Analysis of Noncovalent Complexes between Human Telomeric DNA and Polyamides Containing *N*-Methylpyrrole and *N*-Methylimidazole by Using Electrospray Ionization Mass Spectrometry

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Abstract: Electrospray ionization mass spectrometry (ESI-MS) was used to investigate noncovalent complexes formed between four novel polyamides containing *N*-methylpyrrole (Py) and *N*-methylimidazole (Im), and human telomeric DNA. Of the four polyamides investigated, PyPyPyγImImImβDp (**3**) had the highest binding affinity towards the duplex d(TTAGGGTTAGGG/CCCTAACCCTAA) (D1). Results of competition analysis showed that the polyamides had binding affinities with D1 in the order PyPyPyγImImImβDp (**3**) > PyPyPyPyγPyImImPyβDp (**4**) > PyPyPyβImImImβDp (**2**) ≫ ImImImβDp (**1**). MS/MS spectra confirmed that binding between D1 and the hairpin polyamides is more stable than that with the three-ring polyamides. By contrast, in the case of single-stranded d(TTAGGGTTAGGG)(D2), the binding order changes to ImImImβDp (**1**) > PyPyPyγImImImβDp (**3**) > PyPyPyβImImImβDp (**2**).

Keywords: DNA recognition · human telomeric DNA · mass spectrometry · noncovalent interactions · polyamides

Introduction

Telomerase and telomeres are the focus of much research in the fields of cell immortality and cancer. Telomeres are guanine-rich sequences that possess several functions essential for genome integrity. Human telomeres consist of TTAGGG repeats and have single-stranded termini. The telomerase enzyme is capable of rebuilding the ends of telomeres by replacing these terminal sequences and has been shown to be active in 85–90% of human cancers, but inactive in healthy, somatic cells.[1–6] An active search is, therefore, underway for drugs that can bind to and stabilize telomeres, thus inhibiting telomerase activity.

Recently, polyamides containing *N*-methylpyrrole and *N*-methylimidazole have attracted the attention of synthetic and biological chemists, because of their ability to recognize and bind to the minor groove of DNA.[7,8] Since these polyamides can permeate living cell membranes, they have the potential to control specific gene expression.[9] We have developed a convenient method of polyamide synthesis by employing the haloform reaction and the DCC/HOBt coupling reaction in the solution phase (DCC = dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole).[10] By using this technique, four novel polyamides were designed and synthesized for the study of their recognition of and binding affinity towards human telomeric DNA (Scheme 1).

Within the last decade, electrospray ionization mass spectrometry (ESI-MS) led to a great development in the analysis of biomolecules,[11–13] for example, the analysis of complexes formed between drugs and DNA,[14–20] RNA,[21,22] and proteins.[12] Here, we report the analysis, by using ESI-MS, of the noncovalent interactions between polyamides and DNA molecules containing the TTAGGG sequence, the telomeric repeat in vertebrate somatic cells.

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Results and Discussion

Complexes between polyamides and a duplex oligonucleotide (D1): Following the annealing of two single-stranded oligonucleotides, the ESI-MS spectrum of the free duplex DNA solution was recorded and revealed five main ions at *m*/*z* 1177, 1251, 1457, 1766, and 1877. The ion $[duplex]^{5-}$ at *m*/*z* 1457 could be easily distinguished from ions of the two single-stranded oligonucleotides (*m*/*z* 1177, 1251, 1766, and 1877), confirming formation of the duplex in the annealing

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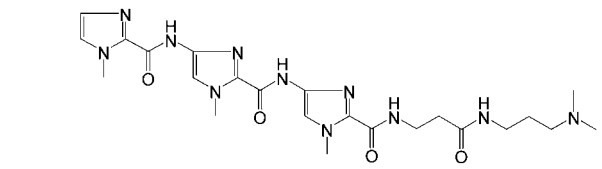
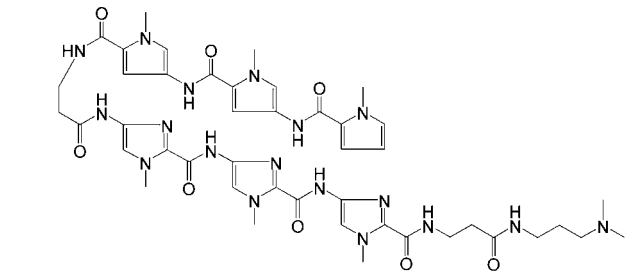


