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Synthesis of a L-lysine-based alternate alpha,epsilon-peptide: A novel linear polycation with nucleic acids-binding ability

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ABSTRACT

A novel nucleic acid compaction device based on a positively-charged alpha,epsilon-poly-L-lysine was realized for the first time. The polycationic peptide was obtained by assembling Fmoc and Boc orthogonally protected L-lysine monomers by solid phase synthesis. The route to the novel polycation is very fast and convenient because it allows for the obtainment of the desired product in few synthetic steps exclusively employing Fmoc chemistry. The purification of the poly-amino acid was performed easily by RP-HPLC on C18 stationary phase while ESI-MS analysis confirmed the identity of the novel basic molecule. The structural characteristics of the novel water-soluble peptide, as well as the interaction with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been investigated by circular dichroism (CD) and ultraviolet (UV) spectroscopy.

1. Introduction

The interactions between proteins and nucleic acids play a crucial role in the organization and maintenance of several nucleoprotein complexes such as chromatin and viruses, and cellular structures such as ribosomes. Binding of peptides to nucleic acids has been intensively investigated (Helene et al., 1981; Puglisi and Doudna, 2008) in order to elucidate the types of interactions controlling the recognition process between nucleic acids and proteins. Insights on factors determining the formation of complexes between proteins and nucleic acids can be obtained by using (1) material derived from natural sources or (2) artificially synthesized peptides which represent simplified mimics of natural molecules. The binding of peptides to nucleic acids is exerted through several intermolecular forces namely: (1) electrostatic interactions between basic amino acids and negatively charged phosphates present on the nucleic acid; (2) stacking interactions, occurring between the side chains of aromatic amino acids and nucleic acid bases; (3) hydrogen-bonding, formed between the peptide amide group, as well as several amino acid side chains, with nucleic acid phosphates, sugar and bases; (4) hydrophobic interactions, formed between the side chains of aliphatic amino acids and the nucleobases. In particular, the electrostatic interactions play a fundamental role in the histone–DNA recognition in eukaryotic cell nuclei (Perico et al., 2005). In this regard, the highly positively-charged nature of histones, aside from facilitating histone–DNA recognition, contributes to the water solubility of histones. Several proteins which play key physiological roles contain regions of positively-charged amino acids. For istance cell surface proteins, G-protein-coupled receptors (Sacks et al., 1989a) and rheumatic disease associated autoantigens (Brendel et al., 1991; Sabbatini et al., 1993) present basic regions. Cationic peptides also play a role in phosphorylation of calmodulin and other proteins (Meggio et al., 1987; Sacks et al., 1989b; Moreno et al., 1993) and in the calmodulin-stimulated cyclic nucleotide phosphodiesterase (Itano et al., 1980). Naturally-occurring or synthetic polycationic peptides are involved in the proteinase-substrate or proteinase-inhibitor recognition processes (Sela and Katchalski, 1959) and some of them are activators of the human erythrocyte multicatalytic proteinase (Mellgren, 1990). Among the many polycationic peptides, synthetic alpha-poly-L-lysine has been largely used as a model substrate to elucidate the mechanism of action of trypsin-like enzymes of bacterial and mammalian origin, and as an inhibitor of plant proteinases (Amato et al., 1995). Interestingly, this homopolymer can exist stably in either alpha-helical (at room temperature) or beta-sheet conformations (at higher temperature) (Sarkar and Doty, 1966) and can be used to prepare cationic hydrogels (Oliveira et al., 2003).

The epsilon-poly-L-lysine is another example of naturally-occurring basic polyamide capable of binding to nucleic acids (Huang et al., 2008). Epsilon-poly-L-lysine is a nonribosomally-produced homo-poly-amino acid characterized by peptide bonds linking the carboxyl and the epsilon-amino groups of L-lysine residues. This peptide is stable at high temperatures, under alkaline

