, coilings of its structure are formed, so that charges are shielded

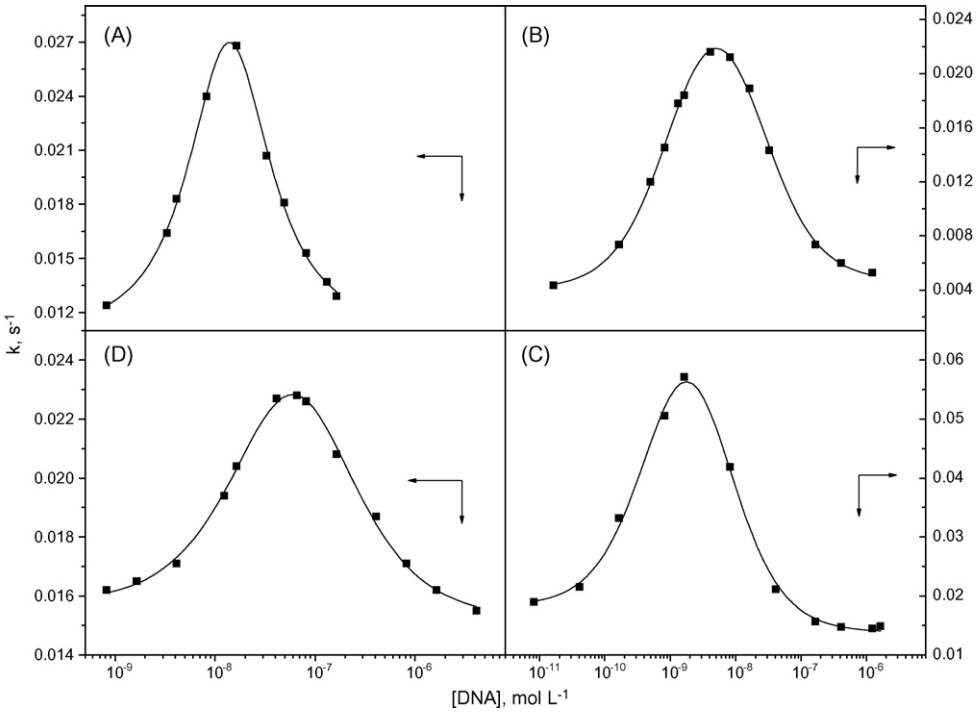


Fig. 4. Kinetic constant values for the process (II) in aqueous solution as a function of DNA concentration at a fixed concentration of (A) PAHy–CPTA$_{60\%}$ (4.2×10^{-7} mol L^{-1}), (B) PAHy–CPTA$_{48\%}$ (4.6×10^{-7} mol L^{-1}), (C) PAHy–CPTA$_{15\%}$ (6.4×10^{-7} mol L^{-1}), and (D) PAHy–CPTA$_{23\%}$ (5.8×10^{-7} mol L^{-1}), $T = 37.0$ °C. (■) Experimental and (–) calculated data by means of Eq. (6).

and the DNA/PAHy–CPTA interaction become unfavorable and slow down. However, a synergic effect could be taken into consideration.

Moreover, it can be seen that the greatest value of k is included in the range of DNA concentration about between 10^{-9} and 10^{-8} mol L^{-1}.

By observing the graphics reported above, it can be seen that PAHy-CPTA$_{15\%}$ shows higher kinetic constant values, while the other copolymers show lower values which, indeed, are comparable each other. Nevertheless, since the values of polycation molecular weights reported in Table 1 are different to each other, it is not possible to connect the four polycations at different charge density. In this case, in fact, the observed rate constant value does not depend only on charge density but also on the way, how this charge is distributed on different polycation fragments. The system becomes more and more complicated for concentration values higher to that giving the greatest values of kinetic constant. At these values, in fact, the viscosity contribution becomes significant as well as some different DNA conformations due to the high concentration values. All these factors make the systems much more complicated.

As for the kinetic study of the process (I) in buffered solution, similar trends to those mentioned above were obtained on changing the reaction medium (Fig. 5).

