FULL PAPER

Thermal Stability of Modified i-Motif Oligonucleotides with Naphthalimide Intercalating Nucleic Acids

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In continuation of our investigation of characteristics and thermodynamic properties of the i-motif 5′-d[(CCCTAA)$_3$CCCT)] upon insertion of intercalating nucleotides into the cytosine-rich oligonucleotide, this article evaluates the stabilities of i-motif oligonucleotides upon insertion of naphthalimide (1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione) as the intercalating nucleic acid. The stabilities of i-motif structures with inserted naphthalimide intercalating nucleotides were studied using UV melting temperatures (T_m) and circular dichroism spectra at different pH values and conditions (crowding and non-crowding). This study indicated a positive effect of the naphthalimide intercalating nucleotides on the stabilities of the i-motif structures compared to the wild-type structure which is in contrast to a previous observation for a pyrene-intercalating nucleotide showing a decrease in T_m values.

Introduction. – DNA was the first target for anticancer drugs [1]. However, with the advent of new molecular targets, such as kinases and cell surface receptors that promised greater selectivity for treating cancer cells, the interest in DNA-targeted drugs faded, even though they are still the mainstay of most treatment regimens [2]. As a result of research on telomeric G-quadruplexes and cellular consequences of targeting them with small molecules that stabilize these structures [3], their biological and therapeutic significance is well appreciated and they continue to be an active field for drug discovery [4–9]. Guanine/cytosine (G/C)-rich sequences were found to exist in biologically important regions of DNA, *e.g.*, the ends of chromosomes (telomeres), immunoglobulin switch regions, and regulatory regions of oncogenes [10–12]. Recently, the structures of synthetic oligonucleotides corresponding to either G- or C-rich strands have been investigated extensively because of their unusual functions [13–17]. Several reports indicated that some repetitive DNA sequences adopted unusual pairing arrangements with their complements other than the *Watson–Crick* pairing [18]. The tandem-repeated sequences, with three or four contiguous sequences, are known to form polymorphic quadruplexes containing G-quartets stabilized by *Hoogsteen* H-bonding [18][19]. C-Rich oligomers were also shown to self-associate at acidic or even neutral pH into an intercalated structure called the i-motif (*Fig. 1*) [20][21]. Several studies on the stabilities and biological activities of G-rich quadruplexes have been published, but only a few reports on the modification of the i-motif by insertion of unnatural DNA building blocks can be found. Therefore, herein, we report the stability of the C-rich i-motif by intercalation of naphthalimide (1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione). Naphthalimides belong to a group of promising DNA-targeting anticancer agents [22]. They are used as intercalating moiety on DNA/DNA duplexes and give high stability in comparison with other heterocyclic compounds [23]. Locked nucleic acid (LNA)-modified i-motif structures show different electrophoretic mobilities as compared to the DNA i-motif [24]. On the other hand, there are some NMR studies on i-motif oligonucleotides [25], but only a few on heterocyclic modified i-motif structures. Pairs of oligonucleotides with pyrene-modified deoxyadenosine (^{Py}A) units induce a stable interstand i-motif structure that can be characterized by a change in fluorescence (λ_{max}) [26]. Fluorescence intensity, anisotropy, and energy transfer properties of dimethylaniline (DMA)-modified deoxycytidine (^{DMA}C) can be used to track conformational changes in real time [27]. Single insertions of a modified nucleotide (nt) into the

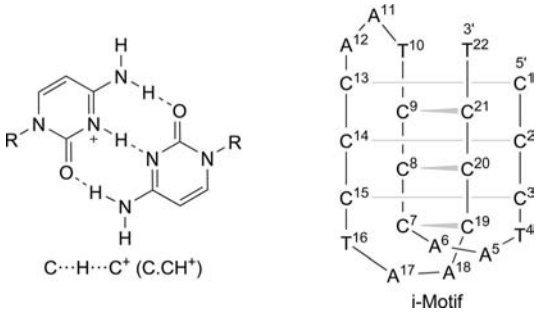

Fig. 1. *Hemiprotonated C···H···C$^+$ base pair and i-motif of 5-d[(CCCTAA)3CCCT)]*

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i-motif of single-strand oligonucleotides have also been investigated [28 – 31]. In this article, the stacking effect of naphthalimides when inserted in i-motif sequences is investigated.

