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DNA detection by dye labeled oligonucleotides using surface enhanced Raman spectroscopy

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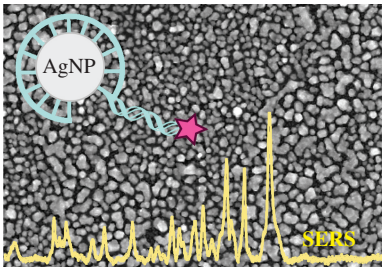
Dye labeled oligonucleotides have been applied to detection of DNA with high sensitivity using surface enhanced Raman spectroscopy (SERS). Duplex formation of dye labeled oligonucleotides with DNA targets, followed by selective conjugation with silver nanoparticles, made it possible to determine the optimal distance between the SERS label and the silver surface, which was necessary for strong signal enhancement. For all Rh6G labeled oligonucleotides the limits of detection were 3 fmol dm^{-3}, while for Cy3 labeled oligonucleotides the signal enhancement depended on the distance between Cy3 dye and silver nanoparticles.



Keywords: optical sensors, surface enhanced Raman spectroscopy, silver nanoparticles, dye labeled oligonucleotides, DNA detection, specific sequence, promoter of human telomerase catalytic subunit gene.

Genome stability is known to be crucial for all organisms, while DNA mutations occur regularly due to external and internal influence causing aging, neurodegenerative diseases and cancer. Thus, identification of such changes in DNA with required sensitivity and accuracy allows one to perform reliable diagnostics and predict various diseases.[1,2] The methods currently used for mutation diagnostics are based on preliminary amplification of DNA fragments using a polymerase chain reaction (PCR) followed by hybridization with labeled probes and detection of the resulting fluorescence.[3,4] However, considerable DNA fragmentation in extracellular fluids hampers an identification of cell-free DNA through the preliminary amplification, resulting in a bias.[5] Besides, damage of nucleotides leads to a decrease in the hybridization efficiency and to blocking the primer elongation in PCR. All these factors constrain the detection of small amounts of DNA as well as the DNA samples extracted from long-stored biomaterials.[6] Moreover, the errors during PCR amplification lower the accuracy of multiplex sequence determination and the sensitivity of single nucleotide polymorphism detection. As a result, the detection limit for identification of mutations increases together with the probability of false positive results. Thus, the established techniques for mutations determination in individual DNAs are time consuming and have poor reproducibility, therefore the limitations mentioned above evoke the search for new approaches to the determination of short damaged DNA in biological fluids with no amplification step. So, the development of sensor systems for biomedical diagnostics application is now of high interest.

The surface enhanced Raman spectroscopy (SERS) technique provides a simple, reliable, high-throughput and rapid detection of various analytes.[7–9] Due to the utilization of surface-distributed clusters, typically nanoparticles, the enhancement of inelastic scattering signal of optical radiation for target molecules can reach 2–12 orders of magnitude. A particular use of SERS is governed by plasmonic properties of nanoparticles.[10] Thus, silver nanoparticles (AgNPs) are well-known robust SERS signal enhancers.[11] In the area of detection and characterization of biomolecules, the advantages of SERS include selectivity,[12] ability to multiplex due to the narrow band width of 1–2 nm,[13] the possibility to detect single molecules,[14] flexibility in the choice of SERS labels[8] as well as the absence of signal quenching by water,[15] oxygen or other species.[7,13] In spite of the existence of various synthetic approaches to the preparation of stable SERS-active nanoparticles, there is a high demand for robust techniques for the synthesis of AgNPs aggregates without additional stabilizing reagents, which can generate Raman signal interfering with a signal of the target analyte.[16–19]

Several SERS approaches were proposed for the detection of nucleic acids.[20,21] It is known that the sensitivity of Raman DNA detection can be improved by the presence of molecules having an electronic transition in resonance with laser excitation, these molecules being called SERS labels. In this case, the surface enhancement is accompanied by an additional resonant intensity gain up to ~10^3 resulting in the phenomenon of surface-enhanced resonance Raman scattering (SERRS).[22,23]

In this work, we have developed dual domain oligonucleotides for conjugation with AgNPs by their anchor domain and for binding to the target dye labeled oligonucleotides by their promoter domain (Table 1). As a target we used a specific sequence located in the promoter of a human telomerase catalytic subunit gene. We have determined optimal conditions for the