ImImImβDp (**1**)



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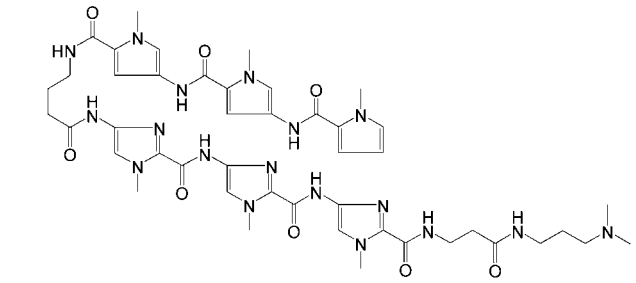
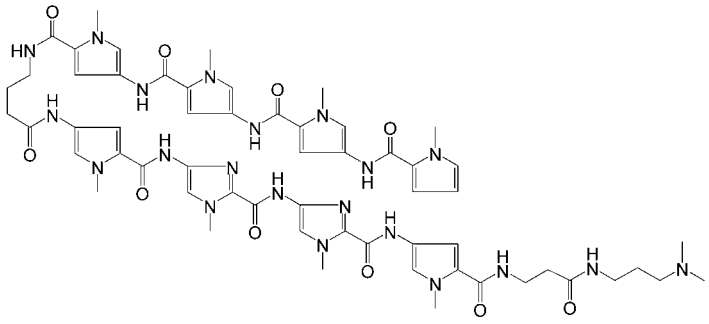


PyPyPyγImImImβDp (**3**)



PyPyPyPyγPyImImPyβDp (**4**)

Scheme 1. The four DNA-recognizing polyamides used in this study: Im = *N*-methylimidazole; Py = *N*-methylpyrrole; Dp = *N*,*N*-dimethylpropyldiamine, which increases the polarity of the polyamide; β = β-alanine, which increases polyamide–DNA binding affinity; γ = γ-aminobutyric acid, which facilitates the formation of a γ-turn.

process. Therefore, we chose to study mainly the ions with the 5− charge state, that is, $[duplex]^{5-}$.

Figure 1 shows the results of the binding of polyamides **1**–**4** with the duplex oligonucleotides in a 1:1 molar ratio. Table 1 summarizes the effect of the molar ratio of D1 to polyamides on binding, in which the relative abundance was normalized to 100% for each spectrum. In the case of ImImImβDp (**1**), since it targets the two GGG sites of D1, we found not only the 1:1 and 1:2 complexes (*m*/*z* 1562 and 1668, respectively), but also the higher molar ratio complexes $[D1+3P]^{5-}$ (D1/polyamide = 1:3, *m*/*z* 1773) and $[D1+4P]^{5-}$ (D1/polyamide = 1:4, *m*/*z* 1879). This corresponds to three or four molecules of **1** binding in the minor groove of the duplex DNA in a side-by-side, antiparallel fashion. For the six- or eight-ring polyamides, the most abundant ion in each spectrum could be assigned to the 1:1 complex (except for in the case of the highest molar ratio of polyamide to duplex). In addition, the signal of the duplex (*m*/*z* 1457) decreased dramatically as the concentration of the six- or eight-ring polyamides increased. This decrease was significantly greater than that observed for **1**, and demonstrates how the connection of the carboxyl and amino termini of two polyamides through either a γ-butyric acid linker or a β-alanine linker can greatly increase the DNA-binding affinity.[23]

Table 1. Effect of the molar ratio of D1 to polyamides on binding.

