Biosensors and Bioelectronics 26 (2010) 1605–1611

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A dual surface plasmon resonance assay for the determination of ribonuclease H activity

Hana Šípová [a], Hana Vaisocherová [a], Josef Štěpánek [b], Jiří Homola [a,*]

[a] Institute of Photonics and Electronics, Academy of Sciences of the Czech Republic, Chaberská 57, 182 51 Prague, Czech Republic
[b] Charles University in Prague, Faculty of Mathematics and Physics, Ke Karlovu 3, 121 16 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form 16 July 2010
Accepted 4 August 2010
Available online 10 August 2010

Keywords:
Biosensor
Surface plasmon resonance
Antisense
Ribonuclease H
Enzyme activity assay

ABSTRACT

There is a demand for efficient tools for the monitoring of RNase H activity. We report on a new assay which allows for simultaneous (1) real-time monitoring of RNase H activity and (2) detection of cleavage reaction products. The dual assay is implemented using a multichannel surface plasmon resonance (SPR) biosensor with two independently functionalized sensing areas in a single fluidic path. In the first sensing area the RNA cleavage by RNase H is monitored, while the products of the cleavage reaction are captured in the second sensing area with specific DNA probes. The assay was optimized with respect to AON concentration and temperature. A significant improvement was obtained with special chimeric probes, which contain RNA substrate for RNase H and a longer deoxyribonucleotide tail, which enhances the SPR signal. It has been shown that RNase H stabilizes the RNA:DNA hybrid duplex before the cleavage. The potential of the assay is demonstrated in the study in which the ability of natural and modified oligonucleotides to activate RNase H is examined.

1. Introduction

Ribonuclease H (RNase H) is an endonuclease that specifically hydrolyzes the RNA strand in the RNA:DNA hybrid duplex. This activity occurs in a number of cell processes such as DNA replication or transcription. An abundance of RNase H can be found in eukaryotic as well as prokaryotic cells (Wintersberger, 1990). Moreover, RNase H constitutes an essential functional domain of the reverse transcriptases of retroviruses, hepadnaviruses and transposons and therefore represents an important target for the development of antiviral drugs (Schultz and Champoux, 2008; Veal et al., 1998). Antisense oligonucleotides (AONs) are a therapeutic paradigm (Bennett and Swayze, 2010) based on the selective genetic suppression of disease-relevant proteins (Baker et al., 2001; Dean and Bennett, 2003). One of the most promising AONs strategies is the RNase H-mediated action (Zamaratski et al., 2001). A hybrid duplex formed by a DNA-mimicking AON and a complementary mRNA segment is recognized by the RNase H as a substrate and cleaved at the mRNA strand. This mechanism allows for the destruction of multiple mRNA copies per single AON and thus substantially enhances the effectiveness of the antisense therapy.

AONs are supposed to meet rather complex requirements, including good hybridization properties, binding specificity, resistance towards nucleases and sufficient uptake into cells (Juliano et al., 2008, 2009). Addressing these challenges has driven an extensive search for new oligonucleotide analogs. The development of modified AONs with diverse novel chemical modifications calls for fast and efficient methods allowing for assessment of their properties, including their ability to activate RNase H. Traditional assays for RNase H activity almost exclusively utilize substrates with tags, such as RNA with H^3 tag prepared by *in vivo* transcription (Kanaya et al., 1991) or synthetic RNA oligonucleotides tagged with ^{32}P (Kanaya, 2001), dioxigenine (Rychetsky et al., 1996), or fluorescent dyes (King et al., 2004; Parniak et al., 2003; Rizzo et al., 2002). Other methods used for analysis of cleavage reaction products such as HPLC (Hogrefe et al., 1990) require rather high (micromolar) concentrations of the substrate. Moreover, all of these approaches are indirect and time-consuming. Most of these methods are focused on product detection of RNase H cleavage and cannot observe the RNase H activity in real-time.

In recent years, surface plasmon resonance (SPR) biosensor method has been successfully employed to investigate kinetics of RNase H binding to RNA:DNA hybrid duplexes (Haruki et al., 1997) and the cleavage of RNA in the hybrid duplex (Fang et al., 2005). In this paper, we report on a dual real-time assay based on the use of an SPR biosensor which allows for simultaneous (1) monitoring of RNA cleavage with RNase H and (2) detection of the cleavage products. In this assay, probes mimicking the mRNA are immobilized on the SPR chip surface. Then, formation of their heteroduplexes with antisense oligonucleotides is monitored. The hybridization is

* Corresponding author. Tel.: +420 266 773 448; fax: +420 284 681 534.
E-mail address: homola@ufe.cz (J. Homola).

