Adductomics

Deutsche Ausgabe: DOI: 10.1002/ange.201507682
Internationale Ausgabe: DOI: 10.1002/anie.201507682

Ultrasensitive Direct Quantification of Nucleobase Modifications in DNA by Surface-Enhanced Raman Scattering: The Case of Cytosine

Judit Morla-Folch, Hai-nan Xie, Patricia Gisbert-Quilis, Sara Gómez-de Pedro, Nicolas Pazos-Perez, Ramon A. Alvarez-Puebla, and Luca Guerrini**

Abstract: *Recognition of chemical modifications in canonical nucleobases of nucleic acids is of key importance since such modified variants act as different genetic encoders, introducing variability in the biological information contained in DNA. Herein, we demonstrate the feasibility of direct SERS in combination with chemometrics and microfluidics for the identification and relative quantification of 4 different cytosine modifications in both single- and double-stranded DNA. The minute amount of DNA required per measurement, in the sub-nanogram regime, removes the necessity of pre-amplification or enrichment steps (which are also potential sources of artificial DNA damages). These findings show great potentials for the development of fast, low-cost and high-throughput screening analytical devices capable of detecting known and unknown modifications in nucleic acids (DNA and RNA) opening new windows of activity in several fields such as biology, medicine and forensic sciences.*

Chemical modifications of the canonical nucleobases of nucleic acids (especially cytosine variants for DNA) play a major role in the increasing of the genetic diversity and thus the codification of biological information.[1] In fact, the vastly different function and properties of cells inside higher organisms demand the specific silencing and activation of cell-type-specific genes which is achieved through an additional layer of epigenetic information beyond simple genomic sequence.[2] Recently, this chemical diversity in DNA has been demonstrated to be greater than initially predicted. These discoveries suggest that there may be additional underestimated modifications that contribute to the gene regulation.[3] In addition to epigenetic modifications, an ample variety of nucleobase lesions (such as alkylation, oxidation, deamination, and cross-linking) can take place as a result of carcinogen attacks to DNA.[4] Accumulation of such mutations in genes controlling cell growth, proliferation, programmed cell death, and cell differentiation is likely to cause cancer.[5]

Recent discoveries of new nucleotide variants with epigenetic functions have directed an intense research toward the development of novel methods to detect, profile, and sequence these base modifications in the genome and transcriptome.[2] These strategies span from pure detection and quantification methods (such as immunological detection,[6] ^{32}P-postlabelling[4a] and liquid chromatography–mass spectrometry (LC-MS)[7]) to genome-wide profiling methods and single-base-resolution sequencing methods.[8] A relatively new field in this exciting area is the screening of DNA samples for unknown or unanticipated lesions, which is referred to as "adductomics".[9] Nowadays, this sort of analysis is primarily performed with high-resolution LC-MS.[4a,9] This technique relies on the fragmentation of protonated modified nucleobases which are then differentiated according to their molecular masses.[4a,9] However, DNA adduct analysis by mass spectrometry is costly and time-consuming as it requires prior sample preparation which normally involves several standard steps: DNA denaturalization, hydrolysis into the corresponding monomers, enrichment of the adducts, removal of unmodified nucleobases and addition of the appropriate internal standard.[9] In addition, extreme caution must be paid to avoid artificial generation of DNA lesions during these processing steps.

Surface-enhanced Raman scattering (SERS) spectroscopy has emerged as a rapid and effective method for identifying unknown species, finding direct application in biomedical analysis, forensics, art and cultural heritage and homeland security.[10] In the very recent years, we have witnessed an upsurge of interests in the direct SERS analysis of DNA boosted by the efforts from several groups addressing the reproducibility issues associated with the DNA interaction with plasmonic metal colloids.[11]