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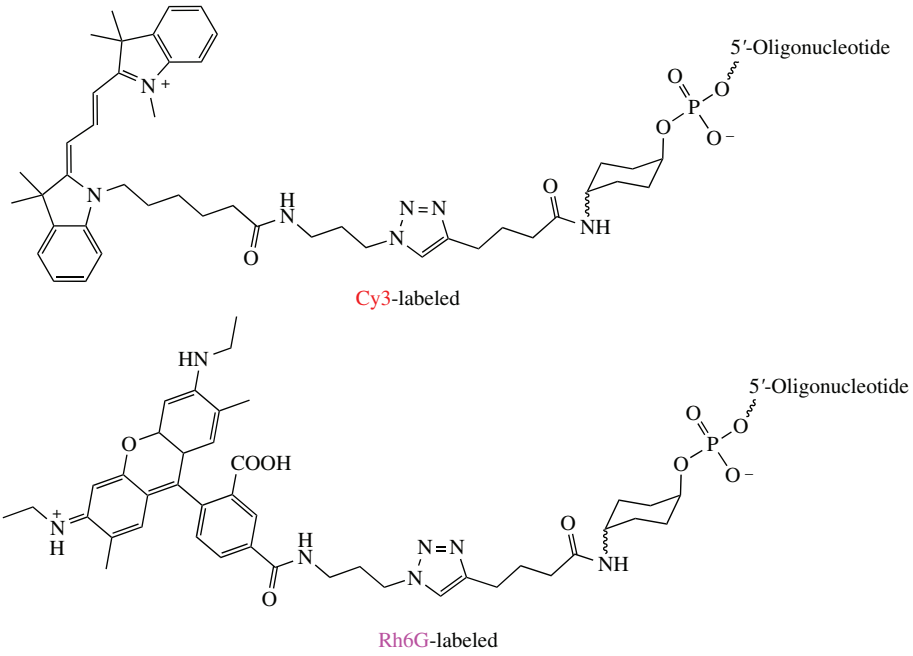


Table 1 Oligonucleotide sequences used in this work. Oligonucleotides A–D were covalently labeled by Rh6G or Cy3 at the 5′ end.

Oligonucleotide	Sequence, 5′ → 3′
Anchor 1	TGCCTTTTGGGGACGGATA
Anchor 2	CACCGCTTTTGCCTTTTGGGGACGGATA
Reverse complement Anchor 2	TATCCGTCCCCAAAAGGCAAAAGCGGTG
Reverse complement Promoter	GAGGGCCCGGAGGGGGCTGGGCCGGGGACCCGGGAGGGGTCGGGACGGG
Anchor 1–Promoter	TGCCTTTTGGGGACGGATACCGTCCCGACCCCTCCCGGGTCCCCGGCCCAGCCCCCTCCGGGCCCTC
Anchor 2–Promoter	CACCGCTTTTGCCTTTTGGGGACGGATACCGTCCCGACCCCTCCCGGGTCCCCGGCCCAGCCCCCTCCGGGCCCTC
A/Dye	Dye–AAAGGGCCCGGAGGGGGCTGGGCCGGGGACCCGGGAGGGGTCGGGACGG
B/Dye	Dye–AAAGGGCCCGGAGGGGGCTGGGCCGGGGACCCGGGAG
C/Dye	Dye–AAAGGGCCCGGAGGGGGCTGGGCCG
D/Dye	Dye–AAAGGGCCCGGAG

conjugation of the dual domain nucleotides with AgNPs, for their following hybridization with target oligonucleotides and have defined the distances needed for enhancement of SERS signals of the dye labeled oligonucleotides.

First, we obtained a surface formed by AgNPs as a SERS sensor using the pyrolytic decomposition of an aqueous silver(I) oxide–ammonia complex aerosol.[24,25] Thermal reduction of silver(I) using ultrasonic silver rain (USSR)[16,24] on preheated to 280–300°C thin glass substrate resulted in a pure nanostructured silver coatings (Figure 1). Each aerosol droplet of 1–5 μm diameter served as a micro reactor and provided an interlacing 'coffee' ring structure [Figure 1(*c*)] of AgNPs clusters of 90 ± 5 nm size. This pyrolytic synthesis of AgNPs did not require any stabilizing or reducing agents, which was important for their further application as stated above. A valuable feature of the developed SERS sensing silver substrate is the wide plasmonic band, which covers the whole visual wavelength range starting from *ca.* 420 nm. This allows one to record strongly enhanced Raman signal from most common dyes, including fluorescein, rhodamine, cyanine and BODIPY. However, for successful SERS/SERRS application, the coincidence of the following parameters is highly desirable: (i) plasmonic band of NPs, (ii) absorbance range of the analyte and (iii) laser wavelength. As commercially available Ar^+ and He–Ne lasers have wavelengths in the visible range, *viz.*, 514.5 and 633 nm, respectively, we selected and synthesized dye labeled oligonucleotides bearing rhodamine 6G (Rh6G, $\lambda_{max} \approx 524$ nm) and Cy3 ($\lambda_{max} \approx 550$ nm) moieties as had been described.[26] Using the dye labeled oligonucleotides A–D (see Table 1) complementary to different regions of the oligonucleotides bound to AgNPs, we could control the distance between SERS-active AgNPs and the dye moiety. For details, see Online Supplementary Materials.