		1:1	1:2	1:4	1:8
	$[D1]^{5-}$	100.0	100.0	100.0	100.0
	$[D1+P]^{5-}$	30.6	56.7	68.8	89.5
1	$[D1+2P]^{5-}$	21.4	31.8	30.3	83.5
	$[D1+3P]^{5-}$	5.2	10.5	29.4	57.2
	$[D1+4P]^{5-}$	N.D.[a]	7.7	10.8	38.5
	$[D1]^{5-}$	89.5	31.2	N.D.[a]	N.D.[a]
2	$[D1+P]^{5-}$	100.0	100.0	100.0	100.0
	$[D1+2P]^{5-}$	N.D.[a]	N.D.[a]	10.0	185.9
	$[D1]^{5-}$	17.8	N.D.[a]	N.D.[a]	N.D.[a]
3	$\mathbf{[D1+P]^{5-}}$	**100.0**	**100.0**	**100.0**	**100.0**
	$[D1+2P]^{5-}$	N.D.[a]	12.4	72.7	134.5
	$[D1]^{5-}$	24.1	5.2	N.D.[a]	N.D.[a]
4	$[D1+P]^{5-}$	100.0	100.0	100.0	100.0
	$[D1+2P]^{5-}$	N.D.[a]	7.0	27.7	80.6

[a] N.D. = not detectable.

Comparison of the data for these six- or eight-ring polyamides shows that PyPyPyγImImImβDp (**3**) had the highest and PyPyPyβImImImβDp (**2**) the lowest binding affinity towards D1. Moreover, the data shows that 1:2 complexes of **2** (D1/polyamide **2**, *m*/*z* 1843) became predominant at the highest concentration used. This result may be explained as follows: for **2**, the β-linker is too short to enable the polyamide to fold into a hairpin motif and to fit into the minor groove of the DNA molecule. However, two molecules of **2** probably form a side-by-side, antiparallel motif; therefore, the formation of this 1:2 complex is preferred to that of the 1:1 complex. In the cases of PyPyPyγImImImβDp (**3**) and PyPyPyPyγPyImImPyβDp (**4**), it is very likely that their longer γ-linker enables them to bind to DNA in a hairpin-like conformation, conserving the side-by-side alignment of the Im and Py subunits. This also facilitates the antiparallel pairing of Im/Py, which targets a G–C base pair, and Py/Py, which recognizes either an A–T or a T–A base pair.[24–27] Therefore, compound **3**, which targets a GGG(A/T) sequence, was the optimal, matched binder for D1, whereas **2** and **4**, which target (A/T)(A/T)(A/T)(A/T)GGG and (A/T)GG(A/T)(A/T), respectively, were mismatched binders. The highest binding affinity towards D1 was, therefore, dem-