Herein, we exploit positively charged silver nanoparticles coated with spermine (AgNP@Sp) as an effective plasmonic substrate for DNA analysis. The electrostatic adhesion of the phosphate groups of the DNA backbone with the spermine bound to the silver surface promotes the nanoparticle aggregation into stable clusters in solution. This aggregation traps the nucleic acids into highly efficient electromagnetic hot spots at the interparticle junctions (Figure 1 A). As a result, highly intense and reproducible SERS signals are obtained and can be then applied to the challenging task of quantifying structurally analogous nucleobase variants

[*] J. Morla-Folch, Dr. H.-n. Xie, P. Gisbert-Quilis, Dr. S. G.-d. Pedro, Dr. N. Pazos-Perez, Prof. Dr. R. A. Alvarez-Puebla, Dr. L. Guerrini
Medcom Advance, Viladecans Business Park, Edificio Brasil
Bertran i Musitu 83–85, 08840 Viladecans, Barcelona (Spain)
J. Morla-Folch, P. Gisbert-Quilis, Dr. N. Pazos-Perez, Prof. Dr. R. A. Alvarez-Puebla, Dr. L. Guerrini
Universitat Rovira i Virgili and Centro Tecnológico de la Química de Catalunya, Carrer de Marcel·lí Domingo s/n
43007 Tarragona (Spain)
Prof. Dr. R. A. Alvarez-Puebla
ICREA, Passeig Lluís Companys 23
08010 Barcelona (Spain)
E-mail: ramon.alvarez@urv.cat
luca.guerrini@ctqc.org

Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201507682.

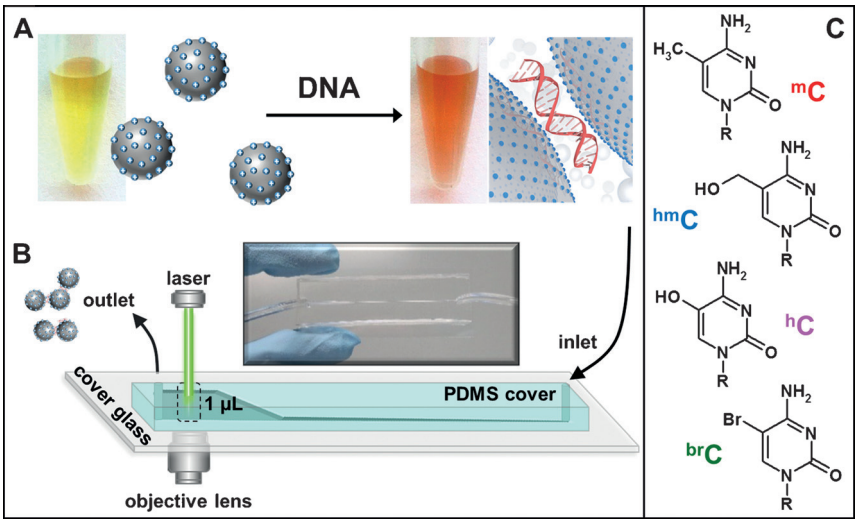

Figure 1. A) Outline of DNA-driven aggregation of AgNP@Sp into stable clusters in suspension. The yellowish colloids immediately turn to deep orange upon DNA addition. B) Representation of the on-line detection of DNA via SERS-microfluidic system. C) Molecular structures of the cytosine modifications: 5-methylcytosine (^{m}C), 5-hydroxymethylcytosine (^{hm}C), 5-bromocytosine (^{br}C) and 5-hydroxycytosine (^{h}C).

through direct SERS analysis. Demonstration of the direct SERS recognition and relative quantification of different cytosine modifications in both single- and double-stranded sequences (ss and ds, respectively) is carried out for: 5-methylcytosine (^{m}C) and 5-hydroxymethylcytosine (^{hm}C) as the most important epigenetic modifications in mammalian DNA;[12] 5-bromocytosine (^{br}C) as the representative inflammation-induced DNA damage;[13] and 5-hydroxycytosine (^{h}C) as the example of oxidative DNA lesion[14] (Figure 1 C). Each of these cytosine variations imposes a characteristic alteration of the SERS spectral profiles of ss and dsDNA, as also revealed by partial least-squares discriminant analysis (PLS-DA). Notably, the spectral changes can be quantitatively correlated with the relative content of modified nucleobases. Finally, the combination of direct SERS detection and microfluidics (Figure 1 B) allows us to perform a reliable ultrasensitive analysis of DNA samples at the picogram regime (therefore removing the need of pre-amplification/enrichment steps) while offering the possibility to develop simple low-cost devices for real time automated DNA screening analysis.

