Molecular assembling of DNA with amphipathic peptides

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Abstract The self-assembling of double-stranded DNA with short synthetic peptides has been analysed using the fluorescent properties of the intercalating dye, ethidium bromide. Two membrane-active peptides with appropriate sequences of lysine and leucine amino acids and a short polylysine have been probed. The results revealed that the secondary structure of the peptide decisively aimed the peptide-DNA complex formation: only the longest peptide, which is the only one to exhibit an α-helical structure in solution, could achieve DNA compacting before charge neutralisation. The obtained complex retained a significant membrane activity as demonstrated by calcein leakage experiments. This shows that short synthetic peptides of elementary sequence can combine both membrane activity and DNA-condensing properties. The potential of these constructs as DNA carriers will be discussed.

Key words: Dye exclusion; Fluorescence; DNA; Alpha-helical peptide; Self-association; DNA carrier

1. Introduction

Active research in the field of drug delivery and gene therapy has demonstrated the importance of creating discrete molecular assembling of DNA with condensing agents to transfect cells. The DNA-condensing effects of a number of polycations and cationic proteins such as polylysine, spermine or histones have been well known for decades (e.g. [1–4]) and collection of polycation-based vectors for gene transfer has been tested (e.g. [5–11]). While almost all of them were able to collapse DNA around the charge neutralisation point, unequal results were obtained with the transfection efficiency [7]. Moreover, the ability of polycation-DNA complexes to transfect cells appeared to be highly dependent on the presence of lysosomotropic effectors such as chloroquine which partially bypass the lysosomal degradation of DNA [12–14]. To find a rational and controlled way to escape the endosome now appears to be the next step forward in the elaboration of nucleic acid carriers. An attractive idea consists in incorporating a membrane-active compound, such as a peptide, in the carrier construction and several groups have already begun to test this concept in transfection experiments [14–18]. However, the achievement of this approach requires the identification and the understanding of the key parameters that govern the DNA-peptide self-assembling structures and their activity. In this paper, we investigated the ability of short synthetic amphipathic peptides, already known for their membrane activity [19], to condense DNA, envisaging the possibility for the complex to evade the lysosomes owing to the peptide membrane activity. We examined model peptides constructed on the basis of only two different amino acids, a hydrophilic one bearing a positive charge, lysine (K), and a hydrophobic one, leucine (L). The sequence was chosen to provide ideal parallel amphipathy along the helix. A 9-residue (KLLLKLLLK) and a 15-residue (KLLKLLLKLLLKLLK) peptide were studied together with a short 4–5-residue polylysine.

2. Materials and methods

2.1. Materials

The two peptides, $^{+}H_3$N-Lys-Leu-Leu-Leu-Lys-Leu-Leu-Leu-Lys-COO^{-} (LK9) and $^{+}H_3$N-Lys-Leu-Leu-Leu-Lys-Leu-Leu-Leu-Lys-Leu-Leu-Leu-Lys-Leu-Leu-Leu-Lys-COO^{-} (LK15) were synthesised by Neosystem (Strasbourg, France) and were more than 95% pure. Stock solutions were prepared in methanol. Further dilution was done in a water:methanol (5:1) mixture. The short polylysine (PLL), 4–5 residues long, was from Sigma. Calf thymus double-stranded DNA was purchased from Sigma and precipitated in ethanol solution. Ethidium bromide (EtBr) was from Molecular Probes. All experiments were performed in 30 mM Tris buffer added with 120 mM NaCl. Egg yolk phosphatidylcholine (EPC) was purified according to Singleton et al. [20].

2.2. Vesicle preparation

Large unilamellar vesicles (LUV) were prepared by freezing-thawing of a swollen phospholipid film followed by extrusion through a 200 nm calibrated polycarbonate filter as previously described by Mayer et al. [21] except the film was hydrated with a 4×10^{-4} M calcein solution instead of buffer. Untrapped calcein was removed by passing the vesicles over an AcA 54 Ultrogel column, equilibrated with Tris-NaCl buffer. Phospholipid concentration in the eluted vesicle fraction was measured by titration of inorganic phosphate [22] and adjusted to 5 mM in the stock suspension.