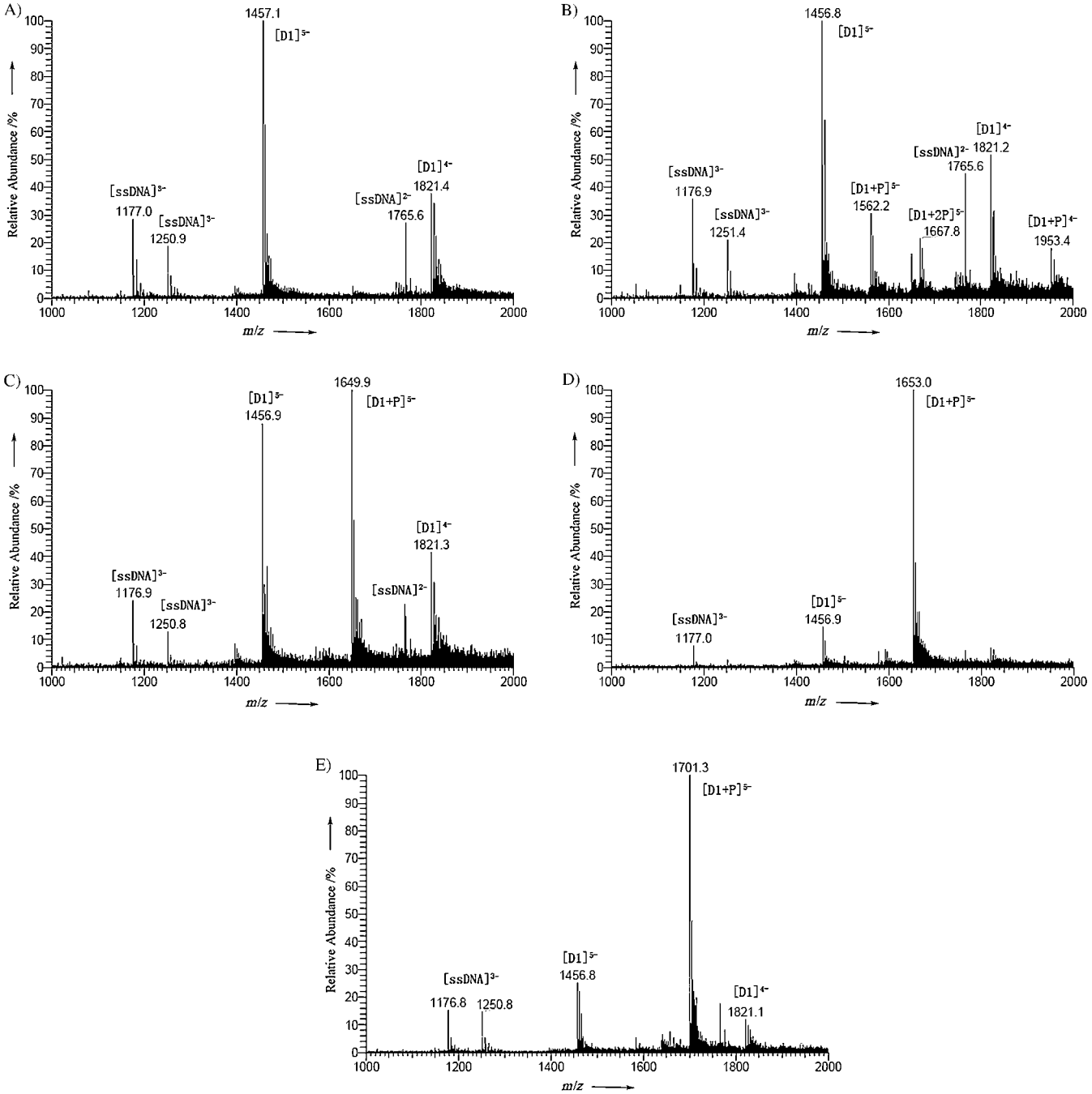


Figure 1. Negative-ion ESI mass spectra of the mixtures containing a 1:1 ratio of D1 to polyamide. A) Free D1; B) ImImImβDp (**1**)+D1; C) PyPyPyβImImImβDp (**2**)+D1; D) PyPyPyγImImImβDp (**3**)+D1; E) PyPyPyPyγPyImImPyβDp (**4**)+D1. (ss: single-stranded.)

onstrated by **3**. These results clearly indicate the relative binding affinities of the polyamides to be: **3** > **4** > **2** ≫ **1**.

Competition experiments of polyamides with the duplex oligonucleotide (D1): A competition study for polyamides **1**–**4** with D1 was performed to directly determine relative binding affinities. In the competition experiment between D1 and **1**, **3**, or **4** (Figure 2A), the 1:1 complex ion (*m*/*z* 1653) of **3** and D1 had the highest intensity (normalized to 100%), whereas the 1:1 complex ion (*m*/*z* 1701) of **4** and D1 represented a relative abundance of 42%, and the complex ion (*m*/*z* 1563) of **1** was not detected at all. In addition, we investigated the competition binding between polyamides **1**, **2**, and **4**. The results suggested that **4** had the highest intensity (normalized to 100%), whereas **2** had a relative abundance of 56%, and **1** had the lowest affinity, with a relative abundance of 11% (Figure 2B).

The results of these competition experiments are consistent with the aforementioned conclusions. Due to different motifs, base-pair binding sites, and sizes, the binding affini-

