RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2016, **6**, 63578

Synthetic approaches to nucleopeptides containing all four nucleobases, and nucleic acid-binding studies on a mixed-sequence nucleo-oligolysine†

Giovanni N. Roviello‡*[a] and Domenica Musumeci‡*[ab]

Received 5th April 2016
Accepted 27th June 2016

DOI: 10.1039/c6ra08765e

www.rsc.org/advances

In this article we describe two solid-phase synthetic routes to obtain a nucleo-oligolysine α-peptide containing all four natural nucleobases. The first one is based on the oligomerization of the nucleobase-containing monomers, easily synthesized as herein described. The second strategy has the advantage of avoiding the solution synthesis of the monomeric building blocks, leading to the final nucleopeptide by direct solid-phase couplings of the suitably protected nucleobases with the free amino groups on the growing peptide chain still anchored to the resin. Both strategies are general and can be applied to the synthesis of nucleopeptides having backbones formed by any other diamino acid moiety decorated with the four nucleobases. We also report the CD and UV studies on the hybridization properties of the obtained nucleopeptide, containing all four nucleobases on alternate lysines in the sequence, towards complementary DNA and RNA strands. The nucleo-oligolysine with a mixed-base sequence did not prove to bind complementary DNA, but was able to recognize the complementary RNA forming a complex with a higher melting temperature than that of the corresponding RNA/RNA natural duplex and comparable with that of the analogous PNA/RNA system.

Introduction

Several artificial oligonucleotide analogues have been recently introduced in the literature, serving *e.g.* as antisense agents.[1,2] One of the most successful in that role, PNA (peptide nucleic acids),[3] is however plagued by poor water solubility and a tendency to self-aggregate. These limits can be overcome by adding L-lysine units at the PNA *termini* or introducing charged units (*e.g.* arginine) into the PNA backbone.[4] This feature, along with the possibility to promote electrostatic interactions with the phosphate moieties of the nucleic acids, recently inspired us to replace the PNA backbone with an oligo-α-L-lysine backbone,[5,6] in which the L-lysine units are decorated on every second residue with carboxymethyl-thymine moieties, thus leading to cationic oligomers, well soluble in water and aqueous buffers. Other examples of nucleic acid analogs based on nucleobase-bearing α-L-lysine units, similar to those employed in our works, were also described by Wada *et al.*[7] who found them able to bind complementary RNA strands.

Our previous studies on the thymine-based oligolysine suggested that base-driven interactions have a major role in the nucleic acid-binding, with respect to the expected electrostatic interactions. Furthermore, CD titrations with both homoadenine DNA and RNA sequences gave a 1 : 1 base/base complex stoichiometry, on the contrary, upon replacing the undecorated lysine spacers between two adjacent thymine-functionalized residues with L-arginine residues, complexes characterised by a 2 : 1 T/A binding stoichiometry were observed.[8]

Nevertheless, these findings did not enable us to obtain detailed information on the nature of the complexes formed between the homothymine oligolysine and the homoadenine nucleic acids. To this purpose, specific hybridization experiments with mixed-sequence nucleopeptides were required to prove the ability of this oligomer to recognize complementary DNA or RNA sequences by sequence-specific hybridization, not aided by the possible formation of a ternary complex stabilized by Hoogsten-type recognition, as in the case of PNA triplexes typically formed when homopurine and homopyrimidine strands are mixed.[9]

In this context, we designed and realized an oligomer with an oligo-α-L-lysine backbone bearing all the four nucleobases, and here reported the CD and UV-binding experiments with complementary DNA and RNA strands. We also described two alternative synthetic approaches for the preparation of

[a]*CNR, Istituto di Biostrutture e Bioimmagini, Via Mezzocannone 16, 80134 Napoli, Italy. E-mail: giovanni.roviello@cnr.it; Fax: +39-81-2534574; Tel: +39-81-2534585*
[b]*Università di Napoli "Federico II", Dipartimento di Scienze Chimiche, 80126 Napoli, Italy. E-mail: domenica.musumeci@unina.it; Tel: +39-81-674143*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6ra08765e

‡ These authors contributed equally to this work.

nucleobase-bearing oligo-α-L-lysines with mixed-base sequences, which could be easily extended to other nucleopeptides with the backbone based also on different amino acids. One of the synthetic routes is based on the solid-phase oligomerization of the nucleo-lysine monomers, *ad hoc* synthesized, coupled in alternate manner with respect to underivatized lysines. The second strategy involves the *on-line* assembly of both the oligolysine backbone and the suitably protected carboxymethyl-nucleobases directly on the solid support, thus avoiding some synthetic and chromatographic steps for the obtainment of the nucleoamino acid monomers.

Results and discussion

Nucleobase-amino acid conjugates, such as nucleoamino amides and nucleopeptides,[10–15] are interesting molecular systems endowed with several biological properties including the ability to interact with nucleic acids. Among others, a very important biological target is poly(A), an important RNA target involved in several biological processes,[16] whose ligands show high potential as therapeutics and are, thus, extensively investigated.[17–20] Diamino acid-based nucleopeptides with homonucleobase sequences are easily obtainable by solid-phase peptide synthesis using protected nucleoamino acid monomers (*ad hoc* synthesized being generally not commercially-available),[5] or by first assembling the desired peptide backbone using *e.g.* Dde/Fmoc protected monomers, followed by final nucleobase decoration, after selective Fmoc removal, using a molar excess of the suitable nucleobase acetic acid.[21] For both strategies, properly protected amino acids can be introduced, whose side-chain groups can be regenerated after the insertion of the bases, in order to have a spacer between two consecutive nucleobase-functionalized amino acids, as well as useful moieties such as the guanidinium or ammonium groups.

