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Spectrophotometric determination of polyethylenimine in the presence of an oligonucleotide for the characterization of controlled release formulations

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Abstract

Polyethylenimine (PEI) is a cationic polymer that can be associated to oligonuclotides to promote their transfection both in vitro and in vivo. The controlled release of oligonucleotide/polyethylenimine complexes from biodegradable systems can result in an increased cellular internalisation of the oligonucleotide and a reduced cytotoxicity of the complex. This effect strongly depends on the amount of PEI loaded in and released from the delivery system. In this work we describe a rapid, sensitive and reproducible spectrophotometric method for the quantitative analysis of PEI by itself or in the presence of an associated oligonucleotide. PEI does not possess chromophores, hence the determination by ordinary spectrophotometry is not possible. However, upon addition of copper (II) ions, PEI forms a dark blue cuprammonium complex that can be detected by UV-vis spectrophotometry. The optimum conditions in terms of optical parameters, copper (II) concentration required for a quantitative PEI complexation, and the most suitable medium for the reaction were ascertained. A linear relationship ($r^2 = 0.9997$) between absorbance and amounts of PEI was found at λ_{max} of 285 nm over the concentration range 5.0–50.0 μg ml^{-1}. The detection limit (QOD) was 4.0 μg ml^{-1}. The method was validated for the quantitation of PEI in the presence of an oligonucleotide, which absorbs at 285 nm as well.

Keywords: Polyethylenimine; Copper (II) complex; Oligonucleotide; Spectrophotometry; Sustained release

1. Introduction

The use of oligonucleotides for the treatment of diseases of genetic, infectious and cancer origins is to date considered a very promising alternative to conventional therapy. However, the development of oligonucleotides as therapeutic agents is still hampered by their short in vivo half-life and limited cellular uptake. In order to achieve a sustained pharmacological activity of oligonucleotides and avoid repeated administrations, the use of systems based on biodegradable polymers such

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as poly(lactic-co-glycolic acid) (PLGA) has been recently investigated [1–3]. Furthermore, the association of viral and non-viral vectors to nucleic acids has been suggested to enhance their intracellular penetration [4–7]. Amid non-viral vectors, polyethylenimine (PEI) (Fig. 1) is a cationic polymer with a high cationic charge density that has been proven to promote an efficient DNA transfection both in vitro and in vivo [8–10].

Recently, we have demonstrated that oligonucleotide/PEI complexes can be encapsulated into PLGA-based microspheres, combining a sustained release of the complex with an improved intracellular uptake of the oligonucleotide [11]. It was suggested that the performance of the delivery system depended upon the amount of PEI encapsulated into microspheres and the rate at which it was released. In order to confirm this hypothesis, a method for PEI dosage in the presence of an oligonucleotide was needed.

PEI does not possess chromophores, hence the determination by ordinary spectrophotometric method is not possible. Upon addition of copper (II) ions, PEI forms a dark blue cuprammonium complex that can be detected by UV-vis spectrophotometry and useful for PEI quantitative analysis [12]. The main limitation of this method is the lack of sensitivity if we conceive it for the quantitation of the little amounts of polycation generally loaded in and released from the microspheres.

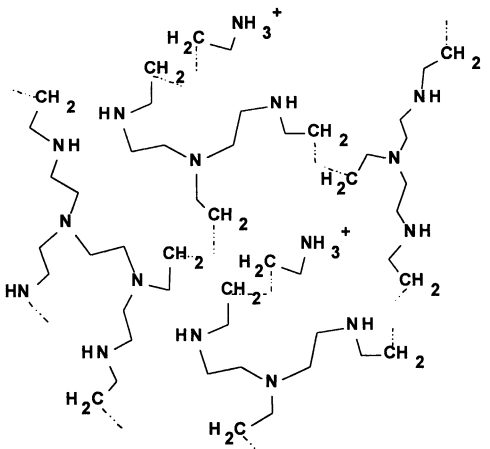

Fig. 1. Chemical structure of branched polyethylenimine.

The present paper describes a simple and reproducible assay for the quantitative determination of PEI in different media in the presence of an oligonucleotide. The proposed method is based on the formation of copper (II)/PEI complexes and is specially developed to quantify PEI loaded in and released from PLGA-based microspheres. Quantitative determination of PEI has been performed in two different media widely used to characterize microspheres, that is 0.5 N sodium hydroxide and phosphate buffer at pH 7.4.