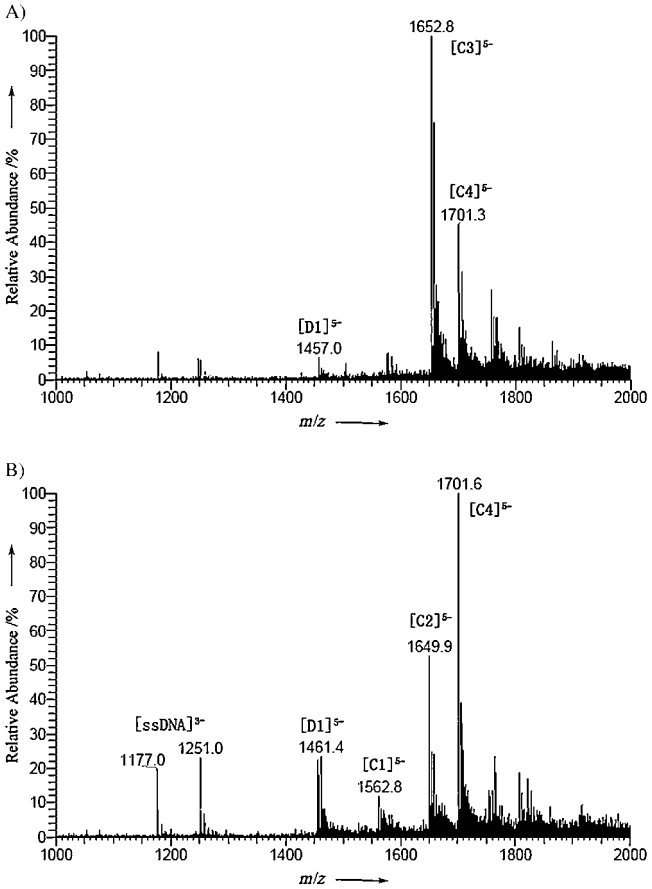


Figure 2. Competition study of polyamides with D1. Complexes of D1 and polyamides **1**–**4** are denoted C1–C4, respectively. A) D1 with polyamides **1**, **3**, and **4**; B) D1 with polyamides **1**, **2**, and **4**.

ties of the polyamides can be easily discriminated according to the specific duplex DNA sequence. As a result, the matched hairpin polyamide (**3**) binds the 5′-TTAGGG-3′ site with the highest affinity and sequence specificity, whereas one-base-mismatched polyamides (**2** and **4**) take second place, and **1** has the lowest binding affinity of the four polyamides. Moreover, because most of the duplex DNA was bound by hairpin polyamides, ions of 1:2, 1:3, and 1:4 complexes of the three-ring polyamide **1**, and even the 1:1 complex, were not observed (Figure 2A).

MS/MS spectra of complexes with D1: To investigate the fragmentation and stability of the complexes, the $[ds+P_n]^{5-}$ ions were activated by collision and the fragment ion spectra were recorded (Figure 3). The $[ds+P_1]^{5-}$ ion dissociated into two noncomplexed single strands and the duplex ion with a charge state of 5−, accompanied by the $[ds+P_1-G]^{5-}$ and $[ds-G]^{5-}$ ions (Figure 3A). However, in the cases of **2**, **3**, and **4**, we have not observed the duplex ion. Instead, the $[ds+P_n]^{5-}$ ions generated only single strands, $[ds+P_n-A]^{5-}$, $[ds+P_n-G]^{5-}$, and $[ss+P_n]^{3-}$ (Figure 3B–D, respectively).

The differences between **1** and polyamides **2**–**4** demonstrated in the MS/MS spectra indicate their distinct binding affinities. Polyamides **2**–**4** had much greater binding affinities than the three-ring polyamide **1**; therefore, as the complexes dissociated, the strong interactions between the DNA strands and polyamides **2**–**4** led to the initial loss of a base group, and the complexes of polyamides and D1 remained as single strands with polyamides. The size of the polyamides also played an important role in dissociation.

These results confirm that the binding between D1 and the hairpin polyamides is more stable than that with **1**.

Complexes between polyamides and single-stranded oligonucleotide (D2): We also studied the binding stoichiometry by mixing D2 (d(TTAGGGTTAGGG), monoisotopic mass = 3756.6) with polyamides **1**–**3** in molar ratios ranging from 1:1 to 1:8. Table 2 summarizes the effects of the molar ratio of D2 to polyamide on binding, in which the relative abundance was normalized to 100 % for each spectrum.

Table 2. Effect of the molar ratio of D2 to polyamides on binding.