2.3. Fluorescence measurements

Double-stranded calf thymus DNA condensation was analysed using EtBr fluorescence properties. When the helix is in random coil state, the dye intercalates between the DNA base pairs and the fluorescence intensity of the dye increases significantly (reviewed by Le Pecq [23]). Upon condensation of the helix the intercalated dye is excluded from DNA resulting in a decrease in fluorescence intensity. Measurements were performed with a Spex Fluoromax spectrometer equipped with a thermostatted cuvette holder maintained at 25°C for all experiments. Excitation wavelength was set at 510 nm where DNA-bound and free forms of EtBr have the same molar extinction coefficient. A 4 nm band pass was used. The spectra, recorded from 525 to 700 nm, made it possible to measure the maximum fluorescence intensity of the dye located around 595 nm (I_{595}) and the intensity of the right Raleigh line bottom scattered at 525 nm (I_{525}). The fluorescence intensity obtained in the presence of the condensing agent (I_{595}) was normalised to the intensity measured in its absence on the same sample (I_{o595}) and called $f = I_{o595}/I_{595}$.

Calcein leakage from EPC vesicles was determined by adding 50 mM $CoCl_2$ in the vesicle suspension to quench external calcein fluorescence [24]. Dye release kinetics were monitored at 515 nm, the excitation wavelength was set at 490 nm.

2.4. Circular dichroism

The spectra were recorded with a dichrograph Mark V from Jobin Yvon (Longjumeau, France).

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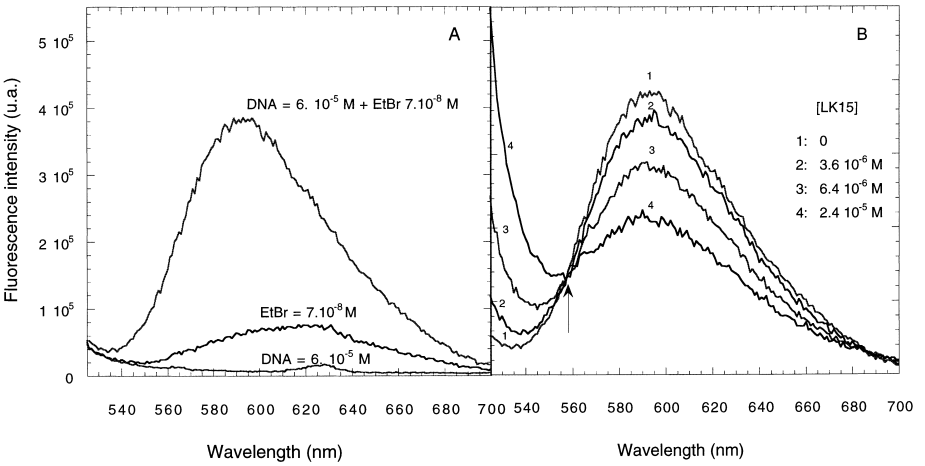

Fig. 1. Ethidium bromide fluorescence spectra. A: EtBr 7×10^{-8} M in the absence and in the presence of 6×10^{-5} M DNA. B: EtBr intercalated in 6×10^{-5} M DNA recorded in the presence of increasing concentrations of LK15. In 20 mM Tris buffer, 130 mM NaCl. Excitation wavelength was set at 510 nm.

3. Results and discussion

A marked increase in the fluorescence intensity of the heterocyclic dye EtBr occurs upon double-stranded DNA binding, depending on the topological constraints of the DNA tertiary structure [23]. This property was used here to probe the interactions of short cationic peptides with double-stranded DNA in so far as these interactions will change the DNA topology. Fig. 1 shows the fluorescence spectra of EtBr either free or bound to the DNA helix together with the spectra obtained with increasing concentrations of LK15. The peptide obviously induced a significant decrease of the fluorescence of the intercalated dye. Interestingly, the intensity of scattered light increased in a closely correlated way as indicated by the presence of an isosbestic point in the wavelength region where fluorescence and scattering overlap (we checked, using an inert emulsion as a scattering source, that in our conditions the light scattered in the Raleigh region did not affect the fluorescence spectra). Fig. 2 shows the changes of the relative fluorescence intensity f and of the Raleigh scattering intensity at 525 nm for labelled DNA compelled with increasing concentrations of LK15, LK9 or PLL, the short polylysine of 4–5 lysine residues. At the pH of the experiments, LK15 and LK9 have 5 and 3 positive charges per molecule, respectively. The data are presented as a function of the concentration of positive charges produced by each peptide, knowing that the DNA concentration corresponded to 10^{-5} M in phosphate, i.e. in negative charges. In each case, the fluorescence decreased to a plateau level which corresponded to a relative fluorescence value, fp, equal to