0956-5663/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2010.08.011

followed with the RNA cleavage by RNase H and the release of AON. Fragments produced during the reaction are detected downstream in another sensing spot. The assay uses biotinylated RNA strand and DNA strand without any label, which makes the assay convenient for investigation of DNA-mimicking oligonucleotides. The assay is optimized with respect to temperature and the concentration of antisense oligonucleotide. Finally, the assay is applied for assessment of the ability of selected antisense oligonucleotides to activate RNase H.

2. Materials and methods

2.1. Materials

The following reagents were used for immobilization of oligonucleotides on the SPR chip. Streptavidin from *Streptomyces avidinii* was obtained from Sigma–Aldrich, USA. Carboxylic ($HS–C_{11}–EG_4–OCH_2–COOH$) and hydroxylic ($HS–C_{11}EG_2–OH$) alkanethiols were obtained from Prochimia, Poland. Ribonuclease H from *Escherichia coli* was purchased from Invitrogen, USA. RNase H from *E. coli* was used as it is the most characterized enzyme from the family of ribonucleases H (Katayanagi et al., 1990). Its structure has been demonstrated to be similar to that of eukaryotic RNase H1 (Nowotny et al., 2005), which plays a key role in antisense therapy. Biotinylated 30-mer chimeric oligonucleotide of the sequence biotin-$(TEG)_2$-5′-dC_7-rA_{16}-dC_7-3′ (cON1) and a short 15-mers complementary to the central polyadenosine sequence: 5′-dT_{15}-3′ (dT_{15}) were purchased from Thermo Fisher Scientific Inc., USA. Biotinylated chimeric oligonucleotides used as a substrate for RNase H: 38-mer biotin-$(TEG)_2$-5′-dC_7-rA_{16}-$d(CCA)_5$-3′ (cON2), 49-mer: biotin-$(TEG)_2$-5′-dC_7-rA_{16}-$d(CCA)_8$ dC-3′ (cON3), oligonucleotides used for coating of reference sensing spot: biotin-$(TEG)_2$-5′-$d(TGG)_5$-3′ ($BdTG_{15}$) and biotinylated oligonucleotides for the detection of products: 25-mer biotin-$(TEG)_2$-5′-$d(TGG)_8$T-3′ ($BdTG_{25}$) were purchased from Integrated DNA Technologies, USA. Phosphorothioate deoxyribonucleotides 5′-$(T^*TTT)_3T^*T^*T$-3′ (PS1) and 5′-$T(T^*T)_7$-3′ (PS2), where * denotes a phosphorothioate bond between the nucleosides, were also obtained from Integrated DNA Technologies, USA. All oligonucleotides were purchased in HPLC-purity grade.

2.2. SPR sensor system

In this work, an SPR biosensor with four sensing spots developed at the Institute of Photonics and Electronics, Prague was used. The sensor is based on the attenuated total reflection method and wavelength spectroscopy of surface plasmons (Vaisocherová et al., 2006). In this sensor, white light is used to excite surface plasmons on a thin gold film in four areas which are interfaced with four separate chambers of a four-channel flow-cell. The sensor fluidics can be configured to provide four independent fluidic channels or selected (or all) fluidic channels may be combined into a single fluidic path.

2.3. SPR chip functionalization

Immobilization of oligonucleotides on the surface of SPR sensor chips is based on the covalent attachment of streptavidin to a ω-carboxyalkylthiol self-assembled monolayer via amide-bond-forming chemistry and the subsequent attachment of the biotinylated oligonucleotide probes. The procedure for the preparation of the mixed self-assembled monolayer of $HSC_{11}(EG)_2–OH$ and $HSC_{11}(EG)_4OCH_2COOH$ alkenthiols as well as *in situ* immobilization of the streptavidin is described in detail in Vaisocherová et al. (2009).

The attachment of biotinylated oligonucleotides was performed in T-NaCl buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.4 at 25 °C). Once a stable baseline was reached, the 100 nM solution of the selected oligonucleotide (cON1, cON2, cON3, $BdTG_{15}$ or $BdTG_{25}$) was flowed across the sensing surface for 10 min. At this point, the amount of immobilized probes reached the equilibrium level. This step was followed by washing the functionalized sensor surface with T-NaCl buffer.