For all the investigated systems, the trend of the kinetic constant as a function of the PAHy–CPTA concentration increases as the PAHy–CPTA concentration increases until a maximum value, over that the effect of the viscosity, the molar mass and the conformational changes of PAHy–CPTA become decisive factors. However, the kinetic constant values calculated in buffered solution are higher than those obtained in aqueous solution. This indicates that the increase of the solvent medium ionic strength makes the charge groups of both DNA and PAHy–CPTA more shielded from the counter ions of the buffer. This effect probably induces conformational changes in the PAHy–CPTA chains and, consequently, the interacting charged groups are more available for the interaction with the DNA so that the process becomes faster.

It must be underlined that the concentration range which gives the greatest kinetic constant value lies in the range 10^{-9} to 10^{-7} mol L^{-1}, with an increasing trend as the charge density increases for the polycations PAHy–CPTA$_{15\%}$ < PAHy–CPTA$_{23\%}$ < PAHy–CPTA$_{48\%}$, (2.24×10^{-7} mol L^{-1}; 1×10^{-8} mol L^{-1}; 8.3×10^{-9} mol L^{-1}, respectively).

An analogous study to that discussed above has been carried out for the process (II) in buffered solution. In Fig. 6 the dependence of the kinetic constant as function of DNA concentration is depicted.

Similar comparison and explanation to those given before could also be done in the case of these kinetic trends.

With the aim at quantitatively explaining the kinetic data, a model of pseudo-phases usually utilized for surfactants both in aqueous and in organic solvents was used [20–22]. This model was also used by Secco [23] for studies regarding processes of electronic transfers in the presence of DNA.

In the case of systems described in this paper, the aggregated pseudo-phase could be constituted by the PAHy–CPTA or the DNA. Then two kinetic pathways contribute to the rate of the total process, in accordance with the reaction (Scheme 1):

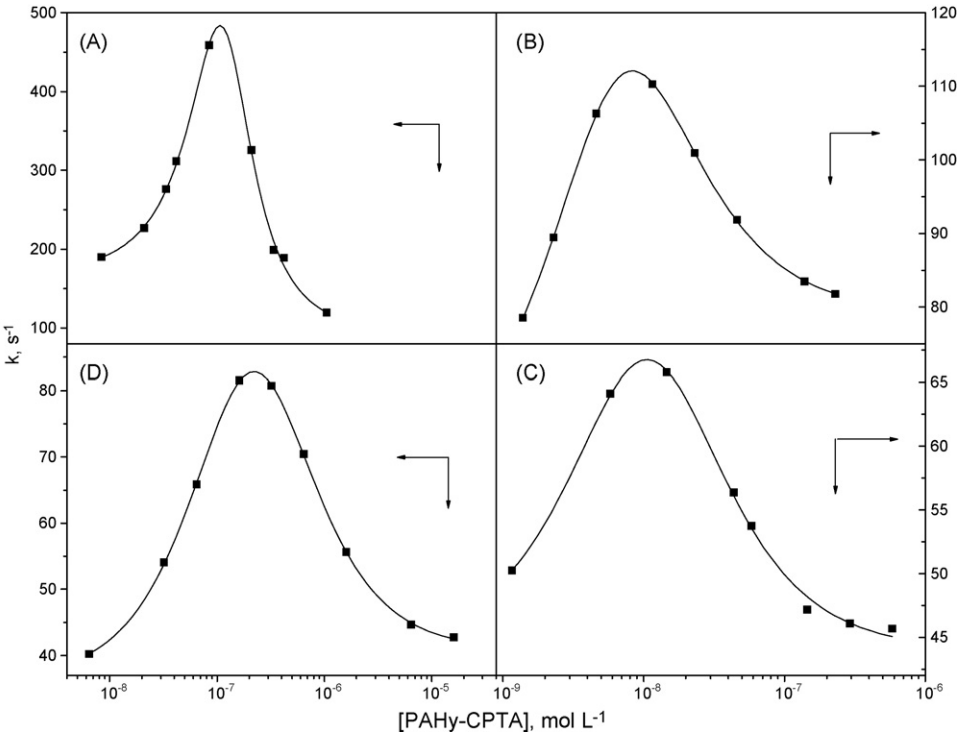


Fig. 5. Kinetic constant values for the process (I) in buffered solution as a function of PAHy–CPTA concentration with (A) PAHy–CPTA$_{60\%}$, (B) PAHy–CPTA$_{48\%}$, (C) PAHy–CPTA$_{23\%}$, and (D) PAHy–CPTA$_{15\%}$; at a fixed [DNA] = 1.6×10^{-7} mol L^{-1}, pH 7.4, T = 37.0 °C. (■) Experimental and (–) calculated data by means of Eq. (5).