The synthesis of a mixed-sequence nucleopeptide is more complex than that of a homosequence oligomer requiring four different, non-commercially available monomers that have to be *ad hoc* synthesized.

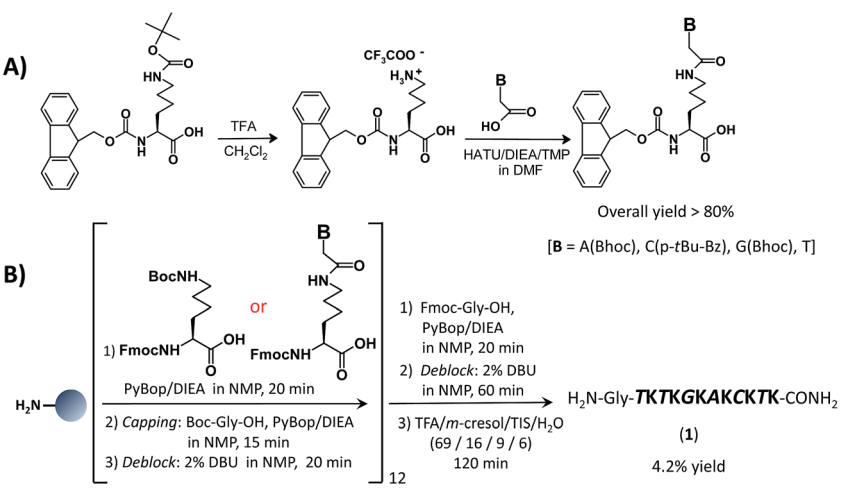
Synthesis of the Fmoc-protected nucleolysines and their use for the nucleopeptide synthesis

In the present study we first realized the new A, C and G nucleolysines (see Fig. S1†) adopting the same synthetic strategy we previously used to obtain the thymine monomer[5] (Scheme 1A). The four nucleoamino acids have been then used in the oligomerization of a mixed-sequence oligolysine by solid-phase peptide synthesis using PyBOP/DIEA as activating system (Scheme 1B).

The LC ESI-MS profiles for the nucleoamino acids and nucleopeptide **1**, both well soluble in aqueous solutions, are showed in Fig. S2 (ESI†) and Fig. 1, respectively. However, the yield for this oligomerization was low (less than 5%) since the coupling of the A, C, G nucleoamino acids was more difficult than with unfunctionalized lysine and thymine nucleoaminoacid, probably due to the steric hindrance caused by the nucleobase protecting groups.

In summary, the above-described synthetic approach had two major disadvantages: (1) it required the synthesis and purification of all the four nucleolysines, and (2) afforded the desired nucleopeptide in low yield. For this reason, special efforts have been subsequently devoted to the design of a more convenient and less time-demanding strategy for the realization of the heteronucleobase-functionalised nucleopeptide (Fig. S3†). Thus, we developed a solid-phase synthetic procedure using as starting materials the carboxymethyl-nucleobases, protected with acid-labile groups on the aromatic amino groups, and two different protected lysine monomers, *i.e.* the commercially available Fmoc-L-Lys(Boc)-OH and Dde-L-Lys(Fmoc)-OH. The proper nucleobase sequence was obtained by an *on-line* solid-phase strategy. More in detail, first, the lysine monomer with the Boc semi-permanent protecting group on its side chain, was anchored onto the amino group of the Rink-amide resin (step *a*, Scheme 2); then, after Fmoc-removal, the coupling with the Dde-Lys(Fmoc)-OH was accomplished (step *b*, Scheme 2).

Successively, a rapid treatment with 2% DBU in DMF allowed the selective removal of the Fmoc group from the lysine side



Scheme 1 Synthetic schemes for the preparation of (A) the nucleoamino acid monomers and (B) the nucleopeptide of mixed-base sequence **1**.

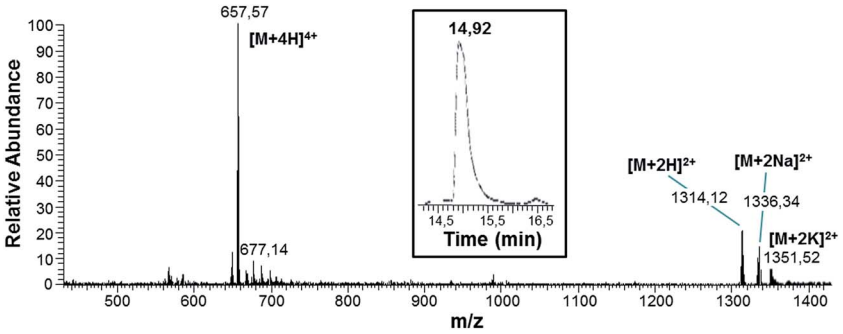
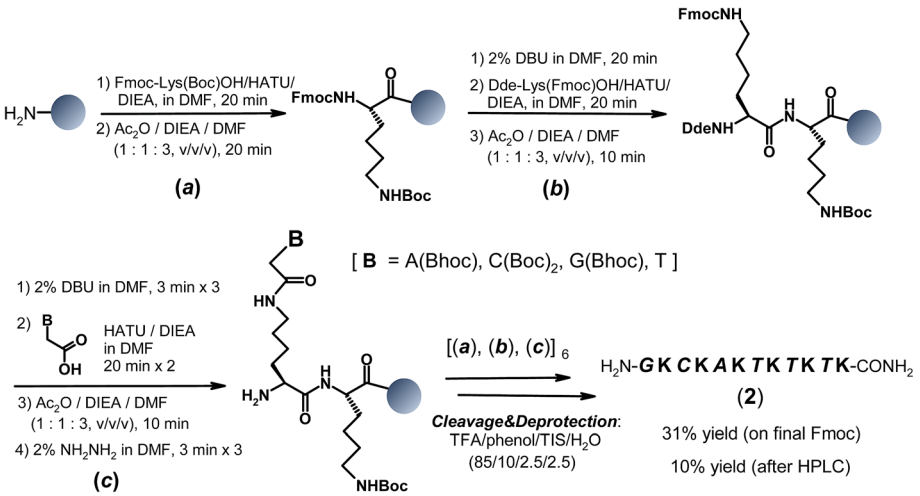