The single-strands selected for this study are listed in Table S1. ssC is an oligonucleotide composed by the canonical four nucleobases (base sequence: CAT CGC AGG TAC CTG TAA GAG), whereas its modified counterparts ss^{mod}Cn (where mod = *m*, *hm*, *h* and *br*) contain one, three or all five cytosine variants ($n = 1, 3, 5$). AgNP@Sp were synthesized via a wet chemical method in which borohydride reduction of silver nitrate was performed in the presence of spermine tetrahydrochloride.[15] The original averaged SERS spectra of all ssDNA sequences are reported in Figure S1 whereas in Figure 2 A we illustrate the baseline corrected spectra of ssC, ss^{m}C5, ss^{hm}C5 and ss^{br}C5 in the most informative spectral regions: 1) the ring breathing range, from 550 to 850 cm^{-1}, which mainly contains the relatively well-separated ring stretching features of purine and pyrimidine residues; 2) the 950–1150 cm^{-1} spectral range, dominated by the intense symmetric stretching vibration of the phosphodioxy moiety at 1089 cm^{-1}; and, 3) the highly overlapped nucleobase bands located between 1150 and 1600 cm^{-1}, containing a complicated pattern of features mainly ascribed to the in-plane vibrations of base residues and carbonyl group stretching vibrations. A general vibrational assignment is also reported in the same figure.[11d,16] Simple visual analysis of the superimposed SERS spectra reveals pronounced alteration of the vibrational patterns, which are mainly circumscribed to the C related bands. For instance, we observe a relative intensity decrease of the C ring breathing band at 790 cm^{-1} (with minor T contribution), and the vibration centered at 595 cm^{-1} which is ascribed to a C ring bending mode.[17] A similar reduction in the magnitude of the C ring breathing mode was also observed in the normal Raman study of 5-chlorocytosine.[17b] Similarly, the C ring bending band centered at 1026 cm^{-1} suffers a marked downshift of 7 cm^{-1} and a drastic variation of intensity, as does the peak height of both the C ring stretching feature at 1246 cm^{-1}[18] and the broad carbonyl stretching band at 1647 cm^{-1}.[18a] Importantly, the extension of these changes differs from nucleobase modification to modification. The ss oligonucleotides were also hybridized to their complementary sequence to yield the corresponding double-stranded structures dsC, ds^{m}C5, ds^{hm}C5, ds^{h}C5 and ds^{br}C5. All investigated 5-modified cytosine nucleobases have the same base-pairing chemistry as cytosine and, thus, the corresponding double-stranded helixes are expected to structurally differ only for the specific cytosine modification. As for ss, the nucleobase modifications mostly alter the vibrational bands with larger C contributions (Figure 2 B and Figure S2). The C/T ring breathing mode undergoes a marked intensity decrease and a 3 nm blue-shift (for ds^{m}C5 and ds^{br}C5). Simultaneously, the C band at 598 cm^{-1} disappears from the spectra of the modified duplexes which also reveal marked perturbations of the C ring features at 1020 cm^{-1} and 1242 cm^{-1}.

Noteworthy, in the case of ss^{br}C new intense features arise at 1060 cm^{-1} and, as a shoulder, at 1622 cm^{-1}. These bands were tentatively assigned to bending modes of the NH_2 group[17b,19] which adopts a co-planar geometry as a result of the Br substitution.[19] However, these bands almost disappear from the SERS of ds^{br}C5. It has been shown that the C5-bromination has little effect on the atomic charge distribution of cytosine[19] as well as on the overall duplex stability.[19–20] Thus, we deduce that in the duplex conformation the NH_2 group geometry is largely determined by the strong Watson–Crick base pairing which significantly reduces the influence of the Br group on the amino conformation (see Supporting Information, Figure S10, for a more detailed discussion).