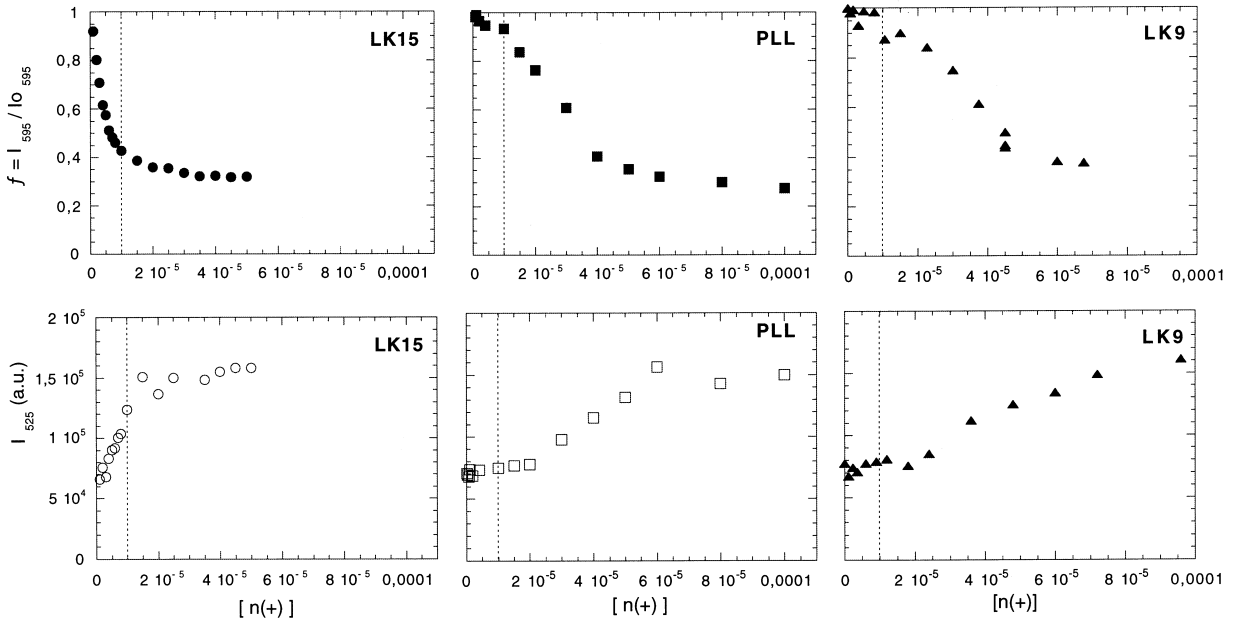

Fig. 2. Fluorescence and light scattering of EtBr intercalated in 10^{-5} M DNA (phosphate concentration) with increasing concentrations of peptides added. The fluorescence intensity was measured at 595 nm (upper panels) and light scattering at 525 nm (lower panels). The dashed lines indicate the neutralisation points. [n+)] represents the concentration of positive charges brought by each peptide, knowing that LK15, PLL and LK9 bear 5, 4–5 and 3 positive charges, respectively.

0.35 ± 0.05. The scattering evolved in a closely correlated way. Other characteristics of the interaction differed greatly between LK15 on the one hand and LK9 and PLL on the other hand. With LK15, the signal showed a steep decrease from the lowest peptide concentrations to a plateau which arose around the charge neutralisation, i.e. one positive charge brought by the peptide for one DNA phosphate. The scattering increased correspondingly as the fluorescence decreased. In contrast, in the case of LK9 and PLL, the fluorescence signal remained unchanged until the peptide concentration reached the DNA charge neutralisation ratio. The scattering increase appeared to be slightly shifted towards the highest concentrations as compared to the fluorescence increase. The fluorescence and scattering plateaus were both obtained for peptide concentrations equal to 5×10^{-5} M, i.e. for a positive to negative charge ratio close to 5, far above the neutralisation point.