Fig. 1 LC-ESIMS (positive ions) of nucleopeptide NH_2Gly-*TKTKGKAKCKTK*-$CONH_2$ (**1**) (RP C18 column, HPLC method: 5 to 40% of B in A over 10 minutes).



Scheme 2 Schematic representation of the *on-line* synthetic route to the oligolysine nucleopeptide of mixed-base sequence **2**.

chain in the presence of the Dde protection: the obtained free ε-amino group was then coupled with the desired carboxymethyl-nucleobase (step *c*, Scheme 2). Removal of Dde from the lysine α-amino group was achieved by treating the resin with a 2% hydrazine solution in DMF in order to perform the nucleopeptide-chain elongation. Steps *a*, *b* and *c* were repeated until the nucleopeptide with the desired base sequence was obtained. Following this protocol, a dodecapeptide oligolysine containing six nucleobases (**2**, Scheme 2) was achieved with a 31% overall yield, in terms of the peptide backbone (as determined by UV measurements of the released Fmoc groups). Since the efficiency of the single nucleobase insertion cannot be determined *on-line* during the solid-phase synthesis, two consecutive couplings were performed to assure the best yields. After deprotection and cleavage of nucleopeptide **2** from the solid support with concentrated TFA, precipitation in cold diethyl ether and lyophilisation of the redissolved crude, HPLC purification on a RP-C18 column allowed to recover about 0.9 μmol of the purified product, *i.e.* 10% of the initial functionalization. ESI-MS characterization confirmed the identity of the desired nucleopeptide, which was well soluble in water as expected from the initial design (see Fig. 2).

The synthetic strategy reported in Scheme 2 is general and can be adapted to obtain other nucleopeptides with mixed nucleobase sequences by using diamino acids (or other amino acids as spacers) with properly protected amino groups (possibly commercially available) and the protected carboxymethyl-nucleobases. In this way, tedious purification steps relative to the synthesis of the nucleoaminoacid monomers can be avoided.

CD and UV spectroscopic studies

Subsequently, we studied by CD and UV spectroscopies the interaction of **2** with complementary DNA and RNA single strands. To this purpose, a two-chamber quartz cell (tandem cell) was used with each of the two reservoirs containing separately the nucleopeptide and the nucleic acid (AAAT[U]GC, DNA or RNA) solution at pH 7.5 and 5 °C. First, the CD profile for the two separate solutions was recorded (Fig. 3A and S4,† black lines).

Successively, we recorded the CD spectrum after mixing the two solutions, (Fig. 3A and S4,† red lines). In case of DNA no appreciable change in the CD profile upon nucleopeptide addition was observed (Fig. S4A†), as it would have been expected in the case of binding with DNA. On the contrary,

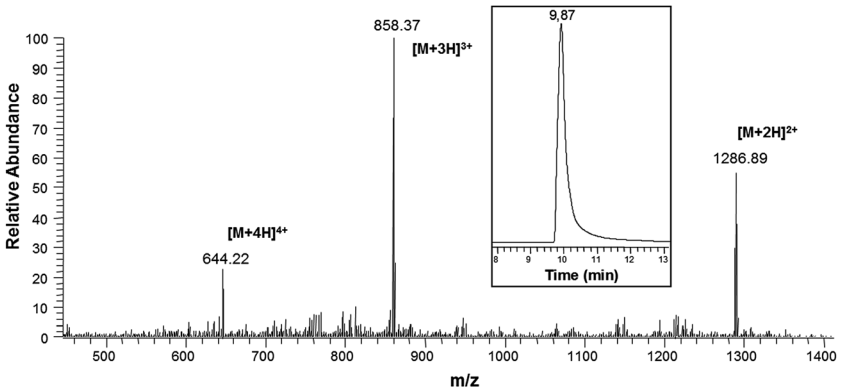


Fig. 2 LC-ESIMS (positive ions) of nucleopeptide **2** (RP C18 column, HPLC method: 5 to 30% of B in A over 15 min) NH_2-*GKCKAKTKTKTK*-$CONH_2$.