Results and Discussion. – The stabilities of naphthalimide-modified 22nt C-rich i-motifs when substituting a nucleotide in the TAA loop (T^{10}, A^{11}, or A^{12}) with naphthalimide was investigated (*Fig. 2*, *Table 1*).

The stabilities of the i-motif structures of these sequences were studied by UV melting method and circular dichroism (CD) experiments. The UV melting curves were recorded at 295 nm in buffer under non-crowding and

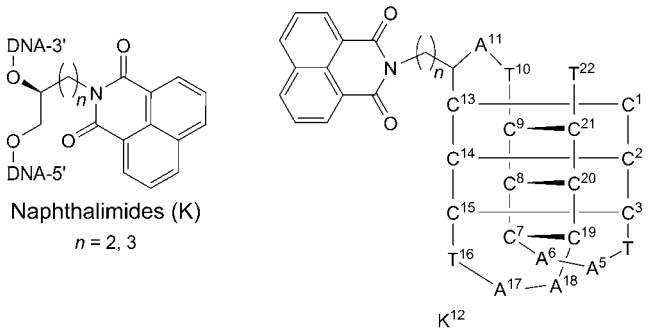

Fig. 2. *Modified i-motif with naphthalimide*

Table 1. *Modified i-Motif Sequences*

ON[a])	Sequence	M_r calc.		M_r found	
		$n=2$	$n=3$	$n=2$	$n=3$
Wild type	d(CCCTAACCCTAACCCTAACCCT)	6504.4	–	6508	–
K^{10}[b])	d(CCCTAACCCKAACCCTAACCCT)	6546.5	6560.5	6543.2	6562.532
K^{11}[b])	d(CCCTAACCCTKACCCTAACCCT)	6537.5	6551.5	6535.4	6552.513
K^{12}[b])	d(CCCTAACCCTAKCCCTAACCCT)	6537.5	6551.5	6535.4	6552.423

[a]) ON, Oligonucleotide. [b]) K^{10} – K^{12}, Naphthalimides.