For the proposed dual assay, the sensing spots were functionalized as follows. Sensing spot 1 was functionalized with $BdTG_{15}$ (reference channel), sensing spot 4 with $BdTG_{25}$ and sensing spots 2 and 3 were functionalized with chimeric cON oligonucleotide (Supplementary material Fig. S1A). Then the sensor fluidics was reconfigured to bring all the sensing spots in a single flow channel. Sensing spot 1 in the flow path was used as a reference to compensate for refractive index changes and potential nonspecific binding of RNase H and AON, sensing spots 2 and 3 were used for monitoring duplex cleavage by RNase H, and sensing spot 4 for downstream detection of cleaved fragments released in sensing spots 2 and 3.

2.4. The SPR measurements of RNase H activity

All SPR experiments were carried out in a flow-through format (flow rate: 20 μL/min). The measurements were performed either in the conventional configuration (a single sensing spot functionalized with cON probes) or in the dual assay configuration. Initially, the RNS buffer (75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 2.5% glycerol, pH 8.3 at 25 °C) was introduced to establish a baseline. Antisense oligonucleotides (AONs) complementary to the cON-R sequence were then flowed along the sensor surface for 5 min and the formation of the hybrid cON-R*AON complex was monitored. Then, to avoid the dissociation of cON-R*AON duplex in the buffer, the sensor surface was immediately exposed to a solution with the same concentration of AONs and with RNase H added. Finally, the sensor surface was washed with RNS buffer.

2.5. Analysis of the SPR measurements

In the SPR sensor used, the sensor response is directly proportional to the mass of biomolecules attached to the surface (Homola, 2006). Upon injection of a biomolecular solution, the SPR signal starts to increase till an equilibrium state is reached. The final increase of the SPR signal then indicates the amount of attached biomolecules. Using the calibration procedure described in Homola (2006) we can determine the surface densities of the immobilized probes and the subsequently attached molecules. In the case of duplex formation, we can calculate the hybridization efficiency (HE), i.e. the ratio of formed hybrid duplexes to immobilized oligonucleotide probes, as $HE = B \times M_{probe}/(A \times M_{AON})$, where M_{probe} and M_{AON} correspond to the molecular weight of the probe and antisense oligonucleotide, respectively, A is the equilibrium sensor response to the immobilization and B is the sensor response to AON hybridization. Considering the duplex formation as a pseudo first-order process, its equilibrium association constant K is given by the following formula $K = HE/[c_{AON} \times (1 - HE)]$, where c_{AON} is the AON concentration in the solution.

2.6. UV absorption measurements

The hybridization capability of the oligonucleotides was characterized in the UV absorption experiments. The samples contained the total strand concentration of 2 μM in the HEPES buffer with the same cation concentration as in the SPR experiments with RNase H (10 mM HEPES, 75 mM KCl, 3 mM $MgCl_2$, pH 7.4 at 25 °C).

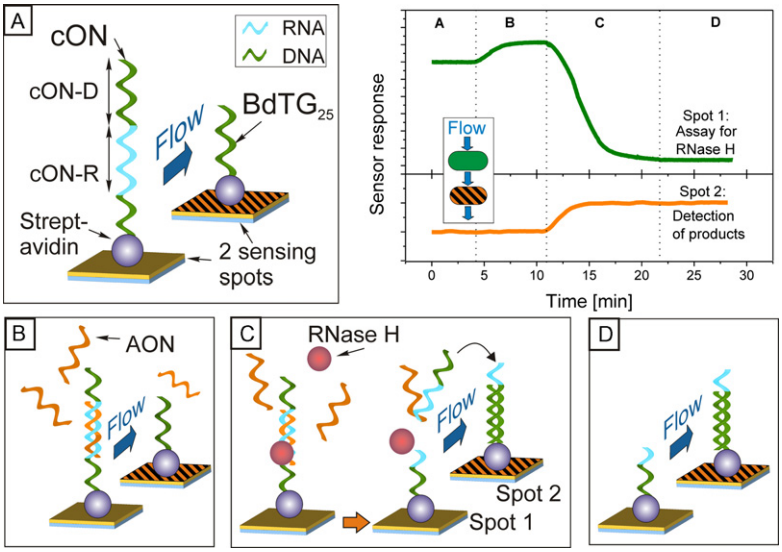

Fig. 1. Concept of the dual SPR-based assay for the study of RNase H activity. The top right graph shows SPR sensor response from the upstream (area with hatch pattern) and downstream (area with no pattern) sensing areas. The former area is functionalized with biotinylated chimeric oligomers (cON) containing the RNA sequence (cON-R) and the DNA sequence (cON-D), while the latter is functionalized with DNA probes complementary to the cON-D sequence. Schemes A–D (in squares) depict the four main stages of the assay. (A) Initial baseline in buffer, no interaction with immobilized oligonucleotides occurs. (B) Tested antisense oligonucleotides (AONs) are injected in the flow path. The formation of the cON-R:AON duplex is monitored in the upstream area, but no sensor response is observed in the downstream area. (C) Injection of RNase H mixed with AONs. Decrease in sensor response in the upstream area indicates the AON-mediated RNase H cleavage of cON-R. Cleaved fragments are recaptured in the downstream area, which causes an increase in the sensor response. (D) Final baseline in the buffer. The differences between the initial and final baselines in both sensing areas (decrease in the upstream and increase in the downstream area) are proportional to the amount of cleaved probes.