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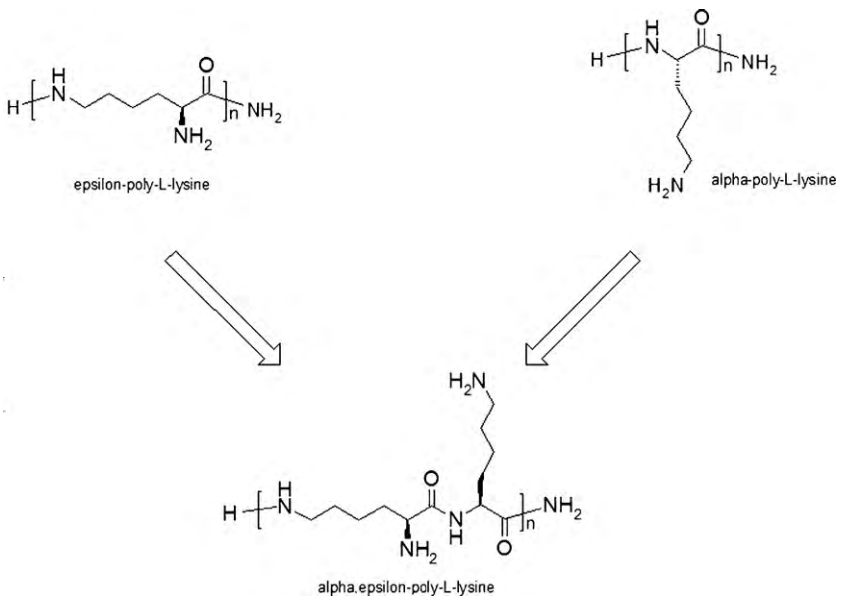


Fig. 1. Comparison of the structure of the artificial alternate alpha,epsilon-poly-L-lysine with those of the natural epsilon-poly-L-lysine, produced by *Streptomyces* bacteria, and alpha-poly-L-lysine.

or acidic conditions, and it shows also a significant antimicrobial activity (Takehara et al., 2008). Due to the latter property, which depends on the ability of the poly-amino acid to adhere to the bacteria cell surface by electrostatic effect due to its polycationic nature, epsilon-poly-L-lysine manufactured by a *Streptomyces* fermentation process (Hirohara et al., 2006, 2007), is used as a food additive.

Other known natural nonribosomally-synthesized poly-aminoacids include: poly(gamma-glutamic acid), cyanophycin, a polymer containing L-arginine and L-aspartic acid residues (Kunioka, 1997; Oppermann-Sanio and Steinbüchel, 2002; Obst and Steinbüchel, 2004), poly(arginyl-histidine) (Nishikawa and Ogawa, 2004a,b) and gamma-poly-L-diaminobutyric acid (Takehara et al., 2008). Besides their occurrence in nature, polylysines can be obtained by chemical polymerization starting from L-lysine or its derivatives. Interestingly, by using two different activating systems and solvents it was possible to obtain selectively the alpha or the epsilon linear polylysines as described recently by Ho et al. (2008). Furthermore, dendrimeric alpha,epsilon-poly-L-lysines were synthesized and employed as delivery agents for oligonucleotides, as described by Eom et al. (2007). However, the synthesis or the isolation from natural sources of linear alpha,epsilon-poly-L-lysines has never been reported up to now. Considering the importance of alpha- and epsilon-L-lysine-based homopolymers, and of their nucleic acid compaction ability (Huang et al., 2008), we prepared the corresponding alternate copolymer, and performed some preliminary studies on its structural characteristics and its nucleic acids-binding ability (Fig. 1).

2. Materials and methods

2.1. Abbreviations

Ac_2O (acetic anhydride), Boc (*tert*butoxycarbonyl), DBU (1,8-diazabicyclo[5.4.0]undecene), DIEA (N,N-diisopropylethylamine), Fmoc (9-fluorenylmethoxycarbonyl), MBHA (4-methylbenzhydrylamine), NMP (4- methylpyrrolidone), poly(U) (polyuridylic acid), PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), PTFE (polytetrafluoroethylene), TFA (trifluoroacetic acid), and TIS (triisopropyl silane).