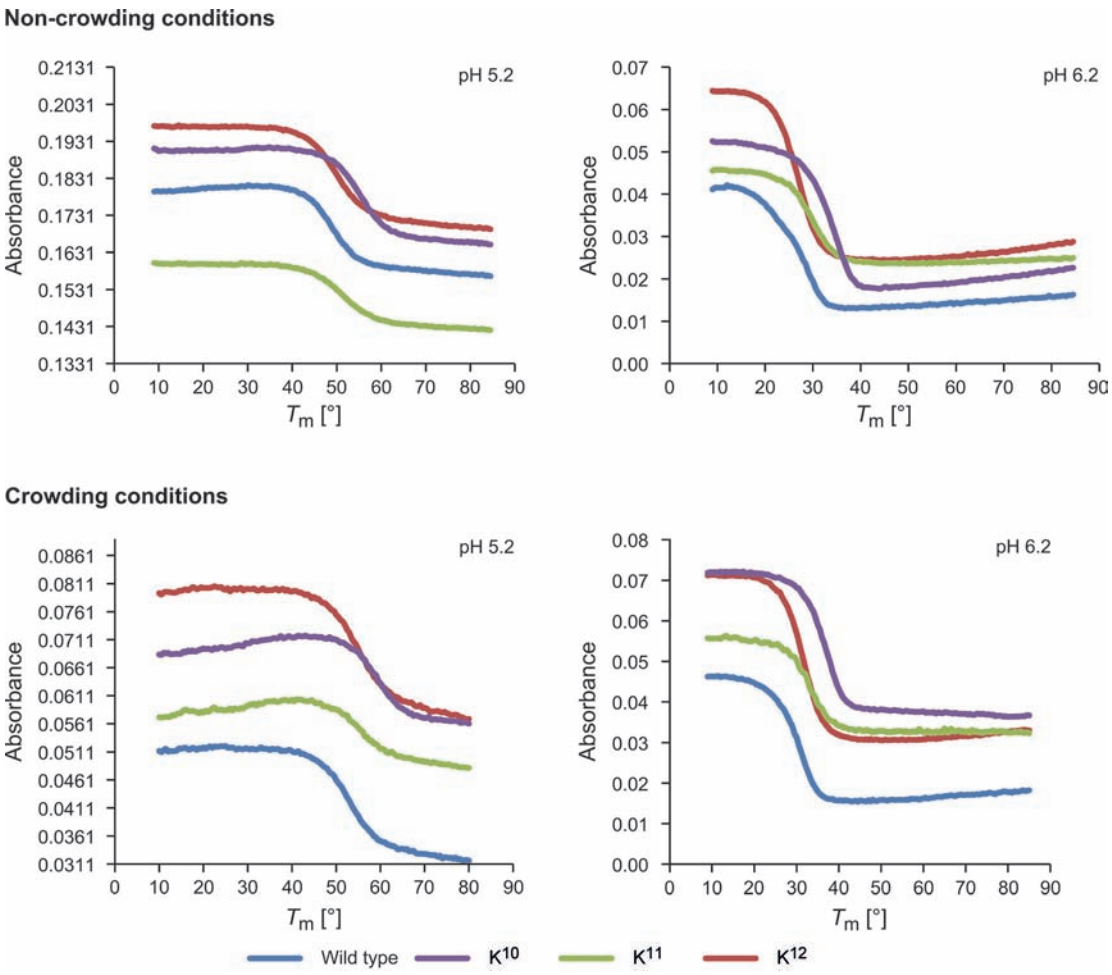

Fig. 3. *Melting temperatures* [°] *of naphthalimide oligonucleotides* K^{10} – K^{12} ($n=2$) *under non-crowding and crowding conditions*

under crowding conditions (with 20% poly(ethylene glycol) 200 (PEG-200)) at pH 5.2 and 6.2 (*Figs. 3* and *4*) in order to evaluate the pH-dependence characteristics of the i-motif structures [32]. The melting curves showed hypochromic changes typical for i-motif behavior [33]. As expected for i-motif structures, the melting temperatures (T_m) at pH 5.2 were significantly higher than at pH 6.2 (*Table 2*).

When the T_m values were determined under crowding conditions (buffer + 20% PEG-200), an increase of 3 – 7° was observed that presumably reflected the relatively high stability of the i-motif (*Table 2*). Since the crowding conditions were assumed to mimic the conditions in cells, this indicates that insertion of an intercalator in a loop sequence is well tolerated. It should be noted that the modified oligonucleotides with the shorter linker ($n = 2$)