3. Results and discussion

3.1. Design of the dual assay

To develop an assay enabling both real-time monitoring of the RNase H activity and sensitive detection of the reaction products a dual SPR assay was designed. The scheme of biomolecular interactions involved in the proposed RNase H assay and corresponding sensor responses are illustrated in Fig. 1. In the first step of the assay, biotinylated chimeric oligonucleotides (cON) containing a ribonucleotide sequence (cON-R) are immobilized on the streptavidin-coated SPR chip surface. These probes contain substrate for RNase H, e.g. the RNA sequence mimicking the mRNA (cON-R), and a short DNA strand conjugated to its 3′-end (cON-D). The antisense oligonucleotides (AON) complementary to cON-R are then injected in the flow-cell and the formation of cON-R*AON heteroduplexes is monitored. In the following step, the solution of RNase H and AON is introduced and the cleavage of cON at the cON-R region is initiated. The cleavage results in a decrease in the sensor response and the fragments containing cON-D region are released to the solution. The reaction stops when all the accessible probes are cleaved. The DNA fragments of cON produced during the reaction are detected downstream at another sensing spot functionalized with probes complementary to the cON-D region.

3.2. Optimization of the RNA probes

For the monitoring of RNA cleavage by RNase H, special chimeric probes were designed containing RNA substrate for RNase H and DNA region conjugated to its 3′-end. The DNA region is not directly involved in the cleavage reaction; instead it is used to enlarge the fragments cleaved by RNase H and thus to enhance the SPR sensor response. After the cleavage reaction, the DNA fragments further hybridize with DNA probes immobilized in another spot and form stable DNA duplexes. The influence of length of the DNA fragments on assay sensitivity was studied using three probes, which contain the same cON-R sequence, but have 7 (cON1), 15 (cON2) or 25 (cON3) DNA nucleotides conjugated to its 3′-end. The probes were immobilized to the streptavidin-coated sensor surface and their surface density was not found to significantly depend on the probe length, falling in the range of 3.5–4.3 $\times 10^{12}$ molecules/cm^2.

In the optimization of the assay, natural 15-mer deoxyribonucleotide dT_{15} was used as a model oligonucleotide mimicking the AON target. Fig. 2 shows the reference-corrected sensor response to dT_{15} hybridization and subsequent RNase H-mediated cleavage of immobilized probes. The hybridization with dissociation phase is shown in Supplementary material Fig. S2. As can be seen from Fig. 2, the amount of bound dT_{15} decreased moderately with an increased probe length. Even though the melting temperatures of the cON1, cON2 and cON3 duplex with the dT_{15} in solution are approximately the same (32.5 ± 0.5 °C), the equilibrium association constants determined from our SPR experiment differed noticeably, being (7.5 ± 0.5) $\times 10^6$ M^{-1} for cON3, (8.7 ± 0.8) $\times 10^6$ M^{-1} for

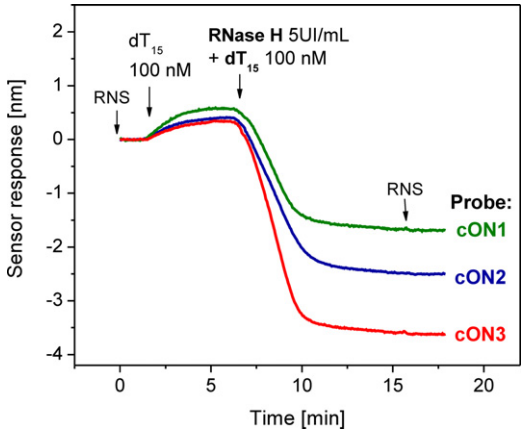

Fig. 2. Sensor response to natural deoxyoligonucleotide (dT_{15}) hybridization to three different probes (cON1–cON3) containing the same ribonucleotide cON-R sequence and 7 (cON1), 15 (cON2) or 20 (cON3) DNA nucleotides conjugated to its 3′-end. The cON-R in cON-R:dT_{15} heteroduplexes are then cleaved by RNase H. Temperature: 20 °C.