2.2. Chemicals

Boc-L-Lys(Fmoc)-OH was purchased from Advanced Biotech Italia, while Fmoc-L-Lys(Boc)-OH was from Iris Biotech Gmbh. Anhydroscan NMP was from LabScan. Solvents for HPLC chromatography and acetic anhydride were from Reidel-de Haën. Diethyl ether was from Carlo Erba. DBU and TIS were purchased from Sigma–Aldrich. dT_{12} (DNA) was from Biomers, TFA and poly(U) (RNA) were from Fluka.

2.3. Apparatus

Centrifugations were performed on a Z 200 A Hermle centrifuge.

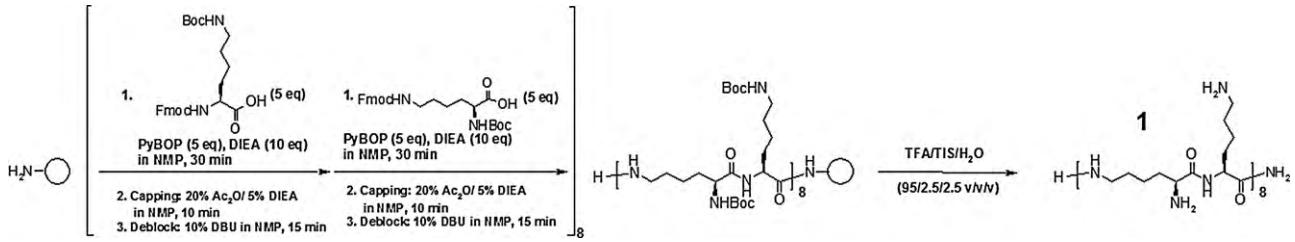
The product was analyzed and characterized by LC–ESI-MS on an MSQ mass spectrometer (ThermoElectron, Milan, Italy) equipped with an ESI source operating at 3 kV needle voltage and 320 °C, and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler, and a PDA detector, by using a Phenomenex Jupiter C18 300 Å (5 μm, 4.6 mm × 150 mm) column. Gradient elution was performed by using increasing amounts of acetonitrile (0.05% TFA, B) in water (0.05% TFA, A), with a linear gradient of 5% (for 5 min) to 40% B in A over 10 min (t_R = 11.7 min), monitoring at 260 nm, with a flow rate of 0.8 ml/min.

Semi-preparative purification was performed on a Hewlett Packard/Agilent 1100 series HPLC, equipped with a diode array detector, by using a Phenomenex Juppiter C18 300 Å (10 μm, 10 mm × 250 mm) column. Gradient elution was performed by building up a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile) with a flow rate of 4 ml/min (monitoring at 210 nm).

Samples were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. UV spectra were recorded on a UV-Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller by using Hellma quartz Suprasil cells, with a light path of 1 cm.

2.4. Solid phase synthesis of (alpha-epsilon-lys)$_8$-NH_2 (**1**, Scheme 1)

Solid phase synthesis was carried out in a short PP column (4 ml) equipped with a PTFE filter, a stopcock and a cap on a Rink



Scheme 1. Synthesis of the alternate *alpha,epsilon-lys*PNA oligomer **1**.

amide resin using the peptide-like Fmoc chemistry. Oligomer **1** was assembled on Rink Amide MBHA resin (0.14 mmol/g, 29 mg, 4 μmol) using the synthetic strategy described in Scheme 1. In particular, a solution of Boc-L-Lys(Fmoc)-OH or Fmoc-L-Lys(Boc)-OH (20 μmol, 5 equiv.), PyBOP (20 μmol, 5 equiv.) and DIEA (7 μl, 40 μmol, 10 equiv.) in 0.3 ml of NMP was added to the NH_2-resin and the mixture was reacted for 30 min under stirring. Each condensation step was followed by a capping step, performed with 20% Ac_2O/5% DIEA in NMP for 10 min, while Fmoc deprotection was achieved with 10% DBU in NMP (15 min). After the final Fmoc removal (UV Fmoc test: 3 μmol, 75% yield) the peptide was then cleaved from the solid support by treatment with TFA/TIS/H_2O (95/2.5/2.5, v/v/v), over 1.5 h and recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The oligomer **1** was purified by semi-preparative HPLC using a linear gradient of 8% (for 5 min) to 25% B in A over 30 min: t_R = 8.9 min; After liophylization, 532 nmol of purified product **1** were collected; ESI-MS (Fig. 2) 1036.19 (found), 1034.93 (expected for $[C_{96}H_{195}N_{33}O_{16} + 2H]^{2+}$); 690.91 (found), 690.29 (expected for $[C_{96}H_{195}N_{33}O_{16} + 3H]^{3+}$).