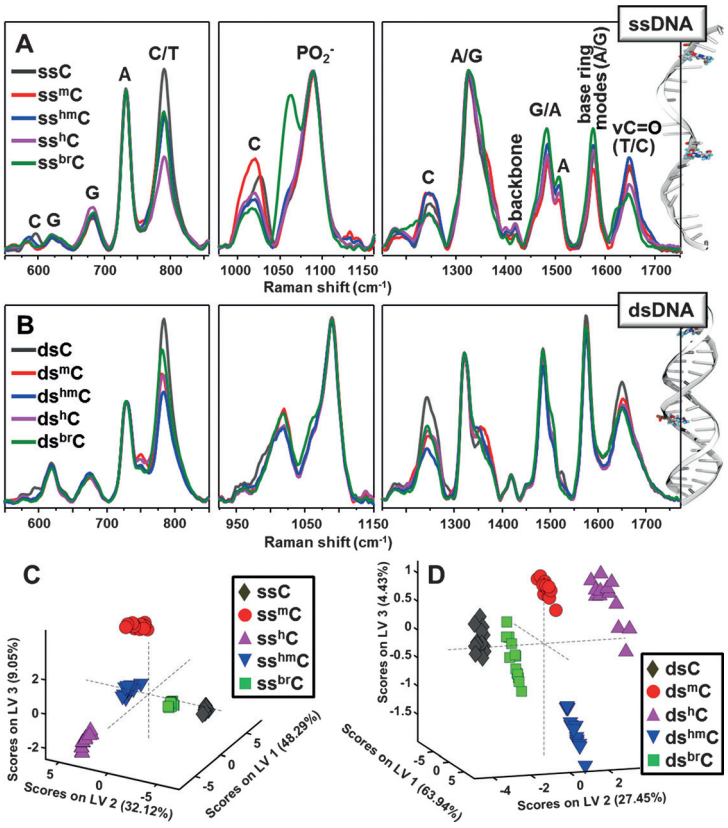

Figure 2. A) Baseline corrected SERS spectra of single-stranded sequences ssC, ss^{m}C5, ss^{hm}C5, ss^{h}C5 and ss^{br}C5 (final concentration in the sample 50 nM). B) Baseline corrected SERS spectra of double-stranded sequences dsC, ds^{m}C5, ds^{hm}C5, ds^{h}C5 and ds^{br}C5, (final concentration in the sample 25 nM). AgNP@Sp concentration was kept constant to ≈0.3 nM for both ss and dsDNA analysis. For comparison, the spectra were normalized, in each of the three spectral range, to: 1) the A ring breathing band at 729-734 cm^{-1}, 2) the ν(PO$_2$) band at 1089 cm^{-1}, and 3) the A + G band at 1321 cm^{-1}, respectively. C,D) Partial least squares discriminant analysis (PLS-DA) of the ss/ds SERS spectra. They are latent number score plot of the obtained SERS spectra. The mean spectra demonstrated here are from averaging more than 30 measurements (6 independent repeats) per sample.