		1:1	1:2	1:4	1:8
	$[D2]^{3-}$	100.0	100.0	100.0	100.0
	$[D2+P]^{3-}$	17	33.4	32.5	53.9
1	$[D2+2P]^{3-}$	8.5	14.3	23.2	61.0
	$[D2+3P]^{3-}$	N.D.[a]	N.D.[a]	12.3	21.1
	$[D2+4P]^{3-}$	N.D.[a]	N.D.[a]	N.D.[a]	41.1
	$[D2]^{3-}$	100.0	100.0	100.0	100.0
3	$[D2+P]^{3-}$	10.3	12.3	27.0	32.3
	$[D2+2P]^{3-}$	N.D.[a]	N.D.[a]	N.D.	28.3
	$[D2]^{3-}$	100.0	100.0	100.0	100.0
2	$[D2+P]^{3-}$	N.D.[a]	10.7	9.0	14.2
	$[D2+2P]^{3-}$	N.D.[a]	N.D.[a]	N.D.[a]	14.9

[a] N.D. = not detectable.

The relative abundances of the polyamide–D2 complexes were lower than those of the D1 complexes, and as the ratio of polyamide to D2 increased, the relative abundance of $[complex]^{3-}$ to $[D2]^{3-}$ slowly increased. For example, in the case of polyamide **1**, the relative abundance of complex $[D2+P]^{3-}$ (m/z 1427) to $[D2]^{3-}$ (m/z 1251) increased from 0.17 to 0.54 as the molar ratio increased from 1:1 to 1:8, and for **3**, the relative abundance of $[D2+P]^{3-}$ (m/z 1577) to $[D2]^{3-}$ increased from 0.10 to 0.32, as the molar ratio increased from 1:1 to 1:8.

The distinct behaviors of D1 and D2 are attributable to their different secondary structures. Since polyamides bind readily in minor grooves of DNA, which exist only in the duplex molecules, the complexes of D1 are much more stable than those of the single-stranded oligonucleotide D2. Furthermore, polyamides **3** and **1** matched the two GGG(A/T) sites of D2, and due to the size effect, **1** had a higher binding affinity than **3**. Polyamide **2** binds preferentially to the (A/T)(A/T)(A/T)(A/T)GGG sequence, which was not present in D2; consequently, **2** had the lowest binding affinity of the three polyamides. Therefore, the order of binding