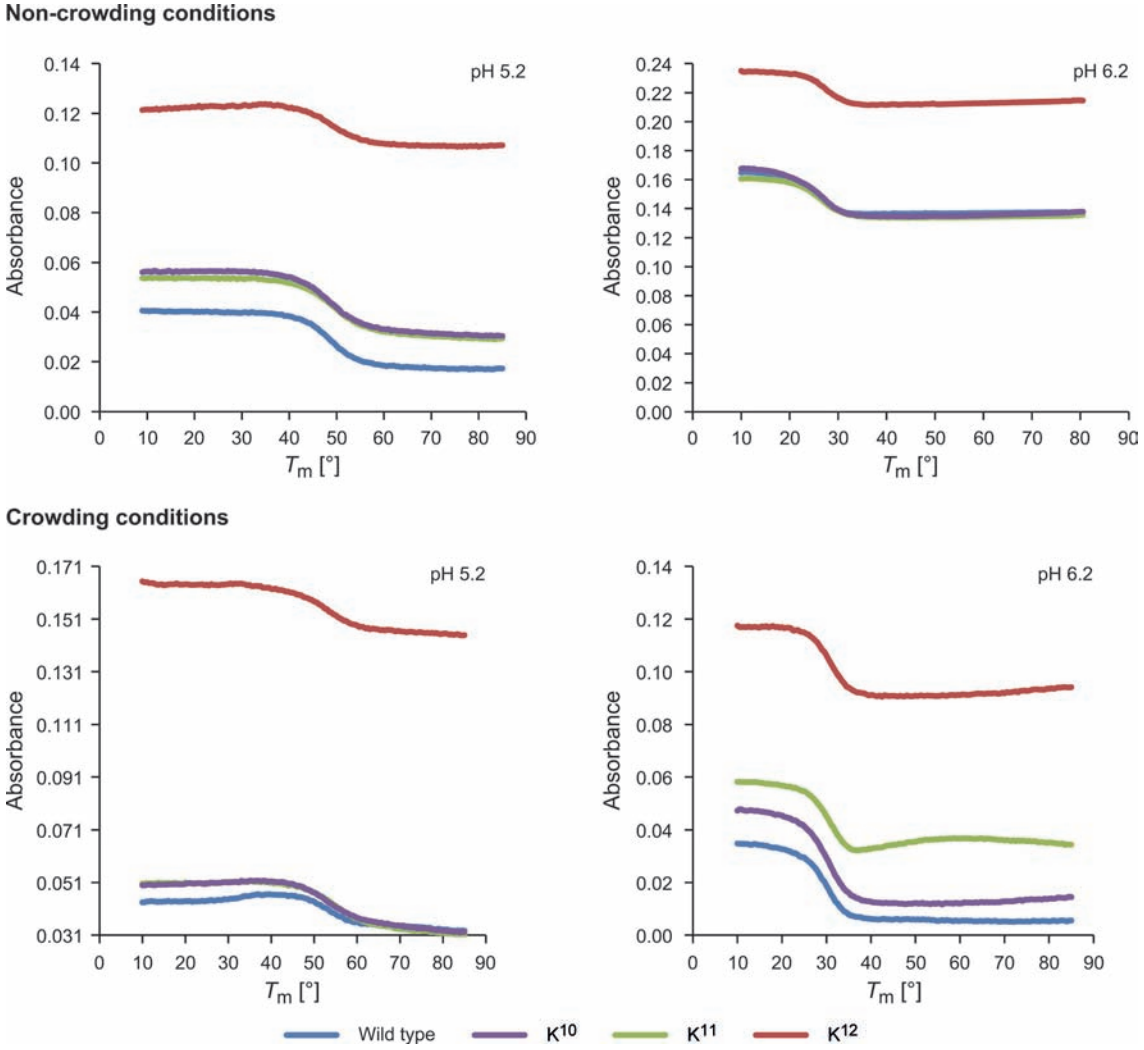

Fig. 4. *Melting temperatures* [°] *of naphthalimide oligonucleotides* K^{10} – K^{12} ($n = 3$) *under non-crowding and crowding conditions*

Table 2. *Melting Temperatures for the Oligonucleotides*[a])

ON[b])	T_m [°]							
	Non-crowding conditions				Crowding conditions			
	pH 5.2		pH 6.2		pH 5.2		pH 6.2	
	$n = 2$	$n = 3$	$n = 2$	$n = 3$	$n = 2$	$n = 3$	$n = 2$	$n = 3$
Wild type	48.9	48.9	25.9	25.9	53.2	53.2	29.7	29.7
K^{10}[c])	55.0	48.9	32.4	25.2	59.0	53.6	36.2	29.8
K^{11}[c])	51.7	49.5	29.6	25.8	55.3	54.8	33.1	29.5
K^{12}[c])	49.8	48.5	26.8	27.2	55.0	52.7	31.6	30.7

[a]) Solution: 100 mM KCl, 10 mM sodium cacodylate, 20% PEG-200. [b]) ON, Oligonucleotide. [c]) K^{10} – K^{12}, Naphthalimides.

