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High-sensitivity label-free electrochemical genosensors for carbon nanotube plasmon-assisted detection of somatic mutations in nucleic acids from formalin-fixed paraffin-embedded tissues

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Highlights

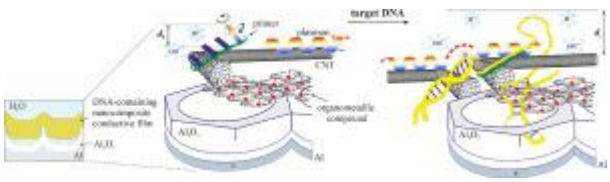
- Novel and highly sensitive non-Faradaic-impedimetric genosensors were developed.
- The label- and enzyme-free EIS sensors discriminate genotype of highly fragmented DNA.
- The genosensors operate on plasmon-assisted effects in DNA-CNT conjugate assemblies.
- The assay is capable of detecting up to 1 aM of single base-mismatched oligonucleotide.

- The method allows to identify allele SNP of native genomic DNA with LOD of 0.33 ng/ml.

Abstract

Currently, molecular genetic testing of somatic point mutations in the genome of cancer tumors for targeted therapy requires high-performance tools for studies of spatial gene expression and spatial genome profiling in formalin-fixed paraffin-embedded (FFPE) tissue samples. We offer new high-performance label-free electrochemical impedimetric deoxyribonucleic-acid (DNA) nanosensors. The sensors based on a platform of crystalline carbon nanotube plasmonic assemblies are fabricated by the Langmuir–Blodgett deposition technique. The carbon-nanotube assemblies are suspended on nanoporous supports. Incorporation of fast Fourier transforms in the impedance spectroscopy led to the introduction of a new information parameter, the integral value of capacitance changes. The allele-sensitive assay based on plasmon-assisted effects in the assemblies of electrically active conjugates between DNA and few-walled carbon nanotubes has been used for discrimination of allele single-nucleotide polymorphisms (SNPs) of Kirsten Rat Sarcoma viral oncogene homologue (KRAS gene). Using 19- and 20-mer single-stranded (ss) probe oligonucleotides and 35- and 47-mer toehold probes, we explored the probe length and the target location. The genotyping technology allows discrimination of single-nucleotide variations in the target 35-base oligonucleotide at the attomolar concentration level. Human genomic DNAs were isolated from FFPE colorectal cancer tumor tissue samples. The KRAS-gene exon 2, codon 12, c.35G>A mutation has been successfully discriminated in the genomic DNAs with a significance level, P, of 0.001–0.02. The assay has a sensitivity with 0.33-ng/ml limit of detection (LOD) for native genomic DNA.

Graphical abstract



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Introduction

Up to now, it has been an understanding that a successful recovery for cancer patients is possible if genetic make-up of the tumor is taken into account during treatment [1]. Emerging in evolution process, descendant tumor clones vary widely in a huge number of mutations (genetic variants, alternative variants of the genome). Such genomic instability of the tumors hampers treatment strategies based on the deoxyribonucleic-acid (DNA) diagnostics [2]. This problem is solved by means of diagnostics of consistent genes with key driver mutations [3]. Among them, the Kirsten Rat Sarcoma viral oncogene homologue (KRAS) mutation is the most frequent one. Allele discrimination of single-nucleotide variations (SNVs) in KRAS-gene plays a key role for personalized targeted therapy prescription for patients with colorectal cancer [4].

The point-mutation percentage in tumor tissue is from more than 1% (metastatic cancer) to 0.01%–0.001% (cancer at early stages) [5], [6], [7] and, correspondingly, a large number of PCR (polymerase chain reaction) cycles are required for sequencing. But in this case even through correcting PCR amplification biases (errors) by attaching common molecular identifiers (CMIs) to molecules in sequencing, such next-generation-sequencing (NGS) technologies as Illumina, PacBio, ONT (Oxford Nanopore Technologies) correctly diagnose CMIs in 73.36, 68.08 and 89.95% of all case, respectively [8].