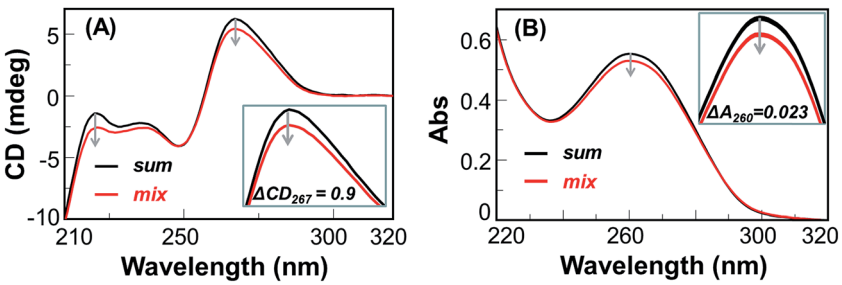


Fig. 3 CD (A) and UV (B) profiles relative to **2** and complementary RNA solutions before (black lines) and after (red) mixing, both at a 8 μM concentration in 10 mM phosphate buffer, pH 7.5, at 5 °C (total volume after mixing = 2 ml).

changes in both shape and intensity (ΔCD_{267} = 0.88 mdeg) of the CD spectrum with respect to the *sum* spectrum were observed in case of the nucleopeptide–RNA binding assay, as showed in Fig. 3A. Analogously, also *sum* and *mix* UV spectra were different in this case (Fig. 3B), and more in detail, a hypochromic effect was observed upon mixing the two ligand solutions, proving that the interaction with the nucleopeptide involved both H-bonding and stacking effects between complementary nucleobases. On the contrary, *sum* and *mix* UV-spectra in the case of the binding experiment with DNA were almost superimposable (data not shown) (Fig. 4).

Furthermore, UV thermal denaturation experiment on the nucleopeptide–RNA complex evidenced a sigmoid-like curve with a T_m of about 26 °C consistent with the nucleopeptide–RNA binding. Remarkably, this T_m is about 10 °C higher than the corresponding melting temperature expected for r(GCAUUU)-r(AAAUGC) and d(GCATTT)-r(AAAUGC) double helices, as estimated by using Oligo-Calculator software (http://biotools.nubic.northwestern.edu/OligoCalc.html). Instead, no cooperative transition was observed in the UV-monitored melting of the system nucleopeptide/DNA (Fig. S4B, ESI†). In addition, a standard PNA of the same sequence as the nucleo-oligolysine (*i.e.* NH_2-gcattt-$CONH_2$) was used as reference for the stability of the RNA complex: UV-melting experiments evidenced a T_m of 28 °C for the PNA/RNA system (Fig. S5 and ESI†), which is similar to that relative to the nucleopeptide/RNA complex.

Finally, we conducted an analogous binding study with a double-stranded DNA (see Fig. S6, ESI†). Upon nucleopeptide addition, the CD spectrum of the duplex was not altered, thus showing that neither strand displacement nor formation of Hoogsteen base pairs occurred. Furthermore, UV thermal denaturation studies showed no variation of its melting temperature (data not shown).

Overall, our findings suggest that: (i) nucleo-oligolysine is able to induce conformational changes upon binding the complementary RNA strand; (ii) the structure of the formed complex is stabilized by W–C base pairs that lead to a duplex structure with stability higher than the corresponding natural counterparts (RNA/RNA and DNA/RNA) and similar to the analogous PNA/RNA system; (iii) the heteronucleobase

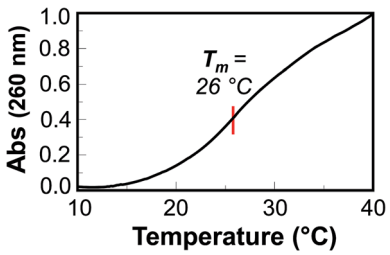


Fig. 4 UV thermal denaturation curve relative to nucleopeptide **2**/RNA complex (6.7/1.5 μM respectively, in 10 mM phosphate buffer–pH 7.5; V = 3 ml).

oligolysine nucleopeptide does not alter the DNA CD bands in the here-studied conditions nor form complexes with DNA with cooperative transition upon UV-monitored melting. The latter result is apparently in contrast with our previous findings for the homothymine oligomer, in which the complex with the homoadeninic DNA was probably based on a more stable triple helix structure (involving both W–C and Hoogsteen base pairings). In any case, the ability to selectively bind RNA, discriminating complementary DNA sequences, is commonly regarded as a highly desirable feature for antisense applications. In fact, we suggest that our antisense nucleopeptide could prove beneficial in those cases in which selectivity in binding RNA and not DNA tracts is required.

Experimental section

Abbreviations

Ac_2O (acetic anhydride); Bhoc (benzhydryloxycarbonyl); CD (Circular Dichroism); DBU (diazabicyclo[5.4.0]-undec-7-ene); Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-ethyl); DIEA (*N*,*N*-diisopropylethylamine); DMF (dimetilformammide); Fmoc (9-fluorenylmethoxycarbonyl); HATU (*O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); *N*-Boc Gly (*N*-(*tert*-butoxycarbonyl)glycine); NMP (4-methylpyrrolidone); NMR (Nuclear Magnetic Resonance); PyBop (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate); TFA (trifluoroacetic acid); TIS (triisopropyl silane); TMP (2,4,6-trimethylpyridine).

Chemicals

Dde-L-Lys(Fmoc)-OH, Fmoc-L-Lys(Boc)-OH, HATU, PyBOP were purchased from Novabiochem. Anhydroscan DMF and NMP were purchased from LabScan. Piperidine was Biosolve. Solvents for HPLC chromatography and acetic anhydride were purchased from Reidel-de Haën. TFA and TMP and Rink-amide resin were from Fluka. RNA and DNA oligomers in single strand form of sequence AAATGC, were purchased from Biomers. CH_2Cl_2, DIEA and TFA (for HPLC) were from Romil. Thyminyl acetic acid, Boc-Gly-OH, *m*-cresol, DBU, diethyl ether, hydrazine solution and TIS were purchased from Sigma-Aldrich. (N^4-bis-Boc-cytosine-1-yl)-acetic acid was synthesised as described by Wojciechowski and Hudson.[22] Bhoc-protected adenine and guanine as well as *p*-(*tert*-butyl)benzoyl-cytosine acetic acids were from ASM Research Chemicals GmbH and Co.