Partial least squares discriminant analysis (PLS-DA) was applied to differentiate between sequences containing the different investigated variations of cytosine bases. The PLS-DA model is based on a supervised classification method for the discrimination of different groups. From the established model, the 5 sample groups are distinguishable from each other with 100% sensitivity and specificity, in the cases of both single and double strands (Figure 2C and Figure 2D, respectively). Next, we investigated the correlation between the extent of the spectral changes, qualitatively discussed in Figure 2A,B, with the number of modified cytosines (1, 3 and 5) in ss and ds (the corresponding SERS spectra are illustrated in Figure S3–S6 and Figure S7–S10, respectively). Most of the relevant spectral changes are localized on the vibrational bands dominated by C contributions. Thus, we monitored the SERS intensities of three of these C-related features (at 784/790, 1026/1020 and 1246/1242 cm^{-1}) as a function of the relative abundance of modified cytosines in each sequence. Additionally, we also included in such comparison the spectral intensity registered at 1060 cm^{-1}, where is centered the new bromo-related band appearing in the ss^{br}C5 SERS spectra (Figure 2A). Each peak height was normalized to the phosphodioxy band at 1089 cm^{-1}, which was used as an internal standard since the content of PO_2^- groups is constant for each sequence. Furthermore, this specific vibrational feature has shown to be scarcely sensitive to structural rearrangements such as the DNA hybridization.[21] The corresponding plots for ssDNA are represented in Figure 3A. The C ring breathing mode undergoes a linear drop in intensity as the variant population increases, even though with a different extent depending on the modification type (i.e. different slopes in Figure 3A). On the other hand, the bromo-related feature at 1060 cm^{-1} only displays a relative increase when ^{br}C nucleotide are included in the strand. Moreover, bromo- and hydroxyl-modifications also promote a progressive decrease of both 1026 and 1246 cm^{-1} bands, whereas the introduction of the methyl and hydroxymethyl groups poorly affects the C ring vibration at 1246 cm^{-1}. In contrast, an increment of ^{hm}C content imposes a linear drop of the 1026 cm^{-1} intensity while an opposite trend is observed for ^{m}C. These results combined are extremely important since they do not only highlight the existence of a quantitative correlation between the detected spectral changes and the relative content of modified cytosine nucleobases but plainly show that, even with the simple analysis of just four of the many vibrational features forming the overall spectrum, each modification perturbs the SERS profile in a unique fashion. Such conclusion was further corroborated by the PLS discriminant analysis of each set of SERS spectra of ssDNA with a different cytosine modification content (Figure S3–S6, that is, statistical analysis was separately performed on ssC, ss^{m}C1, ss^{m}C3 and ss^{m}C5 data, then the classification was repeated in the same fashion for the remaining nucleobase variations as well as for the ds structures). The plots show that each single strand with an increasing number of modified cytosine can be successfully differentiated into a separate group with 100% specificity. Figure 3B illustrates the outcome of the same identical study performed on the corresponding dsC, ds^{m}C5, ds^{hm}C5, ds^{h}C5 and ds^{br}C5 duplexes. The general response of the intensity of the C ring breathing mode to the change in variant contents is fairly close to what observed for the single strands. Similarly, the relative intensity measured at 1060 cm^{-1} exclusively registers an increase in the case of brominated cytosines. Nevertheless, the monitoring of the two remaining C-associated bands at 1020 and 1242 cm^{-1} against the variant content provides the most intriguing results. Once more, their corresponding plots in Figure 3B show a concentration dependent correlation of their intensities but their overall patterns strikingly diverge from those observed for ss (for instance, the change in relative intensity trend of the ≈1020 cm^{-1} band for ^{h}C, ^{hm}C and ^{br}C and the ≈1242 cm^{-1} band for ^{m}C and ^{hm}C). This is consistent with the remarkable structural rearrangement and electronic