Hybridization of a target DNA with oligonucleotides immobilized on solid phase is typically limited by diffusion. Thus, as a rule, a hybridization stage for microarray analysis is performed for 10–20 h.[27] Therefore, we have chosen the probe–target hybridization in solution followed by selective immobilization on AgNPs using specific nucleotide sequence (Scheme 1 and Online Supplementary Materials).[28]

While the target probe was modified with these dyes at the 5′-end, we observed several major bands in the Raman spectra for Cy3 and Rh6G labeled oligonucleotides (see Figure 1). For the Rh6G labeled oligonucleotide sequence, Raman signals at 1183, 1361, 1508, 1572 and 1650 cm^{-1} corresponded to C–H in-plane bending/xanthene ring/NHEt group, C–C stretching/xanthene ring/amine group, xanthene ring/amine group, C–C stretching/phenyl ring and C–C stretching/xanthene ring, respectively.[29] All the peaks in SERS spectra were identical to the corresponding Raman peaks for probes A–D.

Next, we optimized the length of oligonucleotides to enhance the sensitivity of SERS detection. Both anchors were equally efficient for the conjugation to AgNPs (Scheme 1), and we could not observe the differences in their SERS spectra. Then we investigated the dependence of signal intensity on the length of the duplex region after an oligonucleotide binding to the probe and the conjugation with