Apparatus

Samples were analysed and characterized by LC-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 Proteo column (4 μm, 90 Å, 4.6 × 150 mm). Gradient elution was performed by building up a gradient starting with buffer A (0.05% TFA in water) and applying buffer B (0.05% TFA in acetonitrile) with a flow rate of 0.8 ml min^{-1} and monitoring at 260 nm.

MALDI-TOF mass spectrometric analysis of the reference PNA was performed on a TOF/TOF 5800 System in positive mode, using 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Analytical chromatograms were obtained on a Hewlett Packard/Agilent 1200 series HPLC, equipped with a diode array detector, by using a Phenomenex Jupiter C18 column (5 μm, 300 Å, 4.6 × 250 mm). Gradient elution was performed at 25 °C 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 1.0 ml min^{-1} and monitoring at 260 nm. Semi-preparative purifications were performed on a Hewlett Packard/Agilent 1100 series HPLC, equipped with a diode array detector, by using a Phenomenex Jupiter C18 column (10 μm, 300 Å, 10 × 250 mm). Gradient elution was performed at 25 °C 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 4.0 ml min^{-1} (monitoring at 260 nm). Samples were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. Circular dichroism (CD) studies were conducted in analogy to other previous reports.[23–25] CD spectra were obtained at 5 °C on a Jasco J-715 spectropolarimeter with a Tandem Hellma quartz cell. UV spectra and UV thermal denaturation profiles were obtained 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.

Synthesis of Fmoc-protected nucleoamino acids

The synthesis of the Fmoc-protected T monomer was performed according to our previous report,[5] and by analogy with this procedure we here report also the preparation of the A, C and G nucleoamino acids using for the *N*-ε derivatization of the lysine moiety the commercially available nucleobase acetic acids [B = A(Bhoc), T, C(*p*-*tert*-butyl benzoyl) or G(Bhoc)]. Indeed, after Boc deprotection of the Fmoc-Lys(Boc)-OH, the obtained Fmoc-protected diamino hexanoic acid (175 mg, 0.47 mmol) was dissolved in anhydrous NMP (2.0 ml), treated with DIEA (0.9 equiv., 83 μl, 0.42 mmol) and TMP (0.6 equiv., 42 μl, 0.28 mmol), and reacted with BCH_2COOH (2.2 equiv., 1.03 mmol), previously preactivated by addition of HATU (2.0 equiv., 372 mg, 0.94 mmol) and DIEA (2.0 equiv., 175 μl, 0.94 mmol)/TMP (2.0 equiv., 140 μl, 0.94 mmol) in NMP (2 ml) for 2 min. After 1.5 h, the solvent was removed under vacuum and the crude was purified by RP-HPLC (RP C18 column, HPLC method: 25 to 70% of B′ in A′ over 25 min, t_R ~28 min). Yields were higher than 80% for all four monomers.

A nucleolysine. LC-ESIMS (Fig. S2†) *m*/*z*: 711.04 (found), 710.82 (expected for $[C_{42}H_{39}N_7O_7 - CO_2 + H]^+$); 755.66 (found), 754.83 (expected for $[C_{42}H_{39}N_7O_7 + H]^+$); 777.96 (found), 776.81 (expected for $[C_{42}H_{39}N_7O_7 + Na]^+$).

G nucleolysine. LC-ESIMS (Fig. S2†) *m*/*z*: 727.86 (found), 726.82 (expected for $[C_{42}H_{39}N_7O_8 - CO_2 + H]^+$); 770.41 (found), 770.83 (expected for $[C_{42}H_{39}N_7O_8 + H]^+$).

C nucleolysine. LC-ESIMS (Fig. S2†) *m*/*z*: 681.88 (found), 680.79 (expected for $[C_{38}H_{41}N_5O_7 + H]^+$); 702.74 (found), 702.77 (expected for $[C_{38}H_{41}N_5O_7 + Na]^+$).

T nucleolysine. LC-ESIMS (Fig. S2†) *m*/*z*: 313.91 (found), 314.34 (expected for $[C_{28}H_{30}N_4O_7 - Fmoc + H]^+$); 492.57 (found), 491.57 (expected for $[C_{28}H_{30}N_4O_7 - CO_2 + H]^+$); 536.90 (found), 535.58 (expected for $[C_{28}H_{30}N_4O_7 + H]^+$); 558.35 (found), 557.56 (expected for $[C_{28}H_{30}N_4O_7 + Na]^+$).