Clearly, the three peptides studied here were able to cause a drop of the intercalated dye fluorescence. The profiles of relative fluorescence decrease were not influenced when EtBr concentration was changed showing that the probe did not affect the reported process. However, the number of EtBr per base pair was always kept below 1/30. The fluorescence drop was interpreted as a release of the dye from the double helix into the buffer, although a quantum yield decrease due to a change in the structure of the intercalation complex could not entirely be excluded. Nevertheless, whatever the actual molecular mechanism of the fluorescence decrease, it necessarily implies that a change in DNA structure has occurred. The rate of EtBr intercalation mostly depend on topological constraints: for instance negative supercoilings disfavoured intercalation by reducing the possibility of the helix to relax by lengthening [23]. The profiles obtained with LK9 and PLL exhibited a cooperative shape strongly suggesting that the DNA had undergone a conformational transition from the initial random coil state to a condensed phase. This cooperativity has been previously observed with a collection of cationic entities (e.g. [1,25–27]) and analysed within the framework of the counterion condensation theory developed by Manning for polyelectrolytes [28]. This theory predicts that back and forth folding of the helix on itself occurs when the linear charge density of the phosphate backbone has been lowered enough by neutralising above 90% of the DNA negative charges in physiologic ionic strength. The simultaneous increase of light scattering intensity suggests the formation of discrete insoluble entities in agreement with the condensation hypothesis. However, these data do not allow a distinction between mono-molecular or inter-molecular condensates, only the shift in concentration of the scattering profile compared to the fluorescence could lend support to the idea that inter-molecular condensation has taken place. Therefore, the two peptides LK9 and PLL appeared to act only as polycationic entities which neutralise the DNA backbone and induce, in this way, cooperative condensation. The situation is different with LK15, where both fluorescence and scattering turned at a peptide concentration as low as 5×10^{-8} M, i.e. a low positive to negative charge ratio ($r\pm$) equal to 0.025. This peptide was then much more efficient in DNA condensation than the LK9 analogue or the short polylysine which held the same charge number. Surprisingly, the effect on DNA exerted by this peptide did not require charge neutralisation. It also possibly led to a different complex type.

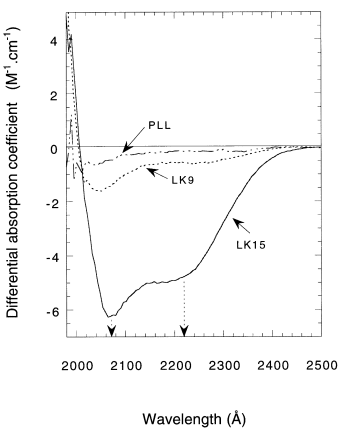

Fig. 3. Circular dichroism spectra of the three peptides LK15, LK9 and PLL, 10^{-5} M in 20 mM Tris, 130 mM NaCl. Background, recorded with the buffer alone, was subtracted in each case.

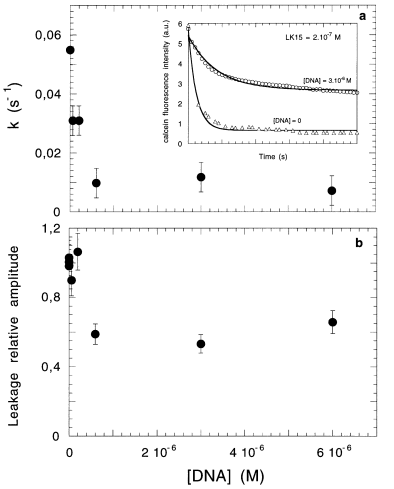

Fig. 4. Calcein leakage from EPC LUVs. The rate constant, k (a) and the amplitude of the decay, A (b), are given as a function of DNA (phosphate) concentration. The insert shows two representative adjustments (lines) of the data (points) according to a mono-exponential decay. $[EPC] = 5\times10^{-6}$ M.

In order to find some clue to explain the behaviour of LK15, we performed circular dichroism (CD) measurements to investigate the secondary structure of the peptides. CD recordings presented in Fig. 3 clearly show that LK15 was the only one whose spectrum exhibited the characteristic features of an α-helical structure, i.e. strong negative bands located at 207 and 222 nm [29]. The same differential absorption, $\Delta\varepsilon$, was observed between 10^{-6} and 2×10^{-5} M peptide, indicating that in this concentration range no structural changes occurred. LK9 showed only a very low level of structuring and none was found with PLL (Fig. 3). In the presence of DNA (data not shown), the spectra were largely dominated by DNA helix signal and did not bring any information on the structure of the peptides within the complex. Together with the fluorescence results, the CD data strongly suggest that the secondary structure of the peptide, opposing polar and apolar faces, plays a central role in its interaction with DNA. Indeed, LK15 presents to interact with DNA not only an ordered array of lysine residues but also a defined hydrophobic domain which is expected to support self-association