2.5. UV and CD studies

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter, while, ultraviolet (UV) spectra were recorded on a UV-Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller, using a Hellma quartz cell with a light path of 1 cm and a Hellma Tandem quartz cell 2 cm × 0.4375 cm (Rocchi et al., 1972; Krzyzanowska et al., 1998). CD and UV spectra were recorded in the 190–250 or 200–320 nm wavelength range.

3. Results and discussion

3.1. Solid phase synthesis of the L-lysine-based hexadecapeptide *1*

The synthesis of the peptide **1** was performed in solid phase by using the commercial Boc-L-Lys(Fmoc)-OH and Fmoc-L-Lys(Boc)-OH aminoacids as starting materials for the realization of the *alpha,epsilon*-peptide backbone. We firstly realized the hexadecapeptide backbone by alternately coupling on the solid support (Rink Amide MBHA) the commercial Boc-L-Lys(Fmoc)-OH and Fmoc-L-Lys(Boc)-OH derivatives using a Fmoc peptide strategy (Scheme 1).

After Boc deprotection and cleavage from the solid support, achieved under acid treatment (TFA/TIS/H_2O = 95/2.5/2.5, v/v/v), precipitation from cold diethyl ether and purification by RP-HPLC, the desired product was obtained in 13% yield. Characterization of **1** was performed by LC–ESI-MS which confirmed the identity of the product (Fig. 2).

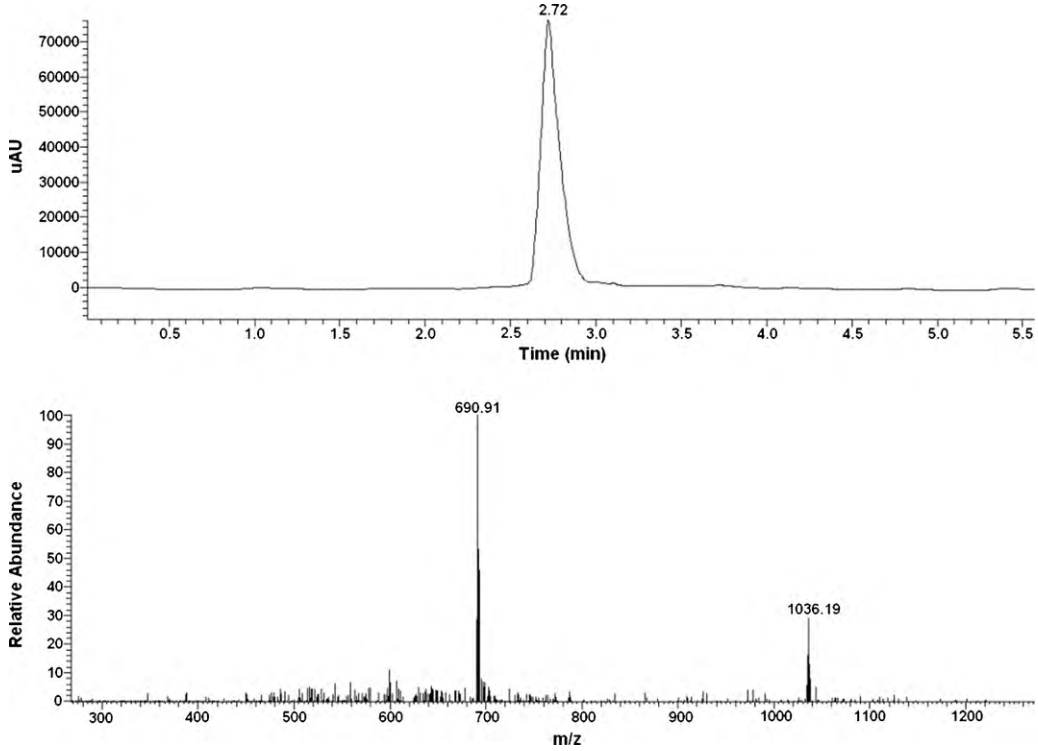


Fig. 2. LC–ESI-MS (positive ions) analysis of the oligomer **1**.