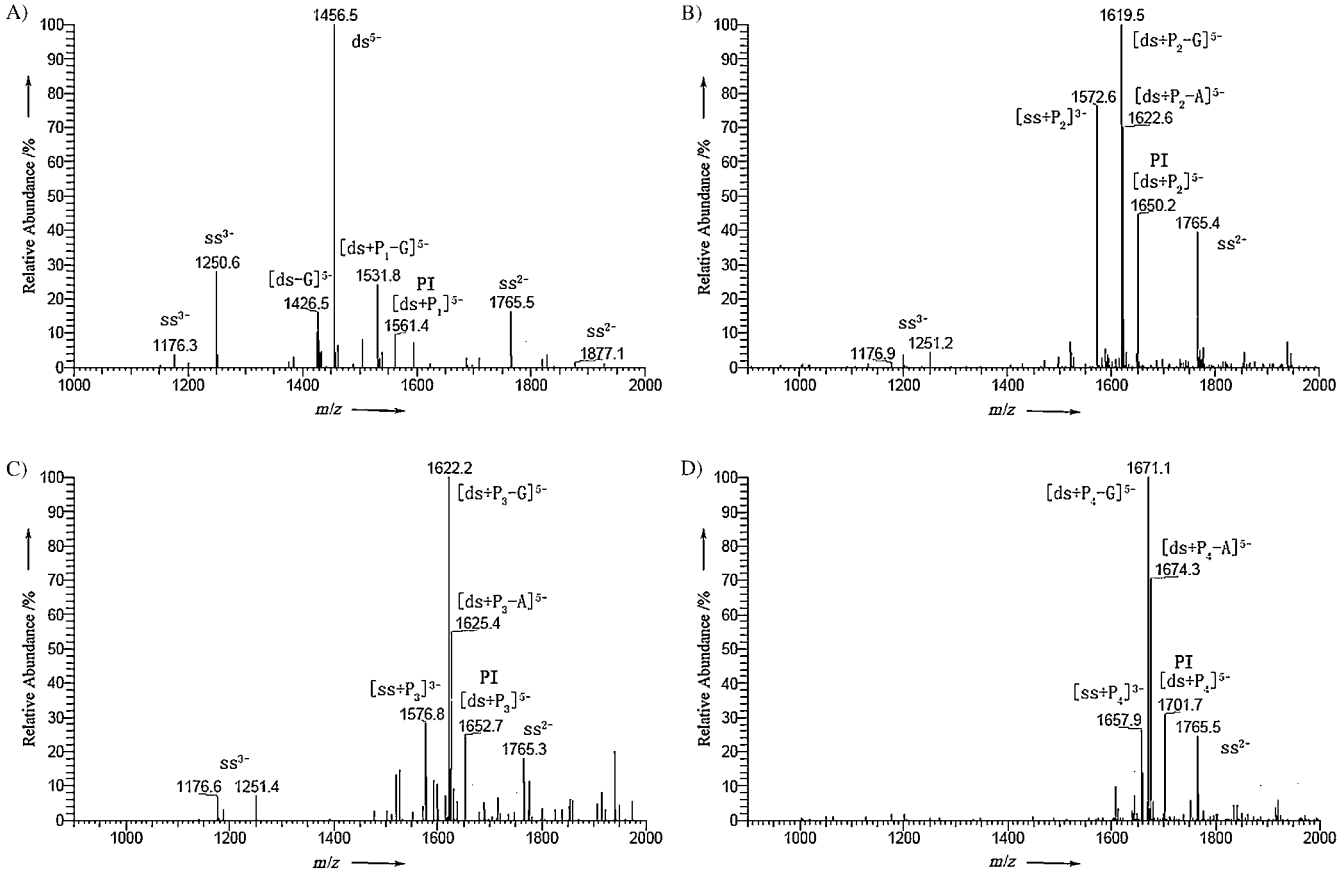


Figure 3. MS/MS spectra of complex ions $[ds+P_n]^{5-}$ for polyamides **1–4** (denoted as P_1, P_2, P_3, and P_4, respectively). A) $[ds+P_1]^{5-}$; B) $[ds+P_2]^{5-}$; C) $[ds+P_3]^{5-}$; D) $[ds+P_4]^{5-}$. (ds: double-stranded; ss: single-stranded; PI: parent ions.)

affinities for the polyamides with D2 is **1**>**3**>**2**. The binding of polyamides to single-stranded oligonucleotides is also affected by other interactions, such as hydrogen bonds and Van der Waals forces. For example, *N*-methylimidazole(Im) is more likely to bind to a guanine base, because its N3 atom can form an additional hydrogen bond with the NH_2 group of a guanine base. Therefore, polyamide **1** exhibits the highest binding affinity of the polyamides **1–3** towards D2.

Conclusion

This study provides strong evidence for the binding of novel polyamides to double- or single-stranded oligonucleotides containing a TTAGGG sequence. Analysis of the stoichiometry and selectivity revealed that, of the four polyamides studied, polyamide **3** (PyPyPyγImImImβDp) had the highest binding affinity towards d(TTAGGGTTAGGG/CCCTAACCCTAA). This research provides insights into interactions between small molecules and telomeric DNA and has potential application in the design and synthesis of novel polyamides that recognize human telomeric DNA.

Experimental Section

Materials and sample preparations: Single-stranded oligonucleotides (d(TTAGGGTTAGGG), d(CCCTAACCCTAA)) were purchased from Sangon (Beijing, China). Oligonucleotides were dissolved in water and then diluted with ammonium acetate solution. For duplex DNA synthesis, two complementary single-stranded oligonucleotides were mixed in equimolar proportions, annealed at 90 °C, and slowly cooled to room temperature (over 4 h) to allow for the formation of the duplex (D1).