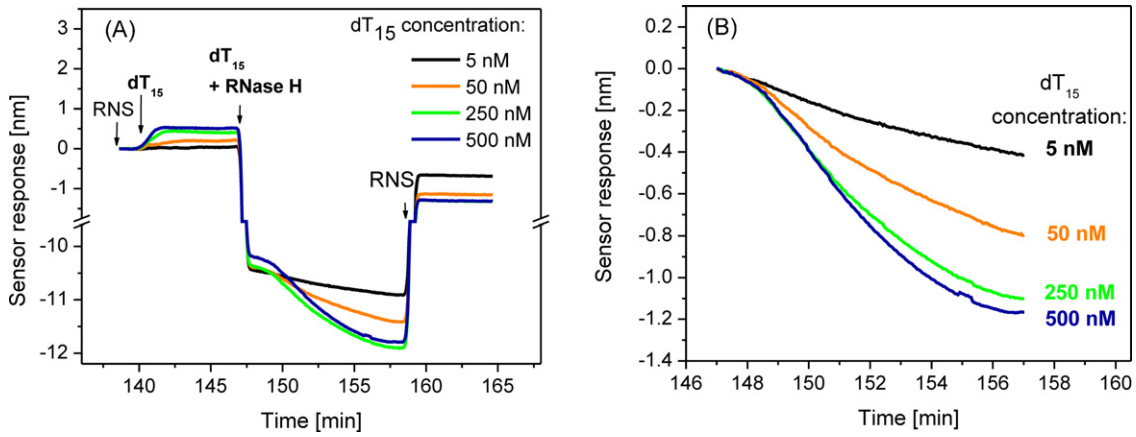

Fig. 3. Influence of the concentration of natural deoxyribonucleotide dT_{15} on RNase H activity. (A) Sensor response to dT_{15} hybridization to the cON1 probes and cleavage of cON1-R in cON1-R:dT_{15}. Arrows indicate injection of the respective solutions. (B) Detail of sensor response to the cON1 cleavage by RNase H (corresponds to section C in Fig. 1). The data were normalized to the same amount of immobilized probes (sensor response of 3 nm). Temperature: 25 °C, concentration of RNase H: 4.2 UI/mL.

cON2 and $(9.2 \pm 0.7) \times 10^6\ M^{-1}$ for cON1 at 20 °C. These differences illustrate the distinct conditions which occur in the solution and on the sensor surface. The oligonucleotides on a chip are densely packed and their negative charges are not sufficiently compensated by small cations, especially in the case of longer strands (Peterson et al., 2001). The repulsion between the surface-bound oligonucleotides then results in destabilization of the duplexes compared to solution.

In contrast, the sensor response to cON-R cleavage by RNase H increased substantially with the size of the conjugated DNA tail due to the larger size of the cleaved fragments. The use of the cON3 probe resulted in an approximately 50% increase in the sensor response to RNase H cleavage compared to the cON1 probe. This enhancement makes it possible to detect subtle changes in RNase H activity and may therefore help identify potential candidates for antisense therapeutics, even those which exhibit a low ability to activate RNase H.

The slow rate of the RNase H cleavage at the end of RNase H action indicates that almost no substrate is left on the surface. By comparing the initial and final baselines of the sensorgrams, it can be determined that, regardless of which probe was used, 75% of the cON-R was cleaved from the surface. The closest cleavage site to the sensor surface is located on average 5.2 ± 0.7 nucleotides away from the 5′-terminus. The calculated value of the cleavage site has mainly statistical meaning and presents an average value (rather than the cleavage site for each oligonucleotide). This observation is in agreement with the previous work by Crooke et al. (1995) who studied the cleavage pattern of *E. coli* RNase H.

3.3. Optimization of AON concentration

The effect of AON concentration in the solution on the RNase H cleavage was investigated using individual sensing spots functionalized with cON1 probes. The concentration of 15-mer deoxyribonucleotide dT_{15} was varied from 5 to 500 nM while the concentration of RNase H (4.2 UI/mL) was kept constant. Fig. 3A shows the respective sensor responses at 20 °C. In the beginning of dT_{15} injection, an increase in the sensor response is observed due to the binding of dT_{15} to the RNA segment of the cON1 probe (cON1-R). Then the dT_{15} hybridization and the sensor response reach equilibrium. The heteroduplexes cON1 with dT_{15} (and with AON in general) may dissociate in buffer (see Supplementary material Fig. S3), which is why the solution of dT_{15} containing RNase H is injected without prior switching back to the buffer. Initially, a steep decrease in the sensor response due to refractive index changes is observed (no reference-correction performed), which is followed by a gradual decrease caused by the cleavage of cON1 probes.