of the peptides with each other. This self-association has already been observed in solution (Castano et al., personal communication) and could very likely be responsible for the distinct interaction observed between LK15 and DNA. In this hypothesis, the self-association of the peptides would create knots within the complexes inducing the topological constraints responsible for the dye exclusion. Being different in nature, this interaction would not require previous neutralisation of the DNA helix to settle but could occur locally at the lowest concentrations. Additional experiments are in progress to explore this hypothesis. Up to now, most reported DNA condensation required quasi-total neutralisation of the DNA backbone by the condensing agent. Wyman et al. [18] have worked with membrane-active peptides which acquired an α-helical structure at pHs higher than 7 but did not report any influence of the secondary structure on the characteristics of the interaction with nucleic acids. Emile et al. [30] noticed a better ability of α-helix structures to bind oligonucleotides as compared to β-sheets; however, this was attributed in their paper to a more adapted geometry of the lysine groups and not to any hydrophobic effect. Also worthy of mention is the fact that, independently of their ability to bind DNA, several peptides holding a well defined secondary structure have been shown to achieve efficient transfer of nucleic acids in cells [14,31–33].

As previous experiments had clearly shown that LK15 was able to form a complex with DNA at low concentrations, we were, at this point, interested in determining how the association of the peptide with DNA affected the membrane activity of the peptide. This activity was evaluated by measuring calcein leakage from EPC LUVs. The kinetic profiles obtained either with LK15 alone or with LK15 bound to DNA within the complex showed that increasing the concentration of DNA, i.e. the degree of peptide association, slowed down the leakage and reduced its final amplitude. The kinetics satisfyingly fitted a mono-exponential decay of the form $F(t) = A \exp(-kt) + Fo$, where A and k are the amplitude of the leakage and its apparent rate constant, respectively. Fo stands for the fluorescence background remaining after complete release of the dye. A and k were determined by parameter adjustment. The rate constant k decreased from 5×10^{-2} s^{-1} (value obtained with the peptide alone) to a plateau value close to 10^{-2} s^{-1} (Fig. 4a) obtained for a DNA concentration near 10^{-6} M, i.e. around the charge neutralisation, identified above as the point where the complex formation was achieved. The relative amplitude of the leakage (Fig. 4b) remained equal to 1 which corresponds to total release of the dye, up to 4×10^{-7} M DNA; then the amplitude dropped steeply to reach a plateau value near 0.5 for DNA concentrations higher than 10^{-6} M. This lends support to the idea that in a first step – DNA concentration lower than 10^{-6} M – the release of the dye was induced by free peptide which remained uncomplexed at these DNA concentrations. The leakage is then total ($A = 1$) and the rate constant of the leakage depends on the free peptide concentration continuously decreasing as DNA increases. Then, when the DNA concentration exceeds 10^{-6} M, corresponding to the charge neutralisation point, the process was no more driven by free peptide, which then had a negligible concentration, but rather by the DNA-peptide complex whose activity was characterised by a rate constant value equal to 10^{-2} s^{-1} and a leakage amplitude equal to 0.5. This actually attests to a membrane activity of the complex itself. This differs from the results obtained by Wyman et al. [18] where a DNA excess totally suppressed the membrane activity of the peptide they used.

4. Conclusion

We have shown above that (i) the α-helix represents an exquisite structural motif for the interaction of peptides with DNA. The interaction appeared to be driven at first by electrostatic interactions but most significant was the fact that the α-helical amphipathic structure allowed topological changes to occur in DNA much before the charge neutralisation point in contrast with other polycationic compounds. (ii) Such complexes acquired a colloidal character as shown by the increase in light scattering, although we cannot at this point state whether we have only a DNA mono-molecular condensation or a multimeric aggregation. (iii) Model experiments have shown that the peptide conserved membrane activity within the complex even though the actual activity of the assembling was reduced.

This work constitutes a first step in the elaboration of active peptide-DNA molecular assembling based on short synthetic peptides. A further step would be to extend our conclusions to peptides conceived to retain the α-helicity and polycationic nature but having additionally a pH sensitivity allowing these properties to befall or not at acidic pH. This would target the activity to the endosome specifically. Another choice would be to entrap the complexes in a colloidal system to add a level of control of the complex's properties like cell surface adhesion then internalisation, or release kinetics.