Solid-phase synthesis of oligolysine nucleopeptides

Route 1. The cationic nucleopeptide **1** (NH_2Gly-*TKTKGKAKCKTK*-$CONH_2$) was assembled on Rink-amide MBHA resin (0.54 mmol g^{-1}, 3.7 mg) by a Fmoc synthetic strategy similarly to other literature examples,[26–28] using the four Fmoc-protected nucleoamino acid monomers, prepared as described above, and classical lysine amino acid [Fmoc-Lys(Boc)-OH]. More in detail, after Fmoc removal by treatment with 25% piperidine in DMF for 30 min, a mixture of the nucleobase monomer (A, T, C or G, 150 μl of a 40 mM solution in NMP, 6 μmol, 3 equiv.), PyBOP (150 μl of a 80 mM solution in NMP, 12 μmol, 6 equiv.) and DIEA (4 μl, 24 μmol, 12 equiv.), or alternatively a mixture of Fmoc-Lys(Boc)-OH (150 μl of a 0.31 M solution in NMP, 46 μmol, 23 equiv.) with PyBOP (150 μl of a 0.32 M solution in NMP, 48 μmol, 24 equiv.) and DIEA (16 μl, 96 μmol, 48 equiv.) was introduced in the reactor and stirred at room temperature. After 20 min the liquid phase was removed from the resin which was washed with DMF, and, subsequently, the unreacted amino groups were capped by treatment with *N*-Boc glycine (45 μmol, 0.1 M solution)/PyBOP (48 μmol)/DIEA (16 μl, 96 μmol, 48 equiv.) in DMF for 15 min. The Fmoc group was removed by treatment with 2% DBU in NMP for 20 min. The above-described procedure for the sequential introduction of the underivatized lysines and nucleoamino acid residues was repeated 6 times. The cleavage from the resin and deprotection of the nucleopeptide was performed after final glycine insertion (Scheme 1) by treatment with 1.5 ml of TFA/*m*-cresol/TIS/H_2O (69/16/9/6, v/v/v/v) solution for 120 min. After precipitation from cold diethyl ether, the sample redissolved in milliQ H_2O, was purified by RP-HPLC using a linear gradient of 10% (for 5 min) to 30% B′ in A′ over 30 min: $t_R = 5.1$ min. The purified product was dissolved in a known amount of milliQ H_2O and quantified by UV absorption at 260 nm by using the molar extinction coefficients of the A, T, C, and G PNA monomers ($\varepsilon_{260ACGT3}$ = 57 800 M^{-1} cm^{-1}): 84 nmol of nucleopeptide **1** (4.2% yield) were obtained; the compound was ≥95% pure by HPLC analysis. ESIMS *m*/*z*: 681.41 (found), 681.02 (expected for $[C_{114}H_{183}N_{49}O_{30} + 4H]^{4+}$); 907.41 (found), 907.69 (expected for $[C_{114}H_{183}N_{49}O_{30} + 3H]^{3+}$); 1361.07 (found), 1361.03 (expected for $[C_{114}H_{183}N_{49}O_{30} + 2H]^{2+}$).

Route 2. The cationic nucleopeptide **2** (NH_2-*GKCKAKTKTKTK*-$CONH_2$) was assembled on Rink-amide MBHA resin using a Fmoc synthetic strategy. In detail, 19.8 mg of resin (10.7 μmol of amino groups; functionalization: 0.54 mmol g^{-1}) were treated with 25% piperidine in DMF for 30 min for the Fmoc removal, and then the coupling with a solution of Fmoc-Lys(Boc)-OH (35.1 mg, 74.9 μmol, 7 equiv.)/HATU (27.6 mg, 72.8 μmol, 6.8 equiv.)/DIEA (26.0 μl, 149.8 μmol, 14 equiv.) in DMF (0.5 ml), pre-activated separately for 2 min, was performed over 20 min at room temperature. After capping of the unreacted NH_2 groups with a solution of Ac_2O/lutidine/DMF (2/1/7, v/v/v) for 2 × 10 min, Fmoc removal was accomplished with a 2% solution of DBU in DMF for 2 × 10 min and monitored spectrophotometrically. Subsequently, the coupling with the lysine monomer Dde-Lys(Fmoc)-OH, followed by the capping step, was performed as previously described. Then, a rapid treatment with a 2% solution of DBU in DMF (3 × 3 min, alternating washing steps) allowed the selective removal from the side chain of the Fmoc group which was quantified by UV measurements. Free amino groups on the resin were coupled with the desired nucleobase acetic acid [T-CH_2COOH, A(Bhoc)-CH_2COOH, C(Boc)$_2$–CH_2COOH, G(Bhoc)–CH_2COOH, 7 eq.], preactivated for 2 min with HATU (7 eq.)/DIEA (14 equiv.) in DMF, for 20 min at room temperature: a double coupling was performed in all cases. Then acetylation of the unreacted side chain amino groups was performed with Ac_2O/lutidine/DMF (2/1/7, v/v/v) for 2 × 5 min. Removal of the Dde groups was performed with a 2% NH_2NH_2 solution in DMF for 3 × 3 min. The above-described procedure for the sequential introduction of the two kinds of lysines followed by the nucleobase coupling was repeated 6 times, until the following protected oligomer attached to the resin was obtained: ResinNH-[Lys(Boc)-Lys(T)]-[Lys(Boc)-Lys(T)]-[Lys(Boc)-Lys(T)]-[Lys(Boc)-Lys(A^{Bhoc})]-[Lys(Boc)-Lys(C^{Boc2})]$_5$-[Lys(Boc)-Lys(G^{Bhoc})]$_6$-NH_2 (from C to N terminus). A 31% overall yield in terms of the peptide backbone was determined by UV measurements of the Fmoc groups released. The cleavage from the resin and deprotection of the nucleopeptide was performed by treatment with 1 ml of TFA/phenol/TIS/H_2O (85/10/2.5/2.5, v/v/v/v) for 90 min, followed by another 1 h treatment with 0.5 ml of the same solution. After TFA evaporation under a N_2 flow and precipitation from cold diethyl ether, the crude pellet was resuspended in milliQ H_2O and lyophilised. Then the crude sample was dissolved in A′ solution and purified by RP-HPLC using a linear gradient of 5% (for 5 min) to 30% B′ in A over 30 min: $t_R = 15.2$ min. UV quantification, performed by dissolving the purified product in a known amount of milliQ H_2O and measuring the absorbance at 260 nm, gave 0.9 μmol of the nucleopeptide **2** (10% yield); the compound was ≥95% pure by HPLC analysis. ESIMS *m*/*z*: 644.22 (found), 643.75 (expected for $[C_{113}H_{180}N_{44}O_{26} + 4H]^{4+}$); 858.37 (found), 858.00 (expected for $[C_{113}H_{180}N_{44}O_{26} + 3H]^{3+}$); 1286.89 (found), 1286.49 (expected for $[C_{113}H_{180}N_{44}O_{26} + 2H]^{2+}$).