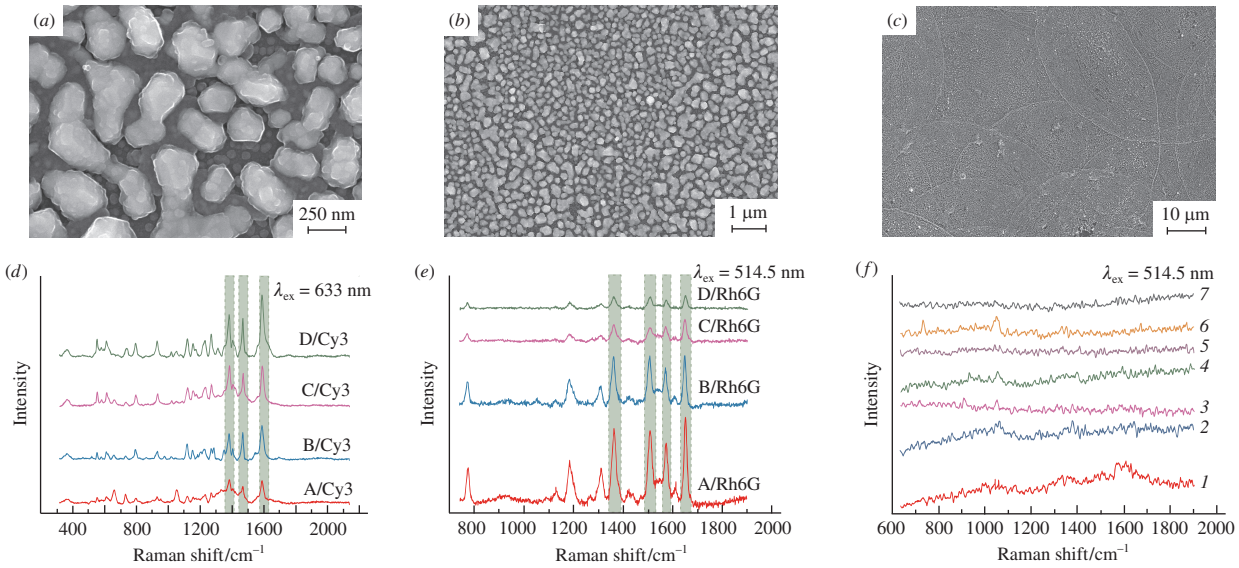


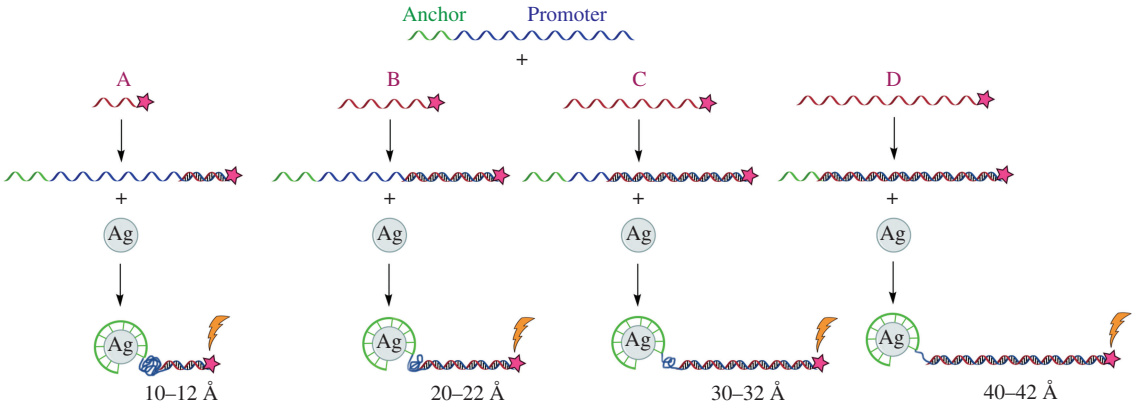
Figure 1 SERS-active USSR hierarchic silver surface structure under different magnification: (*a*) and (*b*) SEM details of silver clusters of different size inside the silver rings; (*c*) rims and interiors of silver rings formed after deposition of a layer of nanostructured silver. SERS/SERRS spectra on the USSR surface: (*d*) for Anchor 1–Promoter duplex with Cy3 labeled oligonucleotides A–D, measured using a 20 mW 633 nm He–Ne laser; (*e*) for Anchor 1–Promoter duplex with Rh6G labeled oligonucleotides A–D, measured using a 17 mW 514.5 nm Ar^+ laser; (*f*) Raman spectra on the silver surface, measured using the 514.5 nm laser: (*1*) no analyte; (*2*) mixture of Anchor 1 with Rh6G–oligonucleotide C; (*3*) mixture of Anchor 2 with Rh6G–oligonucleotide C; (*4*) mixture of Reverse Anchor 2 with Rh6G–oligonucleotide C; (*5*) mixture of Reverse Promoter with Rh6G–oligonucleotide C; (*6*) Anchor 1–Promoter and (*7*) Anchor 2–Promoter.

AgNPs. Double-stranded DNA (dsDNA) part is known to be rigid and thus sets the distance between a dye and AgNP, while single-stranded DNA (ssDNA) part is less rigid. To determine the optimal distance between dyes and AgNPs, we varied the length of double-stranded region formed due to hybridization. As follows from Figure 1(*d*) and (*e*), the dependences of Raman intensity on the distance between dyes and AgNPs were found to be opposite for Rh6G label as compared to the Cy3 one. Then we paid attention to the response of the proposed system with the purpose to avoid false positive data. The comparison of results for (i) oligonucleotide that can bind only to AgNP, (ii) full-size probe with a complementary oligonucleotide, which blocks the anchor part and therefore the interaction with AgNPs (see Scheme 1) and (iii) inverted sequence of the recognition domain, demonstrated the absence of non-specific interactions of signals [Figure 1(*f*)]. The USSR surface itself also demonstrated an absence of peaks with significant intensity [see Figure 1(*f*), spectrum (*1*)]. In the case of Rh6G labeled probes, we achieved the lowest limits of detection (LODs) of 3 fmol dm^{-3}, while LODs for the corresponding Cy3 probes were 1–2 orders of magnitude higher (Table 2). We propose that such a significant difference is a result of better fitting of the laser wavelength to the Rh6G absorption maximum. Enhancement factor (EF) value was calculated as