2. Experimental

2.1. Apparatus

A single-beam UV-vis spectrophotometer (model 1204 Shimadzu, Kyoto, Japan) fitted out with 1-cm quartz cell was used. The instrument was equipped with interchangeable deuterium/tungsten sources operating over a range of 190–750 nm.

2.2. Reagents

Polyethylenimine (PEI, Mw 25 000 Da; branched; water-free), copper (II) sulphate, sodium chloride, sodium hydroxide (NaOH), potassium chloride, and potassium phosphate monobasic were purchased from Aldrich (Germany). Analytical grade glacial acetic acid was supplied by Carlo Erba (Milano, Italy). The oligothymidilate 5′-TTTTTTTTTTTTTTT-3′ (pdT16) was synthesised by CEINGE (Napoli, Italy). Water purified by a Millipore Milli-Q system was used throughout the study.

2.3. Standard solutions

For the preparation of the PEI standard solution, 600 mg of PEI were accurately weighted, transferred into a flask and dissolved under stirring in 100 ml of either phosphate buffer at pH 7.4 (PBS; 10 mM bibasic sodium phosphate, 120 mM sodium chloride; 3 mM potassium

chloride) or 0.5 N NaOH. This solution was stable for at least a week when stored in the dark at 4 °C. Working standard solutions of PEI were prepared by further dilution in either PBS or 0.5 N NaOH.

The standard solution of pdT16 was prepared in water (150 nmol ml^{-1}) and appropriately diluted by PBS or 0.5 N NaOH.

Standard solutions were prepared weekly to avoid any degradation phenomenon.

2.4. General procedure for PEI analysis

A preliminary experiment was run to assess the amount of copper (II) needed for quantitative complexation of PEI. A series of solutions containing a fixed amount of PEI (50 μg ml^{-1}) and increasing copper (II) sulphate concentrations within the range 0–0.250 mg ml^{-1}, were prepared in PBS. The absorbance of the solutions was recorded at λ_{max} (285 nm).

Two milliliters of working standard solutions of PEI were added to 5 ml of a copper (II) sulphate solution (0.145 mg ml^{-1}) in the appropriate reacting medium. As reacting medium, three different aqueous solvents were studied: (i) distilled water; (ii) PBS; (iii) 0.1 M acetate buffer at pH 5.4. The UV-Vis absorption spectrum (200–800 nm) of the solutions was recorded and the wavelength for photometric measurements of PEI/copper (II) complex selected. All measurements were performed in triplicate at room temperature against a reagent blank (copper (II) sulphate solution).

2.5. PEI dosage in PBS at pH 7.4

Five PEI standard solutions within the range 0.02–0.20 μg ml^{-1} were prepared in PBS pH 7.4. Two milliliters of each standard solution were complexed with copper (II) in 0.1 M acetate buffer at pH 5.4 as reacting medium. The absorbance value of each solution was recorded at λ_{max} (285 nm). A calibration curve was obtained plotting absorbance *versus* concentration.

2.6. PEI dosage in 0.5 N NaOH

Five PEI standard solutions within the range 0.09–0.90 μg ml^{-1} were prepared in 0.5 N NaOH. An appropriate amount (0.5 ml) was withdrawn and buffered with an equal volume of 1 M acetic acid. Each resulting solution ($pH = 5.2 \pm 0.2$) was diluted to 2 ml by means of acetate buffer and complexed with copper (II) in 0.1 M acetate buffer at pH 5.4 as reacting medium. The absorbance value of each solution was recorded at λ_{max} (285 nm). A calibration curve was obtained plotting absorbance *versus* concentration.

2.7. PEI dosage in the presence of the oligonucleotide

Five standard solutions of pdT16 (0.4–6.0 nmol ml^{-1}) in either PBS at pH 7.4 or 0.5 N NaOH were analysed at 285 nm (PEI/copper (II) λ_{max}). A calibration curve was obtained plotting absorbance *versus* concentration. The contribution of the oligonucleotide to the absorbance of the mixture at 285 nm was evaluated according to the equation [13]:

$$ABS_{TOT} = \varepsilon_1 \cdot b \cdot C_1 + \varepsilon_2 \cdot b \cdot C_2 + \cdots + \varepsilon_n \cdot b \cdot C_n$$

where ABS_{TOT} is the total absorbance at a given wavelength, the subscripts $1, 2, \ldots n$ refer to components of the mixture, ε is the molar absorptivity of component n, C is the molar concentration of component n (mol l^{-1}), and b is the fixed optical pathlength (cm).