In order to increase the fraction of mutant nucleic acids in a sequencing sample, the selection of tumor tissue sites (dissections) has to be carried out according to morphological features of the cancer clones and their microenvironment. To reveal the tumor tissue morphology, the dissections of tumor tissue are formalin-fixed and paraffin-embedded (FFPE). These sections consist of about 100 mutant clone cells (approximately 5000 KRAS-gene copies) [9]. Unfortunately, the formalin fixation procedure affects the functional cell state depending on the formalin-fixation time [10] and, correspondingly, the determination of tumor-cell microenvironment becomes ambiguous. The sensitivity of modern sequencers is appropriate to genotype the nucleic acid samples in which correct fragments are comprised of sequences with the length of more than **300** nucleotides [11]. Moreover, modern optical and electrochemical sequencing techniques detect the DNA hybridization signal being amplified by enzymes such as DNA polymerase in PCR [12]; DNA ligase, phi29, for isothermal rolling circle amplification [13]; nicking endonuclease [14]. Since (1) nucleic acids from FFPE tissue samples are hardly fragmented through hydrolysis of phosphodiester bonds in the sugar-phosphate backbone, (2) abasic sites are formed due to hydrolysis of N-glycosylic bonds, and (3) formaldehyde presented as a contaminant in DNA samples from the FFPE dissections, may crosslink subunits of the enzymes with each other or with nucleic acids [15], the effectiveness of the enzyme-based NGS methods for genomic DNA from FFPE samples is drastically decreased. The high level of fragmentation of the nucleic acids and the contamination impede sequencing. Due to this, for example, the mutation-specific multiplex PCR allows detection of KRAS mutations in codons 12 and 13 only when more

than 10% of a whole FFPE tissue sample (for example, 25%–30% using Idylla^{TM} RAS-BRAF mutation test) is comprised of metastasizing colorectal cancer (CRC) tumor cells [16], [17].

Damaged-DNA detection using currently devised electrochemical enzyme-labeled or plasmonic genosensors which are based on neutral peptide nucleic acids and utilize single-walled carbon nanotubes (SWCNTs) or gold nanoparticles, respectively, to detect KRAS-oncogene SNV without amplification [18], [19], will also be difficult due to the fact that remaining reactants of the formalin fixing treatment confine nonspecific bio-inclusions by inter- and intramolecular cross-links between amino acids of peptide chains and nucleic acids in the detecting enzyme-containing layer for electrochemical sensor or in the 100-nm region of plasmon near-field distribution for the plasmonic sensor, respectively.

Thus, sequencing methods need novel platforms of high- performance and highly specific SNV detection to solve the problem of tumor genotyping.

Graphene-based materials and, in particular, rolled-up atomically-thin carbon layers called carbon nanotubes (CNTs) with few- nanometers plasmon near-field distribution are promising materials for the development of high-performance electrochemical and optical transducers of DNA-hybridization signals [20].
DNA-sensor techniques based on the non-Faradaic electrochemical impedance spectroscopy (EIS) methods record complex dielectric permittivity of the Helmholtz electrical double layer without the use of both expensive redox couples and devices based on direct currents, e.g., chronoamperometry [21] and differential pulse voltametry [22].

Carboxylated SWCNTs could be used as an adhesive material which confines the target DNA in the Helmholtz layer due to van der Waals stacking interaction of nucleotide bases with SWCNTs. It allows enhancement of the sensitivity of a label-free high-frequency (~ 300 kHz) non-Faradaic DNA sensor up to 1 fM for complementary (perfect) hybridization despite the probe oligonucleotide is covalently bound to SWCNTs and low density and disordered packing of SWCNTs is in the transducer [23], [24]. However, the sensitivity of DNA sensor reduces significantly when a DNA target sequence with a single mismatched nucleotide should be recognized. For example, an increase of the limit of detection value by two and six orders of magnitude for perfect-matched and single base-mismatched DNAs, respectively, was observed when a long DNA probe (21-base oligonucleotide) was used instead of a short DNA (10-base oligonucleotide) [25]. Moreover, two gene alleles (normal wild-type allele and mutant allele) in colorectal cancer tissue are often contained in various concentrations. The allele single-nucleotide

polymorphism (SNP) discrimination is a challenge due to the low selectivity of the impedimetric DNA sensors.