$$EF = \frac{I_{SERS}}{I_{RS}} \cdot \frac{c_{RS}}{c_{SERS}},$$

where I_{SERS} and c_{SERS} were analytical signal intensity and concentration of oligonucleotide on the SERS sensor, respectively, I_{RS} and c_{RS} were analytical signal intensity (normalized by power neutral density filter according to I_{SERS}) and concentration of the dye on the same surface without AgNPs, respectively. EF values are presented for the signal with the specified Raman shift and 0.01 μM concentration of the oligonucleotides on SERS sensor as well as 1 μM concentration of the dyes the same conditions but without AgNPs. Despite the differences in the enhancement gain [Figure 1(*e*)], all the investigated Rh6G labeled oligonucleotides allowed one to detect DNA with equal LOD values. High values of coefficient *b* in the linear dependence (see Table 2) confirm that performance of the developed sensor system is limited by the ability to find location of the target analyte on the AgNP surface.

In summary, we have applied silver nanoparticles as a surface-enhancer together with dye labeled oligonucleotides for an additional resonance gain of SERS intensity to detect and



Scheme 1 DNA detection by SERS spectroscopy. The lightning sign indicates the appearance of an amplified specific signal.

Table 2 Numerical results for Anchor 1–promoter DNA determination using Rh6G and Cy3 labeled oligonucleotides with different lengths.

Oligonucleotide	EF	Raman shift/cm^{-1}	Linear dependence[a] $I = (a \pm \Delta a)\ \lg c + (b \pm \Delta b)$	Linear range/pM	LOD[b]/fM	r
Rh6G labeled oligonucleotides, λ_{ex} = 514.5 nm						
A	*ca.* 2.3×10^4	1650 ± 1[c]	$I = (5.6 \pm 0.4)\times10^3\ \lg c + (9.3 \pm 0.6)\times10^4$	0.01–10	3	0.994
B	*ca.* 2.0×10^4	1651 ± 2	$I = (6.7 \pm 0.5)\times10^3\ \lg c + (9.5 \pm 0.6)\times10^4$	0.01–1000	3	0.975
C	*ca.* 1.0×10^4	1651 ± 2	$I = (3.8 \pm 0.2)\times10^3\ \lg c + (5.2 \pm 0.4)\times10^4$	0.01–1000	3	0.977
D	*ca.* 2.6×10^3	1650 ± 2	$I = (6.9 \pm 0.3)\times10^2\ \lg c + (1.1 \pm 0.1)\times10^4$	0.01–100	3	0.987
Cy3 labeled oligonucleotides, λ_{ex} = 633 nm						
A	*ca.* 7.3×10^3	1590 ± 2	$I = (8.1 \pm 0.6)\times10^2\ \lg c + (2.2 \pm 0.1)\times10^4$	0.1–1000	30	0.992
B	*ca.* 6.5×10^3	1590 ± 2	$I = (5.3 \pm 0.5)\times10^2\ \lg c + (1.8 \pm 0.1)\times10^4$	0.1–100	50	0.981
C	*ca.* 3.7×10^3	1590 ± 1	$I = (4.1 \pm 0.3)\times10^2\ \lg c + (1.1 \pm 0.1)\times10^4$	0.1–100	30	0.987
D	*ca.* 3.5×10^2	1591 ± 2	$I = (2.7 \pm 0.2)\times10^2\ \lg c + (2.9 \pm 0.1)\times10^4$	1–1000	300	0.988

[a] I is Raman intensity of characteristic peaks in SERS spectra (arbitrary units), c is concentration of analyte in the initial analyzed solution in mol dm^{-3}. [b] LOD is limit of detection. [c] Confidence intervals were calculated for n = 5 and P = 0.95 in t-distribution.

quantify DNA bearing a specific promoter sequence of a human telomerase catalytic subunit gene. We have developed an approach to DNA detection with limit of detection of 3 fmol dm^{-3} and optimized the enhancing factor by varying the distance between the SERS label and AgNP. Thus, we propose that the quantification of a specific DNA in biological samples can be carried out by monitoring the SERS characteristic bands and by measuring the intensity of Raman signals according to calibration curves. For Rh6G labeled oligonucleotide, the distance between AgNPs and the dye as low as *ca.* 10–12 Å resulted in gaining simultaneously huge surface and resonant enhancement of Raman scattering, which proved to be enough for oligonucleotide detection at femtomolar concentration. The proposed approach can be applied for the development of highly sensitive analytical methods as well as for accurate input control and identification of DNA, including accelerated analysis of biological fluid samples for monitoring and diagnostics both in laboratory and under point-of-care conditions.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.01.006.

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