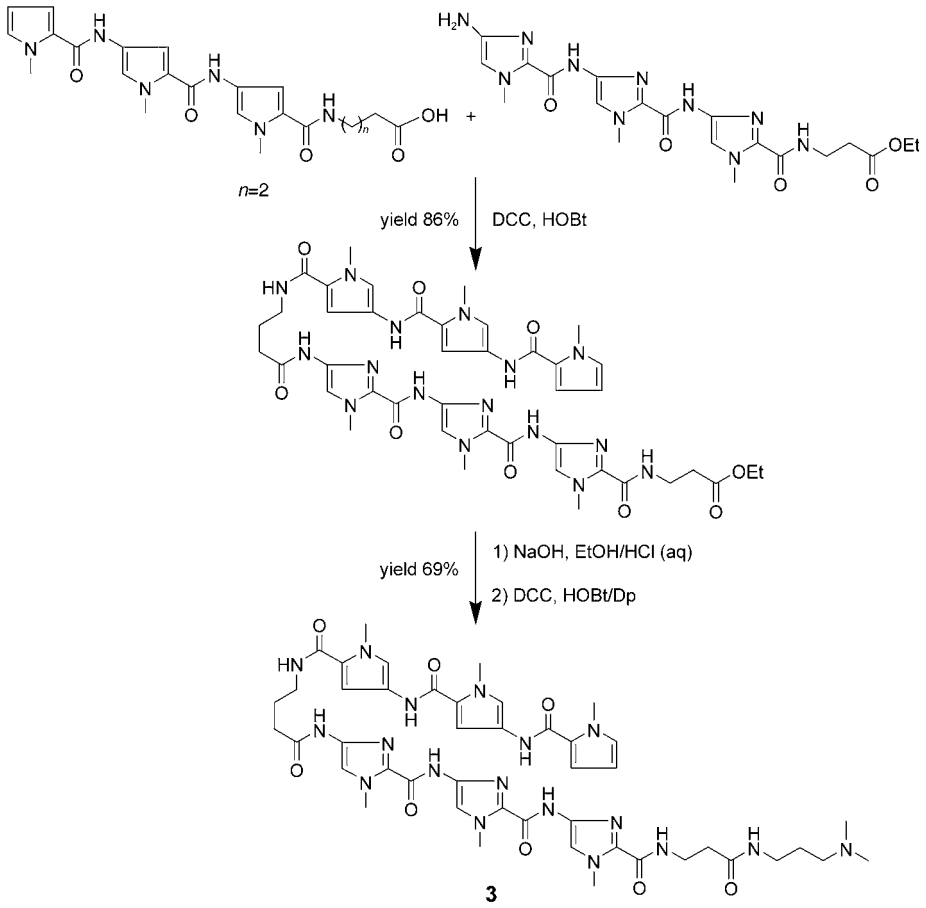
D1: d(TTAGGGTTAGGG/CCCTAACCCTAA) (monoisotopic mass = 7291.0)

D2: d(TTAGGGTTAGGG) (monoisotopic mass = 3756.6)

Desalting was performed three times by using Microcon filters (Amicon, Beverly, USA) with a 3000 Da cut-off. The resulting DNA stock solution was 500 μM in 100–150 mM NH_4OAc.

A haloform reaction was used to synthesize the building blocks that contained two to four heterocycle rings, these building blocks were then joined by means of the DCC/HOBt coupling reaction to produce the polyamides (DCC = dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole).[10] The synthesis of PyPyPyγImImImβDp (**3**) is described as an example of this process in Scheme 2. Polyamides **1**, **2**, and **4** were prepared in the same way. The purification and characterization of polyamides **1–4** was performed according to our previous paper.[10] In all of our experiments, we used the free base form of these compounds.

Polyamides were dissolved in a mixture of methanol and water (50:50, v/v) to a concentration of 500 μM. Aliquots of each DNA solution (2.0 μL) were mixed with the polyamide solutions (2.0–16 μL), and then diluted with methanol/100 mM ammonium acetate (20:80, v/v) to a final volume of 40 μL. Methanol was used as it ensures a good spray.[15,16]



Scheme 2. Synthesis of PyPyPyγImImImβDp (**3**). DCC: dicyclohexylcarbodiimide; HOBt: 1-hydroxybenzotriazole; Dp: *N*,*N*-dimethylpropyldiamine.

Mass spectrometry: ESI-MS spectra were obtained by using a Finnigan LCQ Deca XP Plus ion trap mass spectrometer (San Jose, CA), and all experiments were carried out in the negative-ion mode. We directly infused the complex solution into the mass spectrometer at a flow rate of 2 μL min^{-1}. The electrospray source conditions were optimized to favor the detection of the noncovalent complexes (spray voltage 2.0–2.5 kV, capillary temperature 100 °C). In all experiments, the scanned mass range was set at 1000–2000 u. Data were collected and analyzed by using the Xcalibur software developed by ThermoFinnigan, and 10 scans were averaged for each spectrum.

Acknowledgement

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