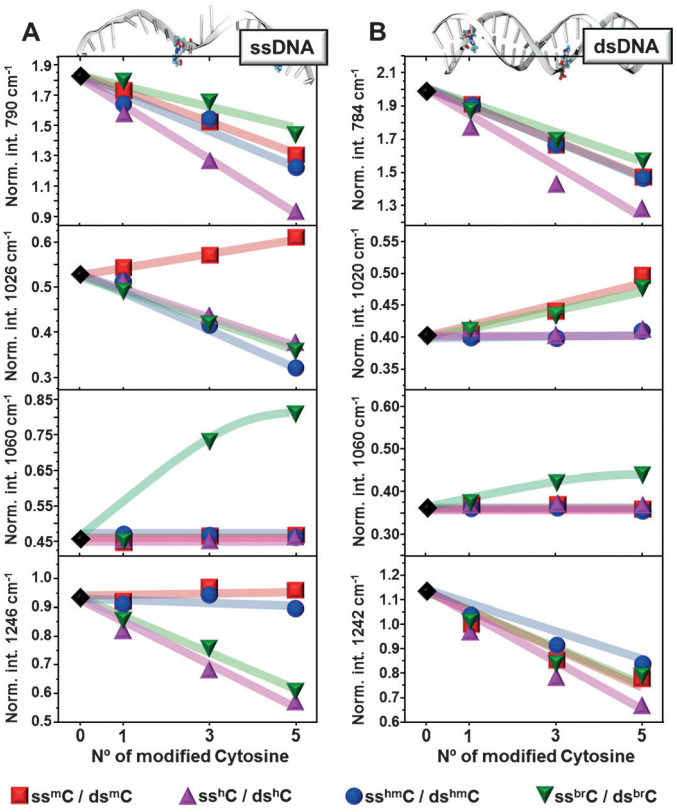

Figure 3. Comparison of the relative SERS intensity of the C spectral marker bands as a function of the nucleobase variant content. A) ssDNA: SERS intensities of the band at 790 cm^{-1}, 1026 cm^{-1}, 1060 cm^{-1} and 1246 cm^{-1} as a function of the content of modified cytosine bases. B) dsDNA: SERS intensities of the band at 784 cm^{-1}, 1020 cm^{-1}, 1060 cm^{-1} and 1242 cm^{-1} as a function of the content of modified cytosine bases. The PO_2^- band at 1089 cm^{-1} was used in both cases as the internal standard.

redistribution imposed by the strong Watson–Crick hydrogen bonding and nucleobase stacking within the double helix. These results show that introduction of nucleobase variants leads to different sets of spectral perturbations depending if such modifications are included either in a single strand or in the duplex. Beyond these considerations, the data illustrated in Figure 3 A,B demonstrate the capability of our direct method to identify and quantify down to one cytosine variant in ssDNA oligonucleotides (21 bases, five of them cytosines), as well as in dsDNA (21 base pairs, 42 bases, 11 of them cytosines). Similarly, PLS discriminant analysis of each set of SERS spectra of dsDNA with different cytosine modification content (Figure S7–S10) was capable to differentiate them into separate groups with 100% specificity.

The reported SERS measurements were performed in a static configuration on colloidal particles in suspension using a long working distance objective. However, under these conditions, the illuminated volume is many orders of magnitude smaller than the actual prepared sample.[11d] Thus, we combined our direct SERS method with a microfluidic device able to manipulate and analyse microscopic volumes of sample in a rapid way (Figure 1 B). Remarkably, the use of microfluidic devices also offers the possibility of automating the whole procedure, being feasible to perform real time analyses in a continuous way at a relatively low cost.[22] To demonstrate the reliability of such approach in differentiating and recognizing nucleobase modifications, we collected SERS spectra of the individual samples ssC, $ss^{m}C5$, $ss^{hm}C5$ and $ss^{br}C5$, together with a binary mixture of two of them, which was used as a blind experiment for our data classification. We set the acquisition time to 3 min for each measurement but well-defined SERS spectra can be obtained even for shorter exposures. Samples were analyzed under a continuous flow rate of 10 $\mu L\,h^{-1}$, which in the described conditions entailed a sample volume of less than 1 μL (corresponding to less than 300 picograms of DNA content). The obtained mixture spectra were compared with the pure spectra of the individual samples and the prediction was made through the non-negative least squares algorithm.[23] The prediction result reveals the composition of the mixture with the precise percentages of each component (39.2% ssC and 60.8% $ss^{hm}C$), perfectly overlapping with the actual constitution (Figure S11).