Fig. 3B shows a detail of the cleavage kinetics corresponding to stage C in Fig. 1. It can be concluded that the kinetics of cON1-R hydrolysis depends substantially on the concentration of antisense oligonucleotides in solution. It is believed that the concentration of AON affects the cleavage rate through the initial concentration of hybrid duplexes on the surface and the rate of their formation. However, at dT_{15} concentrations higher than 250 nM, the interaction kinetics varies only moderately with the dT_{15} concentration. This is likely to be caused by mass transport effects limiting the transport of RNase H to the sensor surface. Therefore, for further experiments, the concentration of dT_{15} was set to 100 nM.

3.4. Optimization of temperature

RNase H activity is known to be temperature-dependent, reaching its optimum at temperatures above 37 °C (Lee et al., 1997). Therefore a series of experiments was performed at various temperatures from 20 °C up to 37 °C (RNase H and dT_{15} concentrations were kept at 5 UI/mL and 100 nM, respectively). The obtained sensor responses are shown in Fig. 4. As follows from Fig. 4, the amount of bound dT_{15} at the end of the hybridization (stage B in Fig. 1) decreases substantially with an increase in temperature as no sensor response to dT_{15} binding is observed at temperatures over

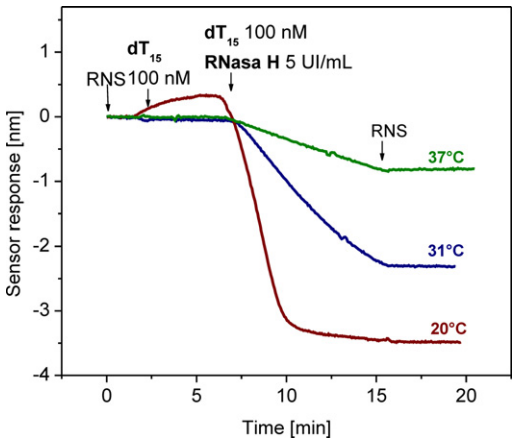

Fig. 4. Influence of temperature on RNase H cleavage of the natural DNA:RNA duplex. Sensor response to dT_{15} hybridization to the cON3 probes and cleavage of cON3-R in cON3-R:dT_{15}. Arrows indicate injection of the respective solutions.

31 °C. This is not surprising; however, as the melting temperature of cON1-R:dT_{15} duplex is 30 °C (determined by UV absorption studies). Moreover, the dense arrangement of oligonucleotides at the sensor surfaces is known to contribute to the destabilization of duplexes (Levicky and Horgan, 2005). The rate of the cleavage process was found to follow a similar trend with the highest value at 20 °C and lower values at higher temperatures. From the initial and final baseline in the buffer, the portion of the probe cleaved-off from the surface during the 10-min period can be determined. After subtracting the part belonging to cON3-D fragments, we determined that 77%, 43% and 8% of the cON3 was cleaved at 20 °C, 31 °C and 37 °C, respectively. The results indicate that the occurrence of rA-dT duplex (i.e. the substrate availability) is the key factor governing the rate of cleavage and outweighing the temperature effect on RNase H activity. Nevertheless, it is important to note that despite the low surface concentration of cON1-R*dT_{15} heteroduplexes at 31 °C and 37 °C, the RNase H cleavage of cON-R still proceeded at a high rate. This finding is in agreement with the work by Li and Wartell who suggested that RNase H may have a stabilizing effect on RNA:DNA heteroduplex formation (Li and Wartell, 1998).

For the study of temperature effect on RNase H activity, a more stable heteroduplex can be used (i.e. with a longer chain or with the inclusion of more G–C base pairs). In our study, we employed 15-mer hybrid duplex which is expected to dissociate immediately after the RNA cleavage. We preferred not to use longer (i.e. more stable) hybrid duplexes, as they could be cleaved multiple times and thus increase the complexity of the cleavage kinetics. The assay can be easily adapted to the development of AON for a specific application by using a probe with a different sequence. In our future study, we will focus on screening for various types and positions of the modifications of the sugar–phosphate backbone. For this reason we used an AON with a sequence of pure thymidine, which has a structure that can be easily modified.