3. Results and discussion

A rapid, sensitive and reproducible spectrophotometric method for the quantitative analysis of PEI in the presence of an oligonucleotide has been developed. This method is intended for the characterization of biodegradable microspheres encapsulating PEI/oligonucleotide complexes. Quantitative determination of PEI has been performed in two different aqueous solvents generally used to dose molecules loaded inside and released from microspheres, that is (i) 0.5 N sodium

hydroxide and (ii) phosphate buffer at pH 7.4, respectively.

3.1. Spectral characteristics

The method is based on the formation of PEI/Cu (II) complexes in aqueous solution. A previously reported spectrophotometric method for quantitative analysis of PEI focused on the absorption band in the visible region that is flat at its maximum [12,14]. The use of this wavelength for quantitative determination of PEI led to good linearity, thus reducing the risk of errors due to failure in reproducing precisely the wavelength setting of the instrument. Actually, PEI/Cu (II) complex exhibited two maxima absorption bands at λ_1 285 nm and λ_2 630 nm, respectively (Fig. 2). As can be seen in Table 1, λ_{630} was significantly lower than λ_{285} (2.47×10^4 and 4.85×10^5 l cm^{-1} mol^{-1}, respectively), in agree with literature data. The limit of quantitation (QOD) (estimated to be ten times the background noise) at 630 nm was 13.5 μg ml^{-1}, that is more than 3-fold higher than the QOD at 285 nm (4.0 μg ml^{-1}). These results suggested that a higher sensitivity and a good linearity could be achieved operating at 285 nm, that was selected for the following studies. It is clear that the reagent blanks had negligible absorption at both 285 and 630 nm.

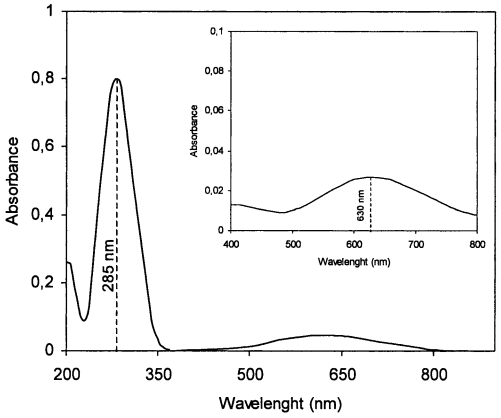

Fig. 2. UV-vis absorption spectrum of PEI/copper (II) complex in water.

Table 1
Optical characteristics and precision data at two different wavelengths

Parameter/characteristic	λ_{max} (nm)	
	285	630
Beer's law range (μg ml^{-1})	5.0–50.0	20.0–200
LOD (μg ml^{-1})	1.1	3.7
QOD (μg ml^{-1})	4.0	13.5
Molar absorptivity (l mol^{-1} cm^{-1})	4.85×10^5	2.47×10^4
Regression equation (y) [a]		
Slope (a)	0.0193	0.0010
Intercept (b)	0.0019	0.0006
Correlation coefficient (r^2)	0.9997	0.9988
RSD[b]	0.75	0.70
F[c]	9383.12	849.87

[a] $y = ax + b$, where x is the concentration in μg ml^{-1}.
[b] Relative standard deviation (%) ($n = 5$).
[c] F test for significance was applied to the regression (CL = 95%).

3.2. Optimum conditions for PEI/copper (II) complexation

Copper (II) can form complexes with PEI at different stoichiometric ratios, depending upon pH of the medium and PEI chemistry. The stoichiometry of the PEI/copper (II) complex under analysis was determined in different media by continuous variation of copper (II) ion concentration, the concentration of PEI being constant. In agree with literature data [12], the plots, obtained by the molar ratio method [15], indicated that copper (II) ions and PEI form a complex at about 1:5 N/Cu ratio (in Fig. 3 results obtained by using water as reacting medium are shown). It can be noticed that a 2-fold molar excess of PEI was required to obtain the saturation of the absorbance of PEI/Cu (II) complex.