In summary, we demonstrated the ability of direct SERS analysis of DNA to accurately identify and quantify structurally analogous nucleobase variants in both single-stranded and duplex structures. This study represents a significant step forward in the ultrasensitive detection, vibrational characterization and quantification of cytosine modifications in DNA by direct SERS, which was limited so far to the qualitative differentiation of methyl- and hydroxymethyl-cytosine variants in short single-stranded sequences at high DNA concentrations (0.1 mM).[24] Classification of the SERS spectra was achieved via PLS-DA. Implementation of the sensing strategy into a microfluidic device further reduced the required amount of DNA per measurement to the picogram level (i.e. no more necessity of pre-amplification or enrichment steps) while enabling fundamentally new applications, such as an increase in automation and parallelization, which paves the way to screening and systematic testing analysis. These findings offer the opportunity for the future development of a fast, low-cost and high-throughput analytical tool capable of detecting known and unknown modifications in nucleic acids (DNA and RNA) and, thus, opening new windows in biology and beyond.[3] For instance, we may foresee a method based on, first, restricted digestion of long genomic DNA into shorter fragments that, secondly, will be analyzed by SERS combined with microfluidics for rapid and ultrasensitive analyte screening. Finally, the acquired spectra will be compared with a previously constructed SERS library comprising the fingerprint vibrational spectra of an ample set of synthetically modified DNA.

Acknowledgements

The work was funded by: European Research Council (CrossSERS, FP7/2013 329131, PrioSERS FP7/2014 623527), Ministerio de Economia y Competitividad (CTQ2011-23167 and CTQ2014-59808R), Generalitat de Catalunya (2014-SGR-480), and Medcom Advance SA.

Keywords: adductomics · cytosine · DNA · nucleobase modifications · surface-enhanced Raman scattering

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 13650–13654
Angew. Chem. **2015**, *127*, 13854–13858