3.5. Dual assay-based study of RNase H cleavage of the DNA*RNA hybrid duplex

The cON3 probe was used for the characterization of the RNase H cleavage kinetics and for the simultaneous detection of the cleavage products. The 25-mer DNA segment at the 3′-end of the cON3-R sequence was detected downstream at the sensing area functionalized with $BdTG_{25}$ of the complementary sequence. The top plot in Fig. 5 shows the sensor response to the formation of the cON3-R*dT15 hybrid duplex and to the cleavage of cON3-R by RNase H. The dT_{15}-induced cleavage of the RNA part of the probe (cON3-R) gives rise to a drop in sensor response due to the depletion of mass at the sensor surface. It should be noted that the reaction kinetics is rather complex and includes contributions from three related and simultaneous processes. These are: (i) RNase H association to the RNA:DNA hybrid duplex and its dissociation (Haruki et al., 1997), (ii) cleavage of the RNA strand, and (iii) the RNA:DNA heteroduplex formation. The last process proceeds in the course of the cleavage due to the consumption of the cON3-R*dT_{15} duplexes by RNase H, which shifts the equilibrium of the hybridization reaction towards the formation of heteroduplexes. A detailed study of the relative contributions of these three parallel processes presents a highly complex issue and will be the subject of our future work.

The downstream detection of products of the cleavage reaction is shown in Fig. 5 (bottom). The increase in sensor response corresponds to the hybridization of fragments of the cON3 probe (i.e. the complementary cON3-D region) to the $BdTG_{25}$ probe immobilized in the second sensing area. During the course of the RNase H action, approximately 10% of the cleaved cON3 mass is captured

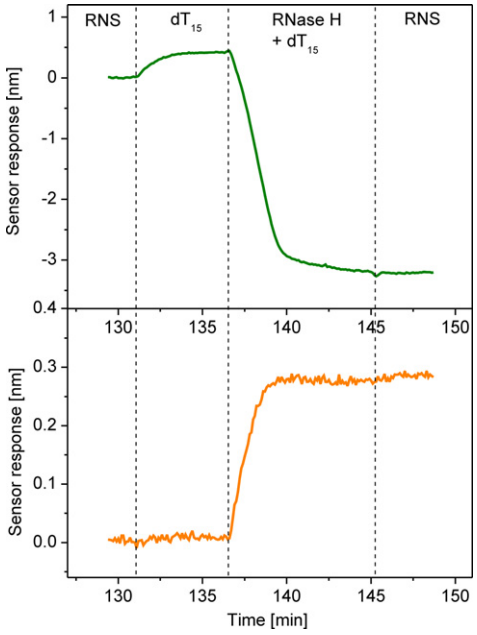

Fig. 5. Real-time monitoring of the cleavage process (reference-corrected data) at 20 °C. Concentration of RNase H: 5 UI/mL, concentration of dT_{15}: 100 nM. (Top) Sensor response to dT_{15} hybridization with the cON3-R segment and to subsequent cleavage of cON3-R by RNase H (upstream area). The sensor response was calculated as an average of measurements in two sensing spots. (Bottom) Sensor response to cON3-D released from the upstream areas by RNase H cleavage of cON3 probes.

in the downstream channel. This is in agreement with the values obtained in typical SPR experiments with high affinity interaction partners (e.g. in Vaisocherová et al., 2006, approximately 10% of the molecules in solution were captured by the receptors immobilized on the sensing surface).

It is clear from Fig. 5 that the temporal sensor response is fastest at the beginning of the cleavage, which indicates that the cleavage starts at a high rate immediately upon the injection of RNase H. About 2 min from the beginning of the cON3-R cleavage by RNase H, the binding rate starts to drop. It is interesting to note that in the third minute of the cleavage process the presence of new cON3-D in the downstream channel is no longer detectable, implying that the cleavage of cON3-R is already almost finished.

3.6. RNase H cleavage of phosphorothioate oligonucleotides

The optimized assay was demonstrated for the evaluation of AON containing phosphorothioates (PS). PS oligonucleotides are one of the first and still widely used AON modifications (for review, see Eckstein (2000)) due to their rare ability to activate RNase H and a higher resistance to cell nucleases compared to natural ONs. The modification consists in the replacement of one of the non-bridging phosphate oxygen atoms with sulfur. We employed 15-mer thymidine strands with PS bonds spread regularly among natural phosphodiester bonds in the ratio of 1:4 (PS1) and 1:2 (PS2). The PS oligonucleotides were compared with natural dT_{15} deoxyribonucleotide under the same experimental conditions (Fig. 6).