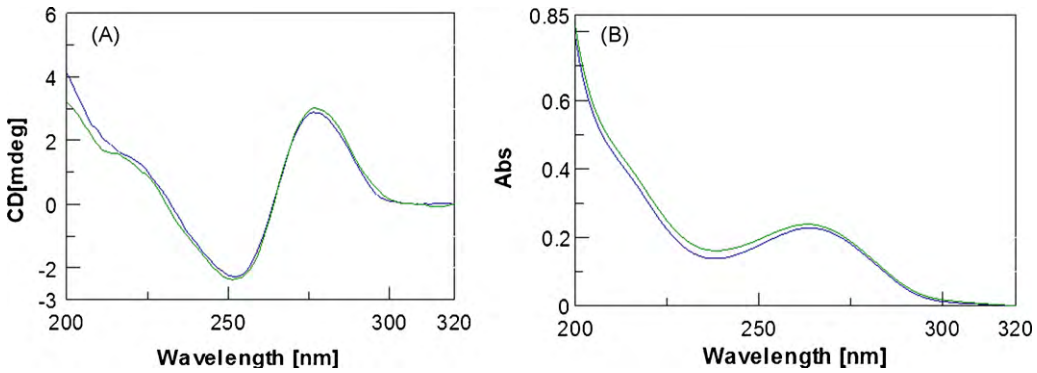


Fig. 3. "Sum" (green line) and "mix" (blue line) CD (A) and UV (B) spectra, recorded at 5 °C by a Tandem cell, relative to dT_{12} (30 nmol in T) and peptide **1** (60 nmol in $-NH_3^+$) dissolved each in 1 ml of 10 mM phosphate buffer, pH 7.5. The volume of solution after mixing was 2 ml (1 ml + 1 ml). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

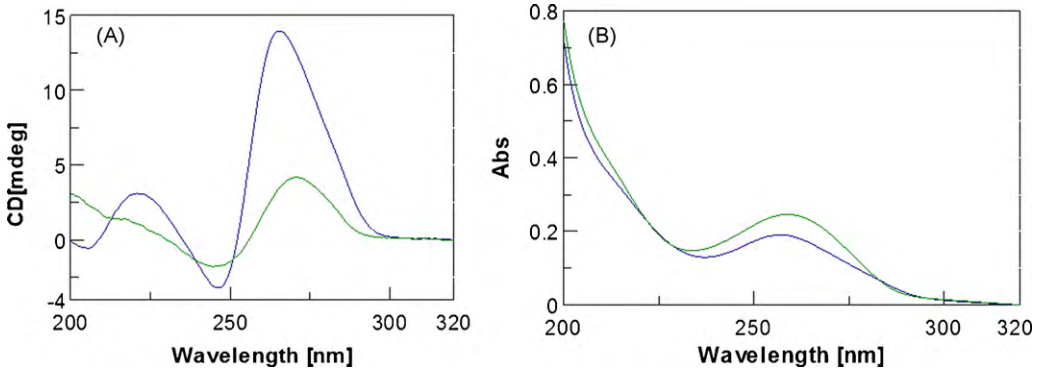


Fig. 4. "Sum" (green line) and "mix" (blue line) CD (A) and UV (B) spectra, recorded at 5 °C by a Tandem cell, relative to poly(U) (30 nmol in U) and peptide **1** (60 nmol in $-NH_3^+$) dissolved each in 1 ml of 10 mM phosphate buffer, pH 7.5. The volume of solution after mixing was 2 ml (1 ml + 1 ml). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The novel *alpha,epsilon*-peptide did not present any tendency to self-aggregate and showed a good water solubility as expected due to its positively-charged nature at physiological pH.