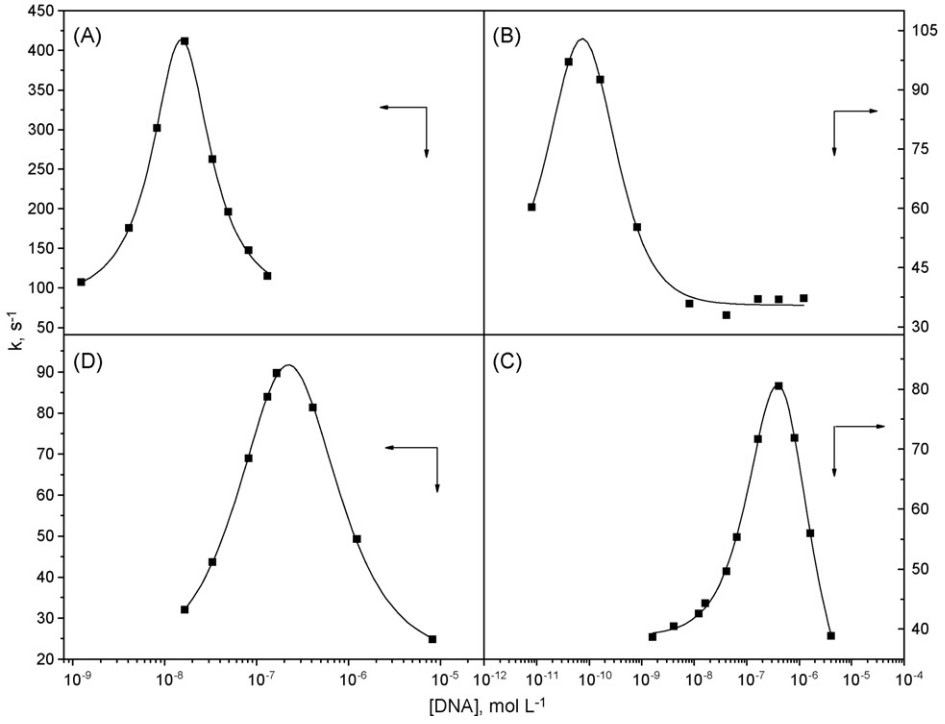
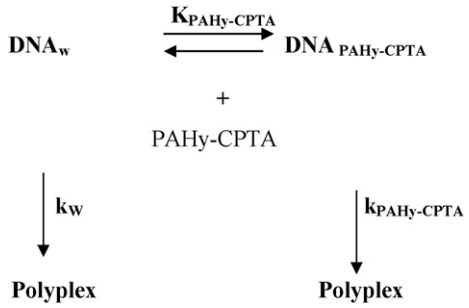
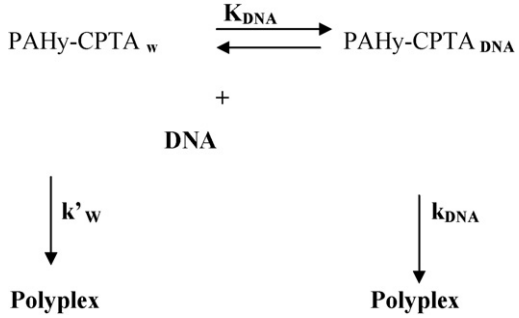


Fig. 6. Kinetic constant values for the process (II) in buffered solution as a function of DNA concentration at a fixed concentration of (A) PAHy–CPTA$_{60\%}$ (4.2×10^{-7} mol L^{-1}), (B) PAHy–CPTA$_{15\%}$ (6.4×10^{-7} mol L^{-1}), (C) PAHy–CPTA$_{23\%}$ (5.8×10^{-7} mol L^{-1}), and (D) PAHy–CPTA$_{48\%}$ (4.6×10^{-7} mol L^{-1}), pH 7.4, T = 37.0 °C. (■) Experimental and (–) calculated data by means of Eq. (6).



Scheme 1.



Scheme 2.

the first path involves both reagents in the aqueous pseudo-phase, while in the second one the DNA is "solubilized" in the PAHy–CPTA pseudo-phase. Thus, the polyplex formation process can be schematized as follows:

$K_{PAHy–CPTA}$ is the "binding" constant of DNA with the PAHy–CPTA ($=[DNA]_{PAHy–CPTA}/[DNA]_w \cdot [PAHy–CPTA]$), k_w is the kinetic constant of the complex formation in the aqueous pseudo-phase and $k_{PAHy–CPTA}$ is the kinetic constant of complex formation in the PAHy–CPTA pseudo-phase.