When dealing with complex ions, the accuracy of the quantitation method depends on the ability of operating without disturbing the complex-forming equilibrium [13,16]. The complex formation can be affected by the aqueous environment in which the reaction takes place. To find a suitable medium for the reaction, that is a medium allowing a linear relationship between absorbance and

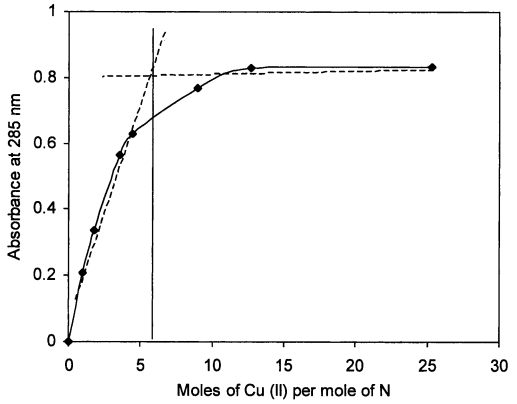

Fig. 3. Effect of copper (II) concentration upon complex formation. PEI concentration was 35 μg ml^{-1}.

Table 2
Optical characteristics and precision data for PEI quantification

Parameter/characteristic	PEI standards aqueous solvent	
	PBS (pH 7.4)	0.5 N NaOH
λ_{max} (nm)	285	285
Beer's law range (μg ml^{-1})	5.0–50.0	5.0–50.0
LOD (μg ml^{-1})	1.1	1.2
QOD (μg ml^{-1})	4.0	4.1
Molar absorptivity (l mol^{-1} cm^{-1})	4.85×10^5	4.55×10^5
Regression equation (y) [a]		
Slope (a)	0.0193	0.0185
Intercept (b)	0.0019	0.0088
Correlation coefficient (r^2)	0.9997	0.9999
RSD[b]	0.75	0.33
F[c]	9383.12	4251.25

[a] $y = ax + b$, where x is the concentration in μg ml^{-1}.
[b] Relative standard deviation (%) ($n = 5$).
[c] F test for significance was applied to the regression (CL = 95%).

concentration, as well as good sensitivity and reproducibility of the response, different reacting media were tested. PEI complexation was studied in: (i) water; (ii) PBS; (iii) acetate buffer. PBS at pH 7.4 and water were not considered suitable media for PEI analysis since a significant shift of the absorption maximum wavelength was observed as the concentration of the standard solution decreased. This wavelength shift was not observed in the case of acetate buffer, that was selected as reacting medium.

3.3. Quantification of PEI

PEI quantification was studied in acetate buffer by determining various optical parameters and precision data in both PBS and NaOH (Table 2). The linearity, slope and intercepts have been calculated using the regression equation $y = ax + b$ where y represents the absorbance at 285 nm, x the concentration of drug in μg ml^{-1} and a and b represent slope and intercept, respectively. Precision and accuracy were tested by carrying out determinations on five replicates and deriving the values of standard deviation (S.D.) and range of error at 95% confidence level. In each case Beer's law was verified over the PEI concentration range of 5.0–50.0 μg ml^{-1}. In PBS, the detection limit (LOD) (estimated to be three times the background noise) was 1.1 μg ml^{-1}. The limit of quantitation (QOD) was 4.0 μg ml^{-1}, a reasonable value of analyte concentration when dealing with dosage of PEI released from a controlled release systems.

Dosage of PEI dissolved in alkaline solution can be critical since precipitation of copper (II) hydroxide can take place. To this purpose, alkaline standards were complexed after buffering by means of acetic acid. PEI standard in 0.5 N NaOH was buffered by an equal volume of 1 M acetic acid, giving a pH value of 5.2 ($\pm$0.2) which did not vary by further additions of acid (data not shown). No significant difference in the calibration plot, LOD and QOD was observed between the analyses of the standards in PBS and in NaOH (Table 2).

To validate daily the performance of the analytical method, the absorbance values of PEI standards in both PBS and NaOH were recorded. The recoveries were always greater than 99%. Replicate analyses of standards at four-day intervals gave

good reproducibility (RSD < 3%), which ensured the repeatability of the analytical method.