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References

[1] Shapiro, J.T., Leng, M. and Felsenfeld, G. (1969) Biochemistry 8, 3219–3232.
[2] Olins, D.E. and Olins, A.L. (1971) J. Mol. Biol. 57, 437–455.
[3] Gosule, L.C. and Schelman, J.A. (1976) Nature 259, 333–335.
[4] Hsiang, M.W. and Cole, D. (1977) Proc. Natl. Acad. Sci. USA 74, 4852–4856.
[5] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413–7417.
[6] Behr, J.-P., Demeneix, B., Loeffler, J.-P. and Perez-Mutul, J. (1989) Proc. Natl. Acad. Sci. USA 86, 6982–6986.
[7] Felgner, P.L. (1990) Adv. Drug Deliv. Rev. 5, 163–187.
[8] Haensler, J. and Szoka Jr., F.C. (1993) Bioconjug. Chem. 4, 372–379.
[9] Behr, J.-P. (1994) Bioconjug. Chem. 5, 382–389.
[10] Boussif, O., Lezoualc'h, F., Zanta, M.-A., Mergny, M.D., Scherman, D., Demeinex, B. and Behr, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 7297–7301.
[11] Gao, X. and Huang, L. (1996) Biochemistry 35, 1027–1036.
[12] Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M. and Roche, A.C. (1993) Nucleic Acids Res. 21, 871–878.
[13] Wadhwa, M.S., Knoell, D.L., Young, A.P. and Rice, K.G. (1995) Bioconjug. Chem. 6, 283–291.
[14] Niidome, T., Ohmori, N., Ichinose, A., Wada, A., Mihara, H., Hirayama, T. and Aoyagi, H. (1997) J. Biol. Chem. 272, 15307–15312.
[15] Legendre, J.-Y. and Szoka, F.C. (1993) Proc. Natl. Acad. Sci. USA 90, 893–897.
[16] Legendre, J.-Y., Trzeciak, A., Bohrmann, B., Deuschle, U., Kitas, E. and Supersaxo, A. (1997) Bioconjug. Chem. 8, 57–63.

[17] Kichler, A., Mechtler, K., Behr, J.P. and Wagner, E. (1997) Bioconjug. Chem. 8, 213–221.
[18] Wyman, T.B., Nicol, F., Zelphati, O., Scaria, P.V., Plank, C. and Szoka Jr., F.C. (1997) Biochemistry 36, 3008–3017.
[19] Cornut, I., Büttner, K., Dasseux, J.-L. and Dufourcq, J. (1994) FEBS Lett. 349, 29–33.
[20] Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53–56.
[21] Mayer, L.D., Tai, L.C.L., Bally, M.B., Mitilines, G.N., Ginsberg, R.S. and Cullis, P.R. (1990) Biochim. Biophys. Acta 1025, 143–151.
[22] Ames, B.N. (1966) Methods Enzymol. 8, 115–136.
[23] Le Pecq, J.B. (1971) Methods Biochem. Anal. 20, 41–86.
[24] Kendall, D.A. and McDonald, R.C. (1983) Anal. Biochem. 134, 26–31.
[25] Wilson, R.W. and Bloomfield, V.A. (1979) Biochemistry 18, 2192–2196.
[26] Widom, J. and Baldwin, R.L. (1980) J. Mol. Biol. 144, 431–453.
[27] Gershon, H., Ghirlando, R., Guttman, S.B. and Minsky, A. (1993) Biochemistry 32, 7143–7151.
[28] Manning, G.S. (1978) Q. Rev. Biophys. 11, 179–246.
[29] Chang, C.T., Wu, C.S. and Yang, J.T. (1978) Anal. Biochem. 91, 13–31.
[30] Emile, C., Bazile, D., Herman, F., Hélène, C. and Veillard, M. (1996) Drug Deliv. 3, 187–195.
[31] Gottschalk, S., Sparrow, J.T., Hauer, J., Mims, M.P., Leland, F.E., Woo, S.L.C. and Smith, L.C. (1996) Gene Ther. 3, 448–457.
[32] Vidal, P., Morris, M.C., Chaloin, L., Heitz, P. and Divita, G. (1997) C.R. Acad. Sci. Paris 320, 279–287.
[33] Wadhwa, M.S., Collard, W.T., Adami, C., McKenzie, D.L. and Rice, K.G. (1997) Bioconjug. Chem. 8, 81–88.