3.2. CD and UV studies

The structural characteristics of the L-lysine-based peptide as well as its DNA- and RNA-binding ability were investigated by CD and UV spectroscopy. Firstly, the CD profile of the nucleopeptide single strand in 10 mM phosphate buffer, pH 7.5, was analyzed in the 190–250 nm range at several temperatures and from these experiments no pre-organization of the single-stranded peptide **1** was evidenced. CD and UV experiments on **1** with DNA and RNA were then performed in order to evaluate its potential as nucleic acid compaction agent. More particularly, direct mixing of dT_{12} DNA and polycation 1, at 5 °C in the presence of 10 mM phosphate buffer (pH 7.5), led to a slight reduction of the magnitude of the CD spectrum (Fig. 3A).

Since the novel peptide **1** did not show any significant absorption in the 248–300 nm wavelength range, this effect could be ascribed to a little conformational change of DNA. Moreover, a small hypochromism in the UV absorption band of DNA was observed in the presence of the novel peptide in the same experimental conditions confirming this hyphothesis. On the other hand, as evidenced by the subsequent CD and UV experiments (Fig. 4A and B), direct mixing of polypeptide with poly(U) RNA at 5 °C in the presence of 10 mM phosphate buffer (pH 7.5), led to a strong interaction which could be associated with the conversion of the polynucleotide to its ordered form in analogy to previous reports (Helene et al., 1981). This transition was reflected in both CD and UV absorption spectra which presented a strong increase in the positive CD band of RNA and a significant UV hypochromic effect, respectively (Fig. 4A and B). The spectral changes induced by peptide **1** were similar to those induced by alpha-poly-L-lysine, Mg^{2+} and Cs^+ (Carroll, 1972; Thrierr et al., 1971).

4. Conclusion

To the best of our knowledge, this is the first report on the linear polyaminoacid alpha,epsilon-poly-L-lysine, whose solid phase assembly and spectroscopic nucleic acids-binding evaluation was presented for the first time in this work. The methodologies adopted for both the synthesis and the spectroscopic studies are similar to those previously reported in literature for various polyamide molecules (Capparelli et al., 2009; Roviello et al., 2009, 2010a,b,c). On the basis of its similarity with alpha-poly-L-lysine and epsilon-poly-L-lysine, this alternate peptide is expected to have biomedical applications similar to those of the naturally-occurring polyaminoacids. Further studies on the antimicrobial and protease inhibitory activity of the alternate (artificial) copolymer are clearly desirable. In this preliminary work, an hexadecapeptide was prepared in solid phase using two commercially-available Fmoc/Boc-protected L-lysine monomers by Fmoc strategy, which could be extended also to peptides with a different number of residues. The novel polymer was purified by RP-HPLC, characterized by ESI-MS (which confirmed its identity) and studied by CD and UV spectroscopies to investigate the presence of a secondary structure of the single-stranded peptide as well as its ability to form complexes with natural nucleic acids. No pre-structuration of the alternate polypeptide was revealed by the CD

spectral results, conducted at different temperatures in water solution (10 mM phosphate buffer, pH 7.5). The CD and UV results showed that alpha,epsilon-poly-L-lysine, which can interact only weakly with dT_{12} DNA, induced a large conformational change of poly(U) RNA, probably shifting its conformation from a single-stranded random coil to an ordered form as already reported in literature (Helene et al., 1981). The results collected suggest that the alpha,epsilon-poly-L-lysine, structurally similar to the antimicrobial epsilon-poly-L-lysine and alpha-poly-L-lysine, is a well-soluble artificial polycation capable of inducing large conformational changes in RNA and can be regarded, in analogy to other basic peptides and nonribosomial poly-amino acids, as a potential delivery agent for RNA molecules. In particular, we plan to investigate this possibility by using complexes of the novel polycation with interesting therapeutic tools such as polyinosinic acid–polycytidylic acid complex (poly I:C), a potent interferon inducer, as well as small interfering RNAs (siRNA), which are useful to silence genes involved in the pathogenesis of various diseases. In conclusion, the well-known oligonucleotide delivery, antimicrobial and hydrogel forming properties of polylysines, support the importance of evaluating also the novel alpha,epsilon-poly-L-lysine as a potential candidate for specific technical applications in various fields of human life such as medicine, food, and chemical materials.

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