were more stable than those with the longer linker ($n = 3$) and both showed higher T_m values than the wild type at pH 5.2. The T_m values were nearly identical at pH 6.2, except for K^{10} with $n = 2$, which showed a substantial higher T_m value than the wild type. Also, at pH 5.2, the highest increase in T_m (5.8 and 6.1° under crowding and non-crowding conditions, resp.) was found for K^{10} with $n = 2$. This is in contrast to what was found, when naphthalimide monomers were inserted into DNA duplexes, where the longer linker led to higher T_m values [23]. In order to gain more insight, the thermodynamic stabilities of the i-motif were studied by CD at pH 4.2, 5.2, 5.7, 6.2, 6.7, 7.2, and 8.6. At lower pH value, the CD spectra of all oligonucleotides showed the characteristic feature of the i-motif with a maximum at 286 – 290 nm and a minimum at 257 – 260 nm. These values were shifted to 273 – 274 nm and 246 – 250 nm, respectively, with a lower amplitude corresponding to the single-strand DNA, at higher pH. In order to get a deeper insight in the pH dependence of i-motif stability, the transitional pH curves for all modified and the wild type oligonucleotides were determined by plotting the amplitude at 289 nm *vs.* pH. The i-motif of oligonucleotides K^{11} and K^{12} for both linker lengths ($n = 2$ or 3) was clearly more stable around neutral pH showing transition midpoints (t_m) for $n = 2$ at pH 6.6 and 6.7, respectively, and for $n = 3$ at pH 6.5 and 6.4, respectively, *vs.* pH 6.4 for the wild type. This effect was even more pronounced under crowding conditions for K^{11} and K^{12} with t_m values at pH 7.0 and 6.9, respectively, for $n = 2$ and for $n = 3$ at pH 6.9 and 6.8, respectively, when compared with the wild type with pH 6.5 (*Table 3*, *Fig. 5*).

The increased stabilities of the i-motifs of K^{11} and K^{12} could be due to improved stacking by the loop nucleotides when 2-deoxyadenosine was replaced by a naphthalimide nucleotide. This is a way to make i-motifs less sensitive to pH around physiological pH. It should be emphasized that the results found here for the naphthalimide nucleotide are much more promising than those reported earlier for a corresponding pyrene nucleotide which showed lower thermal stability than the wild-type oligonucleotide and no substantial improvement in stability at higher pH.

We think that decoys can be developed from i-motifs in the same manner as it was done for quadruplex decoys with