Providing that DNA molecules such as the biotinylated DNA primers are non-covalent immobilized on the electrode insulating layer by streptavidin-biotin affinity binding, a response of non-Faradaic DNA detectors to the signal of hybridization between perfectly matched target and probe (primer) DNAs arises at 10 aM concentration and higher [26].

So, improvement and optimization of the label-free DNA EIS techniques for work in clinical conditions (FFPE tissue samples) is an important and primary issue of research.

The Langmuir–Blodgett (LB) technique has been used for fabricating crystalline CNT assemblies for plasmonic applications [27]. The cross-linking of the highly ordered carbon-nanotube arrays with each other by dsDNA target molecules results in the formation of a conductive network that improves the ability of CNT arrays to screen electric fields [28], [29]. The use of the conductive CNTs vertically and horizontally aligned arrays as electroactive mediator inclusions in the Helmholtz layer could greatly increase the specificity, selectivity, and sensitivity of the impedimetric non-Faradaic DNA sensor.

The main goal of this paper is to develop high-performance label-free enzyme-free electrochemical non-Faradaic sensors for KRAS allele genotyping in FFPE tumor tissue samples.

Section snippets

Reagents and samples

The following single-nucleotide variation mutation: c.35G>A site, p.G12D, located in the exon 2 of the human KRAS gene (NCBI/Gene KRAS genomic DNA sequence NC_000012.12; NCBI/Gene KRAS mRNA var d sequence NM_001369787.1), is recognized by using hybridization DNA probes. The single-stranded 19-base (perfectly-matched $KRAS_w$) and 20-base (single-base-mismatched $KRAS_m$) oligonucleotides, were used as single-stranded DNA (ssDNA) probes. The 35- and 47-base oligonucleotides (named as 35-mer P3 and …

Operating principle of hybridization-mediated SNV detection

Fig. 1a depicts the transducer layers being deposited on Al electrodes. An applied electric field, $\overrightarrow{E}$, is screened by the dipole polarization, $\overrightarrow{P}$, of the near-electrode layer (see Fig. 1a). The near-electrode polarization attenuates this field to $\overrightarrow{E}_{screen}$. The surface-polarization effects originate from the changes in the complex dielectric permittivity, $\epsilon = \epsilon' + i\epsilon''$, of the transducer during DNA hybridization. The discharging (charging) current I_{ch} is produced by the polarization $\overrightarrow{P}$ in the RC …

Discussion

In this study, we have elucidated the carbon nanotube plasmon-assisted detection of point mutations in damaged DNA by high-sensitive non-Faradaic genosensors. The graphene plasmon states are excited by the left- and right-handed circularly polarized electromagnetic radiation [43], [44] from the THz resonances of the cage of water molecules around solvated ions and dipoles [45], [46].

The electrically neutral configurations of the electron–hole plasma (Dirac fluid) are excited in the flattened …

Conclusions

We have proposed novel enzyme-free electrochemical non-Faradaic sensors for genotyping damaged genomic DNA isolated from FFPE tissue samples. The allele-sensitive assay is based on the plasmon-assisted effects in assemblies of electrically active conjugates comprising DNA and FWCNTs. The electrochemical genotyping technologies for KRAS-gene mutation-status detection are promising for applications in molecular diagnostics of colorectal tumors with allele SNVs with accuracy up to attomolar …

CRediT authorship contribution statement

V.P. Egorova: Writing – review & editing, Methodology, Investigation. **H.V. Grushevskaya:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **N.G. Krylova:** Writing – original draft, Software, Investigation. **E.V. Vaskovtsev:** Investigation. **A.S. Babenka:** Writing – review & editing, Investigation, Data curation, Conceptualization. **I.V. Anufreyonak:** Investigation. **S.Yu. Smirnov:** Investigation. **G.G. Krylov:** Writing – review & …

Declaration of competing interest

The authors have no conflicts of interest to declare. …

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