A similar reaction scheme can be proposed in the case of reaction (Scheme 2), hereby the aggregated pseudo-phase is constituted by the DNA.

K_{DNA} is the "binding" constant of PAHy–CPTA with DNA ($=[PAHy–CPTA]_{PAHy–CPTA}/[PAHy–CPTA]_w \cdot [DNA]$)), k'_w is the kinetic constant of complex formation in the aqueous pseudo-phase and k_{DNA} is the kinetic constant of complex formation in the DNA pseudo-phase.

The kinetic constant associated to the reaction (Scheme 1) is given by

$$k = \frac{k_w + k_{PAHy–CPTA} K_{PAHy–CPTA}[PAHy–CPTA]}{1 + K_{PAHy–CPTA}[PAHy–CPTA]} \quad (1)$$

while the kinetic constant associated to the reaction (Scheme 2) is

$$k = \frac{k'_w + k_{DNA} K_{DNA}[DNA]}{1 + K_{DNA}[DNA]} \quad (2)$$

The non-linear least square regression of the kinetic data obtained both in aqueous and in phosphate buffer as a function of PAHy–CPTA concentration, according to the above given Eqs. (1) and (2), provide that the polyplex formation processes do not follow the proposed equation.

Most likely this result implies that the binding constant depends to some extent to the polycation (Scheme 1) and DNA (Scheme 2) concentration. Similar to that proposed by Ref. [23], we can suggest a quadratic dependence from the concentration of PAHy–CPTA or DNA:

$$K_{PAHy–CPTA} = a + b[PAHy–CPTA] + c[PAHy–CPTA]^2 \quad (3)$$

$$K_{DNA} = a + b[DNA] + c[DNA]^2 \quad (4)$$

were parameters a, b and c are constants of the systems and give information about the extent of the association of the polyelectrolite or DNA.

The non-linear least square analysis of the kinetic data by means of the equations obtained by substituting Eq. (3) in (1) and Eq. (4) in (2) allowed us to establish that the quadratic term in Eqs. (3) and (4) is negligible, thus the used equations are

$$k = \frac{k_w + k_{PAHy–CPTA} a[PAHy–CPTA] + k_{PAHy–CPTA} b[PAHy–CPTA]^2}{1 + a[PAHy–CPTA] + b[PAHy–CPTA]^2} \quad (5)$$

and

$$k = \frac{k'_w + k_{DNA} a[DNA] + k_{PAHy–CPTA} b[DNA]^2}{1 + a[DNA] + b[DNA]^2} \quad (6)$$

The obtained parameters are shown in Table 2 for the processes carried out in aqueous solution and in Table 3 for that in phosphate buffer at pH 7.4.

Data analysis suggests that both solvent media (water or buffer at pH 7.4) and the charge present on the polyelectrolyte influence the kinetic parameters. In particular, it is possible to see that for all the studied cases, the kinetic constant values k_w and $k_{PAHy–CPTA}$ resulted to be significantly higher in the presence of phosphate buffer than those obtained in aqueous solution.

Table 2
Kinetic constant values (s^{-1}) of polyplex formation in the aqueous pseudo-phase (k_w) and in the PAHy–CPTA pseudo-phase ($k_{PAHy–CPTA}$), and values of parameters a and b, for the studied polycations in aqueous solution ($T = 37.0\,^\circ C$)

	PAHy–CPTA$_{15\%}$	PAHy–CPTA$_{23\%}$	PAHy–CPTA$_{48\%}$	PAHy–CPTA$_{60\%}$
k_w	0.0058	0.0159	0.0167	0.0127
$k_{PAHy–CPTA}$	0.0127	0.0138	0.0161	0.00130
a	4×10^6	4×10^7	6×10^8	-6.4×10^6
b	3×10^{13}	2.1×10^{15}	2.8×10^{16}	7.1×10^{13}

Table 3
Kinetic constant values (s^{-1}) of polyplex formation in the aqueous pseudo-phase (k_w) and in the PAHy–CPTA pseudo-phase ($k_{PAHy–CPTA}$), and values of parameters a and b, for the studied polycations in phosphate buffer (pH 7.4, $T = 37.0\,^{\circ}C$)

