Integrated planar terahertz resonators for femtomolar sensitivity label-free detection of DNA hybridization

Michael Nagel, Peter Haring Bolivar, Martin Brucherseifer, Heinrich Kurz, Anja Bosserhoff, and Reinhard Büttner

A promising label-free approach for the analysis of genetic material by means of detecting the hybridization of polynucleotides with electromagnetic waves at terahertz (THz) frequencies is presented. Using an integrated waveguide approach, incorporating resonant THz structures as sample carriers and transducers for the analysis of the DNA molecules, we achieve a sensitivity down to femtomolar levels. The approach is demonstrated with time-domain ultrafast techniques based on femtosecond laser pulses for generating and electro-optically detecting broadband THz signals, although the principle can certainly be transferred to other THz technologies.

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1. Introduction

Owing to the exceptional specificity of complementary DNA strands to hybridize, most approaches for rapidly identifying genetic sequences are based on detecting the hybridization of unknown DNA molecules (target) to known single-stranded oligonucleotide or polynucleotide molecules (probe DNA). Hence when we measure the binding state of DNA molecules, i.e., the hybridization of target and probe molecules, genes can readily be identified. Most approaches for hybridization detection are based on the fluorescent labeling of the target DNA and optical detection of the fluorophores. Although fluorescent labeling has given rise to extremely efficient high-throughput diagnostic systems,[1–3] an extremely large interest for label-free detection schemes exists. Labeling not only constitutes an unwanted additional preparatory step complicating the analytic procedures; labeling also possibly modifies the DNA strand conformation[4] and can therefore deteriorate the precision of gene detection. In addition, fluorophore degradation, varying labeling yield, and fluorescence efficiency site dependencies can seriously deteriorate the quantifiability of fluorescence-based genetic diagnostic systems.[5–7] Numerous alternatives for the label-free detection of hybridization have been developed, including dielectric,[8,9] evanescent wave,[10] electrostatic,[11] electrochemical,[12] mass sensitive,[13] acoustic wave,[14] and nanomechanical[15] probing. None of the approaches, however, has reached the maturity necessary to replace standard fluorescence-based approaches.

Theoretical calculations predict numerous intrinsic resonances in the terahertz (THz) frequency range associated with interbackbone excitations of DNA molecules (e.g., propeller-twist, hydrogen-bond breathing, base-roll, and base-shift vibrational modes),[17–19] providing a unique potential of THz-probing technologies for the label-free detection of the DNA binding state. Although investigations by Raman,[20,21] Fourier-transform,[22] or time-domain[23] THz techniques on hybridized DNA molecules have been performed in the past, only a few experiments have addressed binding-state-dependent analysis, e.g., by means of temperature-dependent denaturation analysis.[24] Recently, the interplay between the binding state of DNA and its complex refractive index at THz frequencies has been investigated by us, presenting a proof-of-principle for a new method of label-free gene detection[16]: With a free-space time-domain THz de-

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tection scheme a clear difference between the THz transmission spectra of hybridized (double-stranded) DNA films and denatured (single-stranded) counterparts of DNA molecules was observed, demonstrating a significantly higher refractive index and absorption for hybridized DNA. The major drawback of this free-space approach is the large amount of DNA required for reliable signal responses. Here we present guided THz wave approaches for monitoring the binding state of DNA molecules, which drastically reduce the DNA material requirements, improve the sensitivity, and open the path for integrated THz gene chips. Thin-film microstrip lines[25] are used to guide THz signals in plane through the thin DNA films, thereby drastically enhancing the interaction length of DNA with the THz waves and increasing the detection sensitivity in comparison with our former free-space approach. A further drastic sensitivity increase is obtained by integration of passive THz resonator structures on the waveguides, which amplify the measured DNA-specific differences of the THz signal transmission associated with the binding state of the tested DNA at a specific resonant frequency. The idea of using resonator configurations for monitoring dielectric properties is well known and frequently used at lower frequencies.[26] With the THz resonator approach a sensitivity increase down to femtomolar DNA molecule detection levels for this integrated THz analytic system is demonstrated. Although our approach is demonstrated with time-resolved THz technology, the principle can certainly be transferred to other THz techniques.