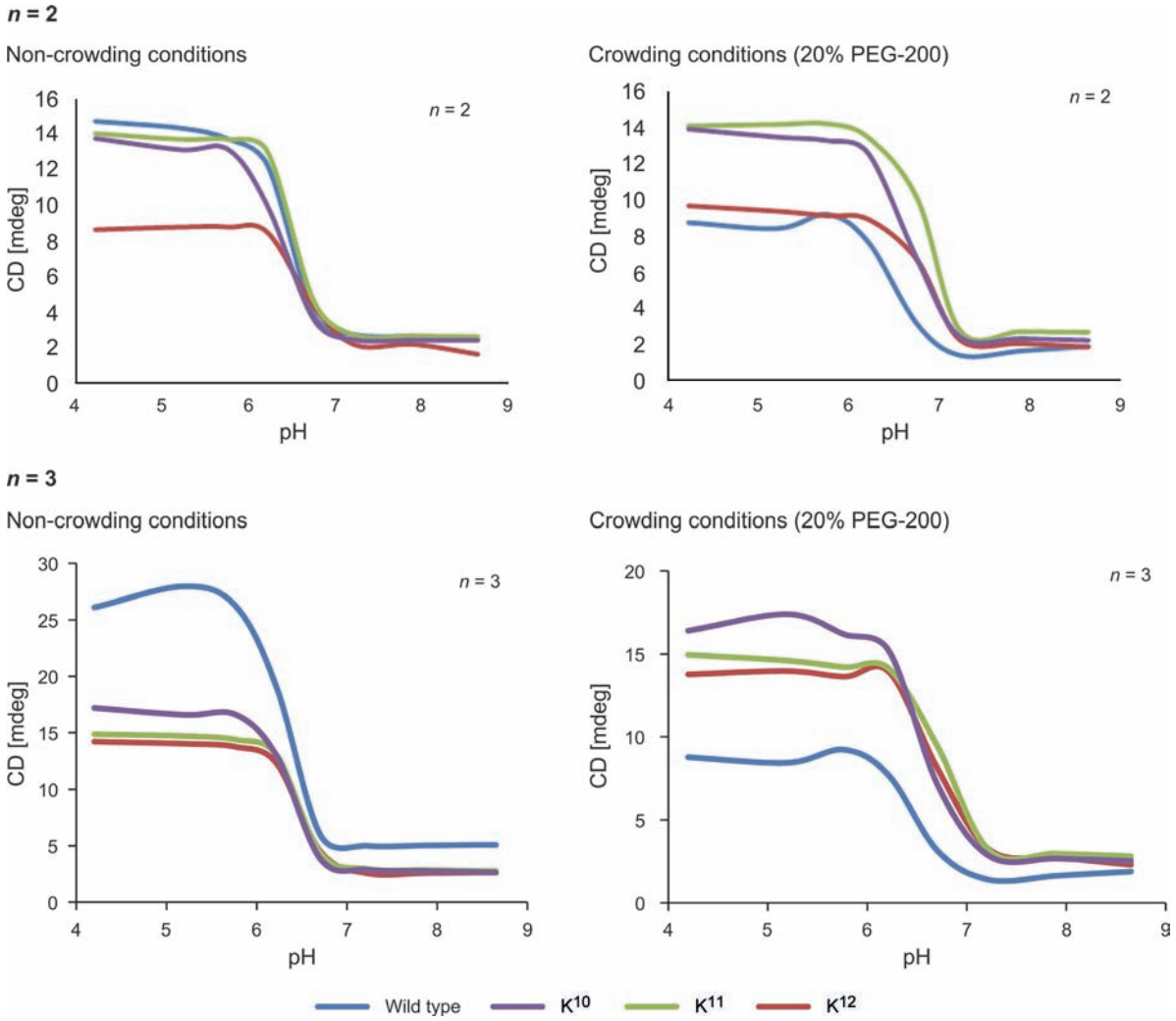

Fig. 5. *Transitional pH curves of naphthalimide oligonucleotides K^{10} – K^{12}*

Table 3. *Transitional pH Curves for the Oligonucleotides*

ON[a])	pH Values of t_m			
	Non-crowding conditions		Crowding conditions	
	$n=2$	$n=3$	$n=2$	$n=3$
Wild type	6.4	6.4	6.5	6.5
K^{10}[b])	6.5	6.4	6.8	6.6
K^{11}[b])	6.6	6.5	7.0	6.9
K^{12}[b])	6.7	6.4	6.9	6.8

[a]) ON, Oligonucleotide. [b]) K^{10} – K^{12}, Naphthalimides.

anticancer activities [34 – 36]. They were developed from quadruplexes by inserting intercalators into their loop sequences. We have now found that insertion of naphthalimide into a loop sequence of i-motifs improves thermal stability and increases the tolerance to pH value in near-physiological range. We therefore think that we have made the idea of developing i-motif decoys more probable.

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Experimental Part

General. The wild-type DNA oligonucleotide was purchased from *Integrated DNA Technologies*. Naphthalimide-modified oligonucleotides were synthesized on an automated DNA synthesizer according to published procedures [23]. Naphthalimide phosphoramidites were synthesized according to reported procedures [23]. The purity of all oligonucleotides was verified by ion-exchange HPLC to be 95% or higher and their composition was verified by MALDI-TOF-MS.

Thermal Melting. Oligonucleotides were melted in two buffers containing 100 mM KCl and 10 mM sodium cacodylate, one at pH 5.2 and the other at pH 6.2, with addition of 20% PEG-200 under crowding conditions and without addition of PEG-200 under non-crowding conditions [37][38]. For calculation of single-strand concentrations was assumed that the naphthalimide extinction coefficient was 17.3 for $n=1$ and 12.6 for $n=2$. The samples were annealed for 5 min at 80°. The measurements were performed in 10-mm (100 µl) quartz microcuvettes. Absorbance *vs.* T_m curves were measured with a heating rate of 0.5°/min by UV spectroscopy at 295 nm in the temp. range 10 – 80° on a *Beckman DU 800* spectrophotometer equipped with a five-position microcell holder and thermoprogrammer.

CD Spectra. CD Spectra were collected on a *Jasco J-600A* spectropolarimeter using 1-ml quartz cuvettes with 5-mm path length. Oligonucleotides were dissolved in a buffer containing 100 mM KCl and 10 mM sodium cacodylate with or without 20% PEG-200 at pH 4.2, 5.2, 5.7, 6.2, 6.7, 7.2, and 8.6 to obtain 3% solns. of samples. All samples were annealed for 2 min at 80° and slowly cooled to r.t. before data collection. The measurements were performed at 10° in the range of 200 – 350 nm. The buffer spectrum was subtracted from the sample spectra. The spectra were smoothened in Microcal Origin 6.0 using *Savitzky–Golay* filter.

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