3.4. PEI dosage in the presence of the oligonucleotide

Oligonucleotide spectrum in the far-UV region typically exhibits a maximum absorption band at about 265 nm. Therefore, when PEI is associated with an oligonucleotide, the latter is expected to interfere in the spectrophotometric analysis at 285 nm. Fig. 4 shows the UV spectra of PEI/Cu (II) complex and the oligonucleotide. As can be seen, PEI/Cu (II) absorbance curve totally overlap the curve of the oligonucleotide. In order to evaluate the contribution of the oligonuleotide to PEI/Cu (II) absorbance, its molar absorptivity at 285 nm was separately evaluated. A calibration curve of the oligonucleotide at 285 nm was obtained and the λ_{285} value derived. The regression equation was $y = 0.0376x + 0.0075$ (drug concentration in nmol ml^{-1}; RSD (%) 0.79; $F = 467.23$) with a correlation coefficient (r^2) of 0.9936 indicating that also at this wavelength a good linearity was achieved. Once the molar absorptivity of pdT16 was calculated ($\lambda_{285} = 3.94 \times 10^4$ l cm^{-1} mol^{-1}), its contribution to the total absorbance of the sample was derived according to the equation reported in Section 2.7. In the case of PEI and oligonucleotide mixture:

$$ABS_{TOT} = \varepsilon_{PEI/Cu\ (II)} \cdot b \cdot C_{PEI} + \varepsilon_{ODN} \cdot b \cdot C_{ODN}$$

where ABS_{TOT} is the total absorbance of the mixture at 285 nm (PEI/Cu (II) in the presence of the oligonucleotide), $\varepsilon_{PEI/Cu\ (II)}$ and ε_{ODN} are the molar absorptivities of PEI/Cu (II) complex and oligonucleotide at 285 nm, b is the fixed optical pathlength (cm), C_{PEI} and C_{ODN} are the molar concentrations of PEI and oligonucleotide (mol l^{-1}), respectively. From this equation C_{PEI} can be simply derived:

$$C_{PEI} = \frac{ABS_{TOT} - ABS_{ODN}}{\varepsilon_{PEI/Cu(II)} \cdot b}$$

ABS_{TOT} and ABS_{ODN} can be measured at 285 nm whereas all the other terms are known.

The accuracy and precision of the proposed spectrophotometric method was demonstrated by performing five replicate determinations of standard solutions containing different amounts of PEI (within the Beer's law range) by itself and in the presence of the oligonucleotide. The range, relative percent error, standard deviation and relative standard deviation (%) at each level are given in Table 3. The relative standard deviation values, which are less than 2% for three level studies, indicate the high reproducibility of the method. The relative percent error less than 3% confirms the accuracy of the proposed procedure.

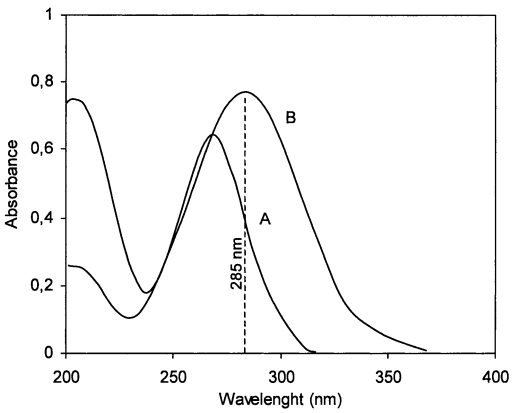

Fig. 4. Far-UV absorption spectra of pdT16 (A) and PEI/copper (II) complex (B).

4. Conclusions

A simple and fast spectrophotometric method for the quantitation of PEI in different media was developed. An appropriate selection of the experimental conditions ensured the sensitivity of the analytical method. The method is reproducible and accurate, and hence can be safely applied for the analysis of PEI in the presence of an oligonucleotide.

Table 3
Accuracy and precision of PEI quantitative analysis in the presence of the oligonucleotide

	PEI standards			PEI/oligonucleotide standards[a]		
Theoretical concentration μ (μg ml^{-1})	11.40	21.55	38.30	11.10	19.80	35.90
Concentration found (μg ml^{-1})[a]	11.22	21.00	38.90	11.35	19.41	34.92
Range (μg ml^{-1})	0.12	0.71	0.47	0.40	0.35	0.94
Error (%)	1.62	2.33	1.57	2.24	1.98	2.74
SD (μg ml^{-1})[b]	0.15	0.37	0.24	0.10	0.25	0.48
RSD (%)[b]	1.32	1.75	0.61	0.88	1.29	1.36

[a] The concentration of the oligonucleotide in the solution was 3.2 nmol ml^{-1}.
[b] Five replicates were analysed ($n = 5$).

Acknowledgements

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