	PAHy–CPTA$_{15\%}$	PAHy–CPTA$_{23\%}$	PAHy–CPTA$_{48\%}$	PAHy–CPTA$_{60\%}$
k'_w	37	44	53	167
$k_{PAHy–CPTA}$	42	49	79	93
a	4.7×10^6	6×10^7	1.4×10^8	-1.1×10^7
b	2.2×10^{13}	8×10^{15}	2.4×10^{16}	7.1×10^{13}

One possibility for this effect is that the increase of the ionic strength induces PAHy–CPTA conformations which facilitate the interactions with the DNA.

As for the k_w values, it is possible to see an increase of these values increasing polycation charge density. This might be attributed to the greater possibility of electrostatic interactions. Also the $k_{PAHy–CPTA}$ values increase as the charge density increases, most likely conformation of polyplexes in the aggregated pseudo-phase facilitates the interactions with further DNA moieties.

If k_w and $k_{PAHy–CPTA}$ values are compared for the three polycations (PAHy–CPTA$_{15\%}$, PAHy–CPTA$_{23\%}$, PAHy–CPTA$_{48\%}$), it is possible to conclude that for PAHy–CPTA$_{15\%}$, the value of k_w is smaller than that of $k_{PAHy–CPTA}$. This implies that the formation process of the polyplex is facilitated in the aggregated pseudo-phase than in the PAHy–CPTA pseudo-phase. This result indicates probable conformation transitions in the PAHy–CPTA chain, giving a polyplex, which evolves quickly to products.

When the charge density increases, the k_w value becomes similar to the $k_{PAHy–CPTA}$ value, indicating that both processes occur at the same rate. Opposite trends were obtained in the case of PAHy–CPTA$_{60\%}$. This evidence further suggests that the reaction mechanism is different. This hypothesis is supported by a and b parameters, which are related to the binding constant.

In the following tables, parameters of Eq. (6) are reported, for the processes in aqueous solution (Table 4) and in the presence of phosphate buffer at pH 7.4 (Table 5):

Analogously to the above-discussed results, the kinetic constant values associated to the process occurring in the aqueous medium results to be significantly lower than those obtained in the buffered solution. However, unlike before, it is not possible to compare the trends of the kinetic constants in both pseudo-phases as the charge density of the polycation varies. In fact, we must consider that besides the variation of the charge density, also the molar mass of the polycation varies. In addition, the PAHy–CPTA$_{60\%}$ gives the same behavior.

At each polyelectrolite concentration (DNA or PAHy–CPTA), for all the analysed systems, the binding constant values were calculated by means of Eqs. (5) and (6). Typical plots are shown in Figs. 7 and 8.

For all systems studied, a linear dependence of K on the polyelectrolyte concentration has been obtained and, for a given concentration, the K value reflects that of the obtained parameters a and b.

Trends of K as function of the polyelectrolyte concentration support the mechanistic hypothesis done above. For all systems studied with exception to PAHy–CPTA$_{60\%}$, it has been found that the K values are in every case positive and rising, while for PAHy–CPTA$_{60\%}$ they are negatives at low concentration and, then, at higher concentration becomes positive. This result implies that for the polycations at lower or equal charge density of the polycation PAHy–CPTA$_{48\%}$, the formation process of the polyplex is always cooperative, while for the PAHy–CPTA$_{60\%}$ from anti-cooperative the process becomes cooperative.

Table 4
Kinetic constant values (s^{-1}) of polyplex formation in the aqueous pseudo-phase (k'_w) and in the PAHy–CPTA pseudo-phase ($k_{PAHy–CPTA}$), and values of parameters a and b, for the studied polycations in aqueous solution ($T = 37.0\,^{\circ}C$)

	PAHy–CPTA$_{15\%}$	PAHy–CPTA$_{23\%}$	PAHy–CPTA$_{48\%}$	PAHy–CPTA$_{60\%}$
k'_w	0.0182	0.0158	0.0040	0.0111
k_{DNA}	0.0138	0.0154	0.00476	0.0113
a	1.6×10^9	2.5×10^7	9×10^8	-5.1×10^7
b	2.9×10^{17}	2.6×10^{14}	4.2×10^{16}	5.3×10^{15}