[1] T. Carell, C. Brandmayr, A. Hienzsch, M. Muller, D. Pearson, V. Reiter, I. Thoma, P. Thumbs, M. Wagner, *Angew. Chem. Int. Ed.* **2012**, *51*, 7110–7131; *Angew. Chem.* **2012**, *124*, 7220–7242.
[2] C.-X. Song, C. Yi, C. He, *Nat. Biotechnol.* **2012**, *30*, 1107–1116.
[3] M. Lee, B. Kim, V. N. Kim, *Cell* **2014**, *158*, 980–987.
[4] a) S. Balbo, R. J. Turesky, P. W. Villalta, *Chem. Res. Toxicol.* **2014**, *27*, 356–366; b) J. K. Nicholson, E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, S. Pettersson, *Science* **2012**, *336*, 1262–1267.
[5] a) X. He, S. Chang, J. Zhang, Q. Zhao, H. Xiang, K. Kusonmano, L. Yang, Z. S. Sun, H. Yang, J. Wang, *Nucleic Acids Res.* **2008**, *36*, D836–D841; b) A. Portela, M. Esteller, *Nat. Biotechnol.* **2010**, *28*, 1057–1068.
[6] G. Jia, Y. Fu, X. Zhao, Q. Dai, G. Zheng, Y. Yang, C. Yi, T. Lindahl, T. Pan, Y.-G. Yang, C. He, *Nat. Chem. Biol.* **2011**, *7*, 885–887.
[7] M. Münzel, D. Globisch, T. Brückl, M. Wagner, V. Welzmiller, S. Michalakis, M. Müller, M. Biel, T. Carell, *Angew. Chem. Int. Ed.* **2010**, *49*, 5375–5377; *Angew. Chem.* **2010**, *122*, 5503–5505.
[8] M. L. Metzker, *Nat. Rev. Genet.* **2010**, *11*, 31–46.
[9] N. Tretyakova, P. W. Villalta, S. Kotapati, *Chem. Rev.* **2013**, *113*, 2395–2436.
[10] a) S. Schlücker, *Angew. Chem. Int. Ed.* **2014**, *53*, 4756–4795; *Angew. Chem.* **2014**, *126*, 4852–4894; b) F. Casadio, M. Leona, J. R. Lombardi, R. Van Duyne, *Acc. Chem. Res.* **2010**, *43*, 782–791; c) R. S. Golightly, W. E. Doering, M. J. Natan, *ACS Nano* **2009**, *3*, 2859–2869.
[11] a) S. R. Panikkanvalappil, M. A. Mahmoud, M. A. Mackey, M. A. El-Sayed, *ACS Nano* **2013**, *7*, 7524–7533; b) L.-J. Xu, Z.-C. Lei, J. Li, C. Zong, C. J. Yang, B. Ren, *J. Am. Chem. Soc.* **2015**, *137*, 5149–5154; c) E. Papadopoulou, S. E. J. Bell, *Angew. Chem. Int. Ed.* **2011**, *50*, 9058–9061; *Angew. Chem.* **2011**, *123*, 9224–9227; d) L. Guerrini, Ž. Krpetić, D. van Lierop, R. A. Alvarez-Puebla, D. Graham, *Angew. Chem. Int. Ed.* **2015**, *54*, 1144–1148; *Angew. Chem.* **2015**, *127*, 1160–1164; e) M. Masetti, H.-n. Xie, Ž. Krpetić, M. Recanatini, R. A. Alvarez-Puebla, L. Guerrini, *J. Am. Chem. Soc.* **2015**, *137*, 469–476.
[12] S. Ito, L. Shen, Q. Dai, S. C. Wu, L. B. Collins, J. A. Swenberg, C. He, Y. Zhang, *Science* **2011**, *333*, 1300–1303.
[13] a) M. Whiteman, A. Jenner, B. Halliwell, *Chem. Res. Toxicol.* **1997**, *10*, 1240–1246; b) C. Badouard, M. Masuda, H. Nishino, J. Cadet, A. Favier, J. L. Ravanat, *J. Chromatogr. B* **2005**, *827*, 26–31.
[14] M. D. Evans, M. Dizdaroglu, M. S. Cooke, *Mutation Res. Rev. Mutation Res.* **2004**, *567*, 1–61.
[15] D. van Lierop, Z. Krpetic, L. Guerrini, I. A. Larmour, J. A. Dougan, K. Faulds, D. Graham, *Chem. Commun.* **2012**, *48*, 8192–8194.
[16] a) J. M. Benevides, S. A. Overman, G. J. Thomas, *J. Raman Spectrosc.* **2005**, *36*, 279–299; b) P. Y. Turpin, L. Chinsky, A. Laigle, B. Jolles, *J. Mol. Struct.* **1989**, *214*, 43–70.
[17] a) M. Shanmugasundaram, M. Puranik, *J. Raman Spectrosc.* **2009**, *40*, 1726–1748; b) V. K. Rastogi, M. A. Palafox, K. Lang, S. K. Singhal, R. K. Soni, R. Sharma, *Indian J. Pure Appl. Phys.* **2006**, *44*, 653–660.
[18] a) W. Suen, T. G. Spiro, L. C. Sowers, J. R. Fresco, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4500–4505; b) C. P. Beetz, G. Ascarelli, *Spectrochim. Acta Part A* **1980**, *36*, 299–313.
[19] M. Alcolea Palafox, V. K. Rastogi, S. Kumar, H. Joe, *Spectrochim. Acta Part A* **2013**, *111*, 104–122.
[20] W. S. Ross, C. C. Hardin, I. Tinoco, S. N. Rao, D. A. Pearlman, P. A. Kollman, *Biopolymers* **1989**, *28*, 1939–1957.
[21] J. G. Duguid, V. A. Bloomfield, J. M. Benevides, G. J. Thomas, *Biophys. J.* **1996**, *71*, 3350–3360.
[22] E. K. Sackmann, A. L. Fulton, D. J. Beebe, *Nature* **2014**, *507*, 181–189.
[23] R. Bro, S. De Jong, *J. Chemom.* **1997**, *11*, 393–401.
[24] A. Barhoumi, N. J. Halas, *J. Phys. Chem. Lett.* **2011**, *2*, 3118–3123.

Received: August 17, 2015
Revised: September 6, 2015
Published online: October 8, 2015