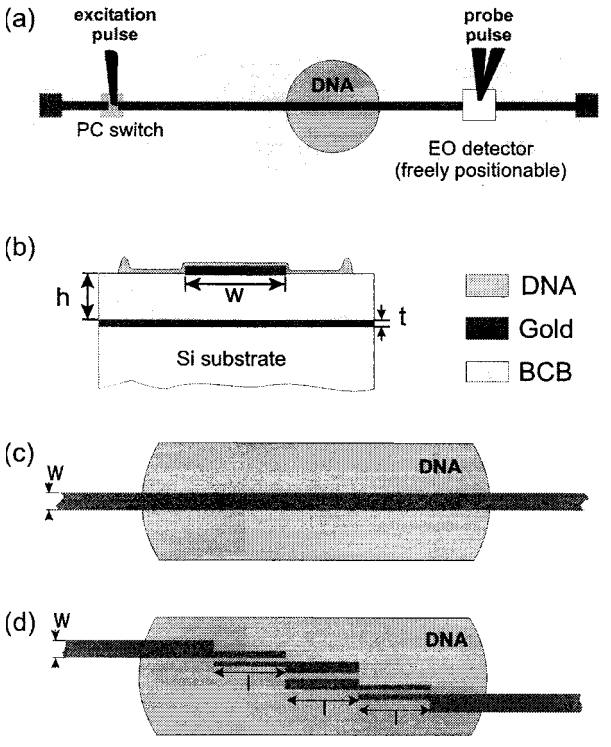

Fig. 1. Scheme of the experimental setup and employed THz probes: (a) operation principle of the analytic system including PC signal generation, microstrip waveguide with DNA spot and free-positionable electro-optic (EO) prober; (b) cross section through the microstrip waveguide; (c) first approach using the microstrip waveguide as signal transducer under the DNA spot; (d) enhanced sensitivity approach using a THz resonator (bandpass filter) as a far more efficient transducer.

2. Experiment

The analytic system used is based on femtosecond laser technology using integrated ultrafast photoconductive (PC) switches of low-temperature GaAs as THz signal sources with a usable bandwidth extending from 20 GHz to 2 THz[25] and time-resolved electro-optic detection.[27] A scheme of the complete test structure is depicted in Fig. 1(a). The integrated biased PC switch is optically excited to generate a subpicosecond electrical pulse that propagates along the attached thin-film microstrip line. The microstrip lines are made from gold with benzocyclobutene (BCB) as dielectric material. A cross section of the microstrip lines is shown in Fig. 1(b). They exhibit a width of $w = 14$ μm, a thickness of $t = 0.5$ μm, and a dielectric layer thickness of $h = 5.9$ μm. The THz signal passes through the DNA sample deposited on the test structure, and the transmitted signal further propagates along the thin-film microstrip line. Electro-optic sampling of the input and the transmitted electromagnetic pulses is applied to evaluate the complex transmission parameters (S_{21}) through the THz DNA sensor.

Denatured and hybridized DNA samples are prepared for testing the capabilities and sensitivity of our hybridization-detection approach. In all experiments presented here we use as DNA molecule the vector pcDNA3 (Invitrogen, Carlsbad, Calif.) with a size of 5.4 kilobase (kb), although the applicability of our method was also confirmed in tests with DNA of various lengths. The DNA is amplified in *E. coli* and purified with a Nucleobond PC500 system. A solution with DNA molecules with a concentration of 5 μg/μl in bidistilled H_2O is prepared. One part of the solution is denatured by means of raising the temperature to 95 °C for 5 min, followed by quenching on ice. Because the DNA strands are large, renaturation is small. Standard analytic techniques in gels confirm the predominance of hybridized and denatured DNA in the respective solutions after this preparation procedure (a cautious estimate yields that more than 90% of the molecules are in the respective state). Approximately 0.5 μl of denatured or hybridized pcDNA3 solutions are pipetted on two identical THz probers at a distance of 1 mm fabricated on the same wafer, to ensure a good intercomparability of all results. After the evaporation of the solution under normal ambient conditions, thin films of DNA with a diameter of ~0.5 mm and a thickness of 40–80 nm are obtained. The influence of the thickness variations is discussed below. A bulge with a height of ~5 μm is formed at the edge of each DNA film during the evaporation of the water.

In a first set of experiments, the microstrip line itself is used as the prober for monitoring the complex