Table 5
Kinetic constant values (s^{-1}) of polyplex formation in the aqueous pseudo-phase (k'_w) and in the DNA pseudo-phase (k_{DNA}), and values of parameters a and b, for the studied polycations in phosphate buffer (pH 7.4, $T = 37.0\,^{\circ}C$)

	PAHy–CPTA$_{15\%}$	PAHy–CPTA$_{23\%}$	PAHy–CPTA$_{48\%}$	PAHy–CPTA$_{60\%}$
k'_w	39	39	20	85
k_{DNA}	34	24	20	86
a	3×10^3	2.1×10^6	2.0×10^6	-8.3×10^7
b	5×10^6	4.9×10^{12}	2.1×10^{13}	4.4×10^{15}

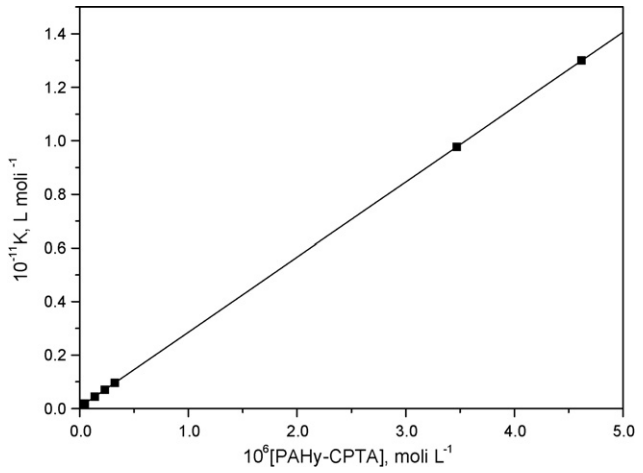


Fig. 7. Binding constant values of PAHy–CPTA$_{48\%}$ to DNA as a function of the PAHy–CPTA$_{48\%}$ concentration in aqueous solution ($T = 37.0\,^{\circ}$C).

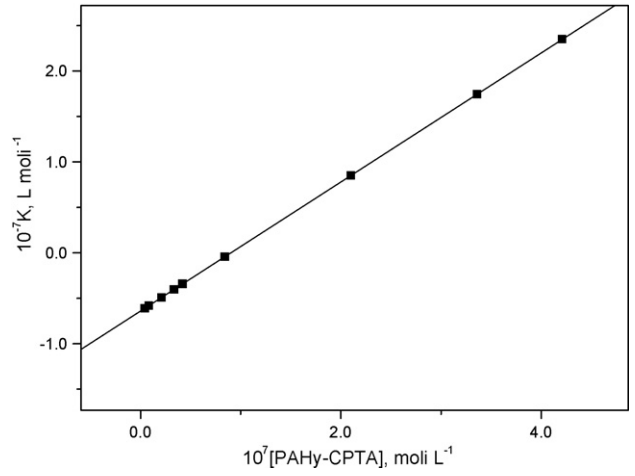


Fig. 8. Binding constant PAHy–CPTA$_{60\%}$ to DNA as a function of the PAHy–CPTA concentration, $Y = 1.5$ in aqueous solution ($T = 37.0\,^{\circ}$C).

4. Conclusions

In the present paper, a systematic kinetic study on interpolyelectrolitic systems, i.e. in general positive-charged polymers physically interacting with DNA, was carried out. In particular, a qualitative–quantitative kinetic study on the interaction between copolymers of the α,β-poly(aspartylhydrazide) and DNA calf thymus filaments was carried out. This study gives a new model starting from a well known "pseudo-phase model", and permits to give a qualitative explanation on the trends of experimentally observed kinetic constants by varying the concentration of one of the two poly-electrolytes. Moreover, this study permits to verify the dependence of the binding constants $K_{\mathrm{PAHy–CPTA}}$ and K_{DNA} from the cationic copolymer (PAHy–CPTA) concentration and from the DNA concentration, respectively, during the formation process of polyplexes, by the kinetic analysis.It was also possible to know the kinetic constants of the complex formation by the proposed kinetic model, both in the aqueous pseudo-phase and in the pseudo-phase formed by the polyelectrolyte in excess. From the parameters (*a*) and (*b*), information on the kinetic nature of the interaction between these electrolytes were obtained (cooperative and anti-cooperative bond).

This work gives a starting important scientific investigation, which could be an useful instrument in the field of the pharmaceutical technology studies on gene therapy.

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