PNA synthesis

Reference PNA (NH_2-gcattt-$CONH_2$) was obtained manually under standard conditions[29–31] on a 2 μM scale. The identity of the product was checked by MALDI-TOF MS: *m*/*z*: 1648.74 (found), 1648.63 (expected for $[C_{66}H_{86}N_{32}O_{20} + H]^+$); 1671.68 (found), 1670.61 (expected for $[C_{66}H_{86}N_{32}O_{20} + Na]^+$).

Conclusions

In the present work two solid-phase syntheses of cationic nucleopeptides based on L-lysine containing all the four bases were described. In one case, we first synthesized in solution all the four nucleoamino acid monomers by a simple route, and

then assembled them in the solid-phase, using Fmoc-based peptide synthesis, by alternate coupling with respect to a standard Fmoc-Lys(Boc)-OH. In the second approach, in order to skip the solution synthesis of the four nucleobase-containing monomers, we developed a synthetic strategy in which the nucleobase-decoration took place *on-line* directly on the peptide chain growing on the solid support: in this case two kinds of differently protected lysines were coupled in alternate manner to the resin [*i.e.* the Fmoc-Lys(Boc)-OH and Dde-Lys(Fmoc)-OH] and the selective removal of the Dde protecting group in the presence of Fmoc was employed for the introduction of the carboxymethyl-nucleobases. The nucleopeptides obtained with both the synthetic strategies were analysed by LC-ESI MS which confirmed the identity of the expected products. These were well soluble in aqueous solutions and did not show any tendency to self-aggregate. The here described synthetic routes for the realization of the nucleo-oligolysines can be easily applied to the synthesis of other nucleopeptides with mixed base sequences starting from any other diamino carboxylic acid to be functionalised with the nucleobases, and using in combination any other natural or synthetic amino acid, possibly charged (*e.g.* L-arginine), as spacer unit.

Furthermore, we studied the binding of the nucleo-oligolysine with mixed-base sequence towards complementary DNA and RNA strands by CD and UV spectroscopies. In the case of DNA we did not observe any significant change of the DNA CD spectrum induced by the nucleopeptide nor effects in the UV absorption spectra, *i.e.* the nucleopeptide did not determine any important structural rearrangement of the nucleic acid. On the other side, we found that the studied nucleopeptide bound a complementary RNA strand provoking changes in the CD spectrum of RNA. Furthermore, UV-thermal denaturation studies demonstrated that the nucleopeptide/RNA complex provided a sigmoid-like melting curve with a T_m higher than that expected in case of the corresponding RNA/RNA or DNA/RNA natural complexes. Overall, our nucleo-oligolysines seem to be promising antisense tools being able to discriminate between RNA *vs.* DNA complementary sequences, and forming with RNA stable complexes. Thus, all these findings encourage us to devote our future efforts on the development of molecular systems of the type described in the present study, but presenting also different nucleobase sequences and/or spacer amino acid units (*e.g.* having arginine in place of lysine spacers), as potential tools in biomedical strategies.

Acknowledgements

We thank Professors Antonio Roviello and Daniela Montesarchio (Federico II University, Naples – Italy) for their precious suggestions, and Mr Leopoldo Zona (IBB-CNR, Naples – Italy) for his invaluable technical assistance. We also thank Regione Campania for the research grant received for the research project "Nuovi sistemi nucleopeptidici per applicazioni diagnostiche" under the research program Legge Regionale n. 5 (annualità 2007).