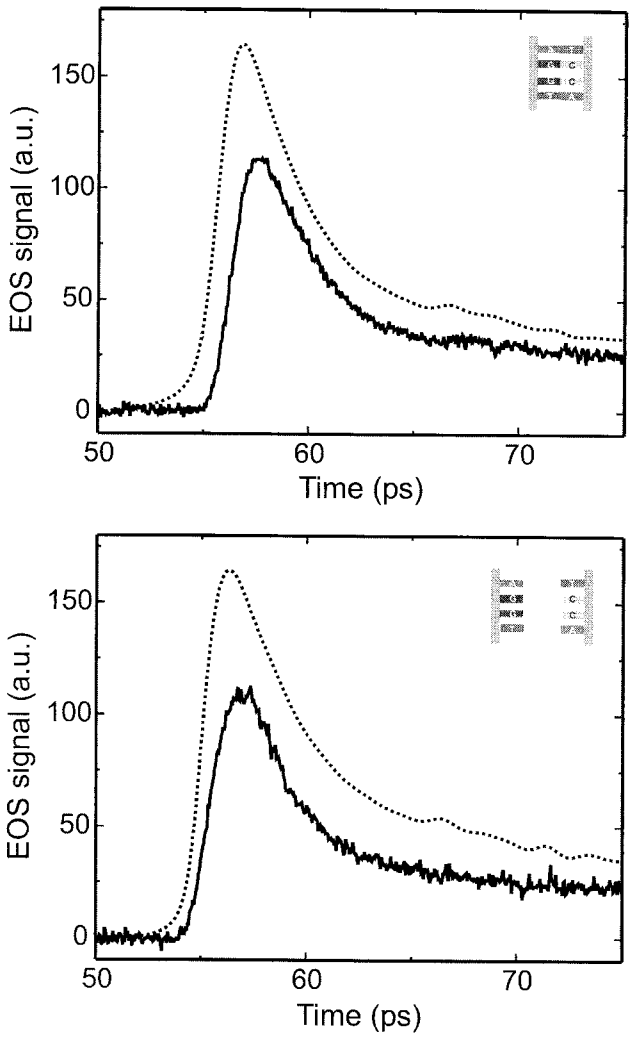

Fig. 2. Experimental transients using the microstrip line signal transducer of the THz pulse transmitted through hybridized (upper plot) and denatured (lower plot) pcDNA3 molecules of length 5.4 kb. The propagation length through DNA spots was 6 mm. Δt is an indication for the DNA induced temporal shift of the signal.

dielectric constant of the DNA material and thereby the DNA binding state, as schematically depicted in Fig. 1(c). As known from a previous free-space analysis,[16] hybridized DNA molecules exhibit a higher refractive index and higher damping at THz frequencies. Consequently, signals propagating along a waveguide loaded with a DNA film are expected to appear later in time and be further damped as a function of the hybridization degree. Several experiments with such waveguide THz sensors were performed, as exemplarily shown for a 40-nm hybridized and a 80-nm-thick denatured pcDNA3 film on microstrip waveguides in Fig. 2. The figure depicts transients of the signal transmitted through the DNA film as well as the respective reference signals without the DNA film. We calculate the references by measuring the propagating THz signal at three positions before the DNA film for precise evaluation of the propagation characteristics of each microstrip waveguide and by extrapolating the signal to the position where the DNA signal is measured. As indicated in Fig. 2 by Δt, the hybridized DNA film exhibits a clear temporal shift of the THz signal induced by the DNA. The shift of the denatured sample is evidently much lower. The difference of the temporal shifts already demonstrates that THz waveguide measurements are potentially attractive for the label-free analysis of the binding state of DNA molecules by sensitive monitoring of variations in the complex refractive index. One should note that in this proof-of-principle experiment the denatured sample is substantially thicker (by approximately a factor of 2) than the hybridized sample, owing to the hybridization-dependent viscosity of the pipetted DNA solution. This implies that the specificity of THz hybridization probing is even larger for an identical sample thickness than observed in these experiments. It is important to stress, nevertheless, that with this approach, extremely careful timing control is crucial. Slight temporal uncertainties, minimal errors in determining the exact probing positions, or errors in calculating the reference signal can inhibit a reliable analysis.

We therefore developed an enhanced and far more reliable probing concept. Because we are interested in monitoring the refractive index of the analyzed DNA material, we can reach a much higher sensitivity by incorporating a resonant structure in the THz waveguide on which the DNA is deposited. This is a well-known approach used at lower frequencies.[26] Since the resonance frequency of a resonator depends sensitively on its dielectric loading, i.e., on the refractive index of surrounding materials, this method allows us to translate the hybridization-dependent refractive index of DNA into a resonance frequency shift, thereby drastically increasing the sensitivity and more importantly avoiding completely the precise timing requirements of our initial approach.

In the experiments performed, an embedded bandpass filter acts as the THz resonator and consequently as the DNA sensor. As indicated in Fig. 1(d), we designed a filter that consists of three coupled microstrip line resonators, in which the crucial length l is a quarter-wavelength at the first passband center frequency f_c in the medium of propagation. The resonators are designed to provide a passband around a center frequency of $f_c = 610$ GHz. The characteristic parameters of the filter, such as center frequency f_c, quality factor Q, and insertion loss, depend sensitively on the relative permittivity and the absorption of the dielectric medium attached. Hence a detuning, damping, and broadening of the output signal that sensitively depends on the binding state of the DNA attached is expected. The resonators are carefully characterized in time domain before DNA pipetting. The frequency-dependent complex transmission coefficient $S_{21}(\nu)$ is derived. Figure 3 shows the transmission spectra of an unloaded resonator, together with the simulated frequency response. A good agreement is observed. The prober homogeneity is excellent, since without DNA the cen-

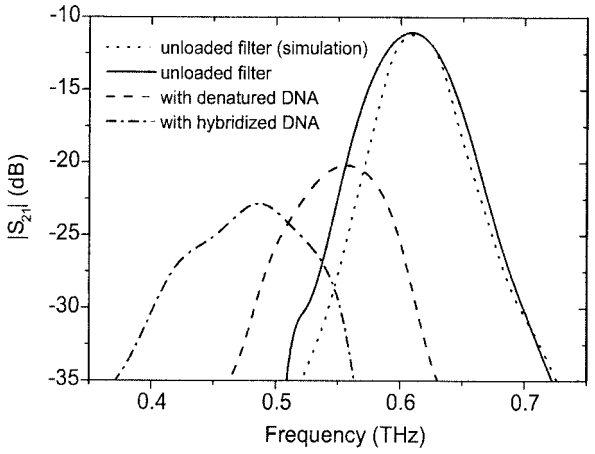

Fig. 3. Enhanced sensitivity results using a bandpass filter as THz resonator under DNA spots of 0.5-mm diameter. The spectra illustrate the calculated and the experimental transmisson spectra through the device prior to the deposition of DNA, and the transmission after deposition of hybridized and denatured pcDNA3 molecules of length 5.4 kb.

ter frequencies of all filters differ less than 0.6%. These repeated measurements of different probers constitute not only a measure for the manufacturing reproducibility; they also yield an experimental verification of the measurement reproducibility and analytic reliability.

Several filters are coated with denatured or hybridized pcDNA3 molecules. Since the refractive index of DNA depends critically on humidity,[23] care is taken to perform all measurements at a constant time interval after sample pipetting and at a constant ambient humidity. To illustrate the resonance frequency shift induced by the DNA loading, one should take into account that dielectric loading leads to an increase of the effective permittivity $\epsilon_{r,\mathrm{eff}}$, which lowers the supported propagation velocity for the THz signals transmitted. As a consequence the structure appears larger and the resonance frequency (f_c) of the filter decreases. In our experiments $\epsilon_{r,\mathrm{eff}}$ increases when we attach DNA material to the resonator in proportion to the hybridization degree. The frequency shift is hence a direct measure of the hybridization degree. The magnitude of the measured transmission parameters (S_{21}) of devices with and without DNA films are compared in Fig. 3. The hybridized DNA clearly generates a stronger shift from the original center frequency than does the denatured DNA. The observed frequency shifts amount to 55 GHz (9%) and 124 GHz (20%) for the denatured and the hybridized material, respectively. Also, a lower quality factor Q and a stronger damping of hybridized samples is observed. This behavior is in good agreement with the free-space measurements in which hybridized DNA samples exhibited an increased refractive index and higher absorption in comparison with denatured samples.[16] Taking into consideration the reproducibility of our analysis (f_c variation $\leq$ 0.6%), the observed large shift of resonance frequency upon the degree of hybridization represents a definite proof for the attractiveness of THz probing as a label-free gene-detection scheme. It is worth noting that as our DNA spots exceed the THz resonator size by far, more DNA material is applied than necessary. The THz signal frequency shift, however, stems exclusively from the extremely small DNA volume defined by the 40–80-nm DNA film thickness and the THz resonator size (265 μm $\times$ 50 μm), since the THz fields do not extend much farther from the microstrip lines than the $h = 5.9$ μm dielectric film thickness between microstrip and backplane. The probed DNA volume corresponds therefore to approximately 1.1 fmol of DNA material. If adequate pipetting and DNA immobilization techniques are introduced, this femtomolar sensitivity makes THz techniques comparably sensitive to standard commercial fluorescence-based systems, which also typically need femtomolars of DNA for a reliable analysis.[3] Because we are not using advanced spotting and immobilization techniques in these experiments but simply pipetting DNA on our THz sensors, again, in all cases the application of DNA solution with denatured DNA molecules resulted in a film with a larger thickness. This variation implies that the specificity of THz hybridization probing will be larger in the case of identical sample thickness than currently observed. The distinct frequency shifts are reproduced well in repeated experiments and confirm conclusively the capability of THz methods not only to identify but also to quantify the degree of hybridization of polynucleotides.

3. Conclusion

In summary our analysis demonstrates that integrated planar THz technologies can efficiently and reliably probe the hybridization of nucleic acids, paving the way for innovative label-free genetic-detection systems. The demonstrated detection sensitivity reaches femtomolar levels. One of the inherent advantages of the applied planar waveguide approach is its scalability, leaving plenty of room for developing high-throughput genetic diagnostic systems. By means of integrating two-dimensional arrays of THz transducers, a large number of genes could simultaneously be analyzed. Further refinement of the technique, e.g., by means of improving the rudimentary pipetting techniques and by immobilizing probe molecules is, however, required before this measurement principle can be transferred to large-scale applications.

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