References

1 J. H. Chan, S. Lim and W. S. Wong, *Clin. Exp. Pharmacol. Physiol.*, 2006, **33**, 533–540.
2 J. Singh, H. Kaur, A. Kaushik and S. Peer, *Int. J. Pharm.*, 2011, **7**, 294–315.
3 P. E. Nielsen, M. Egholm, R. H. Berg and O. Buchardt, *Science*, 1991, **254**, 1497–1500.
4 A. Dragulescu-Andrasi, S. Rapireddy, G. He, B. Bhattacharya, J. J. Hyldig-Nielsen, G. Zon and D. H. Ly, *J. Am. Chem. Soc.*, 2006, **128**, 16104–16112.
5 G. N. Roviello, D. Musumeci, C. D'Alessandro and C. Pedone, *Amino Acids*, 2013, **45**, 779–784.
6 G. N. Roviello, D. Musumeci, C. D'Alessandro and C. Pedone, *Bioorg. Med. Chem.*, 2014, **22**, 997–1002.
7 T. Wada, Y. Inaki and K. Takemoto, *J. Bioact. Compat. Polym.*, 1992, **7**, 25–38.
8 G. N. Roviello, C. Vicidomini, S. Di Gaetano, D. Capasso, D. Musumeci and V. Roviello, *RSC Adv.*, 2016, **6**, 14140–14148.
9 P. E. Nielsen, M. Egholm and O. Buchardt, *J. Mol. Recognit.*, 1994, **7**, 165–170.
10 G. N. Roviello, V. Roviello, I. Autiero and M. Saviano, *RSC Adv.*, 2016, **6**, 27607–27613.
11 G. N. Roviello, A. Ricci, E. M. Bucci and C. Pedone, *Mol. BioSyst.*, 2011, **7**, 1773–1778.
12 G. N. Roviello, S. Di Gaetano, D. Capasso, S. Franco, C. Crescenzo, E. M. Bucci and C. Pedone, *J. Med. Chem.*, 2011, **54**, 2095–2101.
13 G. N. Roviello, D. Musumeci, M. Moccia, M. Castiglione, R. Sapio, M. Valente, E. M. Bucci, G. Perretta and C. Pedone, *Nucleosides, Nucleotides Nucleic Acids*, 2007, **26**, 1307–1310.
14 L. Simeone, C. Irace, A. Di Pascale, D. Ciccarelli, G. D'Errico and D. Montesarchio, *Eur. J. Med. Chem.*, 2012, **57**, 429–440.
15 L. Simeone, D. Milano, L. De Napoli, C. Irace, A. Di Pascale, M. Boccalon, P. Tecilla and D. Montesarchio, *Chem.–Eur. J.*, 2011, **17**, 13854–13865.
16 M. Romano, R. van de Weerd, F. C. Brouwer, G. N. Roviello, R. Lacroix, M. Sparrius, G. van den Brink-van Stempvoort, J. J. Maaskant, A. M. van der Sar, B. J. Appelmelk, J. J. Geurtsen and R. Berisio, *Structure*, 2014, **22**, 719–730.
17 G. N. Roviello, V. Roviello, D. Musumeci and C. Pedone, *Biol. Chem.*, 2013, **394**, 1235–1239.
18 G. N. Roviello, D. Musumeci, V. Roviello, M. Pirtskhalava, A. Egoyan and M. Mirtskhulava, *Beilstein J. Nanotechnol.*, 2015, **6**, 1338–1347.
19 G. N. Roviello, D. Musumeci and V. Roviello, *Int. J. Pharm.*, 2015, **485**, 244–248.
20 A. S. Saghyan, H. M. Simonyan, S. G. Petrosyan, A. V. Geolchanyan, G. N. Roviello, D. Musumeci and V. Roviello, *Amino Acids*, 2014, **46**, 2325–2332.
21 G. N. Roviello, S. Di Gaetano, D. Capasso, A. Cesarani, E. M. Bucci and C. Pedone, *Amino Acids*, 2010, **38**, 1489–1496.
22 F. Wojciechowski and R. H. Hudson, *J. Org. Chem.*, 2008, **73**, 3807–3816.

23 P. L. Scognamiglio, C. Di Natale, M. Leone, M. Poletto, L. Vitagliano, G. Tell and D. Marasco, *Biochim. Biophys. Acta*, 2014, **1840**, 2050–2059.
24 C. Di Natale, P. L. Scognamiglio, R. Cascella, C. Cecchi, A. Russo, M. Leone, A. Penco, A. Relini, L. Federici, A. Di Matteo, F. Chiti, L. Vitagliano and D. Marasco, *FASEB J.*, 2015, **29**, 3689–3701.
25 C. Avitabile, L. Moggio, G. Malgieri, D. Capasso, S. Di Gaetano, M. Saviano, C. Pedone and A. Romanelli, *PLoS One*, 2012, **7**, e35774, DOI: 10.1371/journal.pone.0035774.
26 D. Comegna, I. de Paola, M. Saviano, A. Del Gatto and L. Zaccaro, *Org. Lett.*, 2015, **17**, 640–643.
27 L. L. Vezenkov, C. A. Sanchez, V. Bellet, V. Martin, M. Maynadier, N. Bettache, V. Lisowski, J. Martinez, M. Garcia, M. Amblard and J. F. Hernandez, *ChemMedChem*, 2016, **11**, 302–308.
28 E. Perillo, S. Porto, A. Falanga, S. Zappavigna, P. Stiuso, V. Tirino, V. Desiderio, G. Papaccio, M. Galdiero, A. Giordano, S. Galdiero and M. Caraglia, *Oncotarget*, 2016, **7**, 4077–4092.
29 G. N. Roviello, D. Musumeci, E. M. Bucci, M. Castiglione, A. Cesarani, C. Pedone and G. Piccialli, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 4757–4760.
30 F. Amato, R. Tomaiuolo, N. Borbone, A. Elce, J. Amato, S. D'Errico, G. De Rosa, L. Mayol, G. Piccialli, G. Oliviero and G. Castaldo, *Med. Chem. Commun.*, 2014, **5**, 68–71.
31 F. Amato, R. Tomaiuolo, F. Nici, N. Borbone, A. Elce, B. Catalanotti, S. D'Errico, C. M. Morgillo, G. De Rosa, L. Mayol, G. Piccialli, G. Oliviero and G. Castaldo, *BioMed Res. Int.*, 2014, 610718, DOI: 10.1155/2014/610718.