Fig. 6A shows the hybridization of 100 nM PS1 and PS2 with the cON3 probe and the subsequent cleavage by RNase H. Fig. 6B shows sensor response in the sensing area functionalized for the detection of cleaved fragments. From the equilibrium values of the hybridization one can conclude that PS duplexes with RNA are much less stable than hybrid duplexes with natural deoxyribonucleotide dT_{15}. Details of the hybridization process of PS oligonucleotides without the addition of RNase H are shown in Fig. 6C. The equilibrium

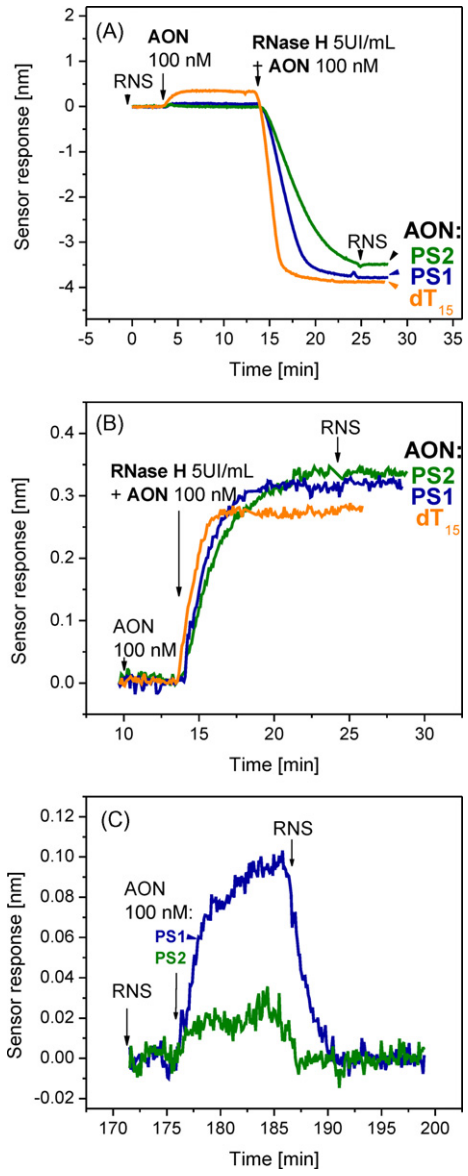

Fig. 6. Monitoring of AON:cON3 duplex formation and cON3 cleavage by RNase H (dT_{15}, PS2 and PS1). (A) Sensing area with cON3 probes. Each sensor response was obtained by averaging the data obtained from measurements on 2 chips and 2 sensing spots (spots 2 and 3). (B) Sensing area with $BdTG_{25}$ for the detection of products. Data averaged from measurements on 2 chips. (C) Hybridization of PS1 and PS2 oligonucleotides with cON3. Temperature: 20 °C.

association constants K_A were determined as 7.5×10^6, 6.25×10^5 and $1.31 \times 10^5\ M^{-1}$ for dT_{15}, PS1 and PS2, respectively (temperature: 20 °C). In control experiments performed with UV absorption spectroscopy, the T_m values of the cON3-R duplex with dT_{15}, PS1 and PS2 were determined to be 31.5 °C, 23.9 °C and 18.8 °C, respectively. This is in agreement with the previous studies, which have reported lower stability of the RNA duplex with oligonucleotides containing a PS bond than with phosphodiester bonds (Ghosh et al., 1993).

The cON3-R cleavage by RNase H induced by AONs with PS bonds was compared to RNase H activation by natural deoxyribonucleotide of the same sequence (dT_{15}). It can be concluded from Fig. 6A and B that the PS oligonucleotides activate RNase H, although a significant decrease in the activation ability was observed with a higher share of phosphorothioate bonds. That is in good agreement with the previously published results (Agrawal et al., 1990; Crooke et al., 1995), which reported inhibition of RNase H by an excess of phosphorothioate oligonucleotides.

4. Conclusions

In this paper we present a novel dual SPR assay for the determination of RNase H activity. The assay enables real-time simultaneous monitoring of (1) RNA cleavage with RNase H and (2) detection of reaction products. Several parameters of the assay were investigated and optimized, including the assay temperature and the concentration of antisense oligonucleotide. In addition, it was demonstrated that the sensitivity of the monitoring of RNase H action can be significantly improved by conjugating a longer DNA strand to the immobilized RNA substrate. The potential of the assay for AON screening was demonstrated in experiments in which ability of selected synthetic modified oligonucleotides to activate RNase H was investigated. In contrast to the methods currently used for the evaluation of RNase H activity, the novel assay does not require labeling of AONs and generates output in real-time allowing for the assessment of kinetic aspects of the involved interactions. Moreover, the assay presents a universal platform which can be adopted for the screening of potential inhibitors of retroviral RNase H activity or for the study of other RNase-dependent antisense mechanisms, such as interference RNA (RNAi).

Acknowledgements

Authors thank Dr. Ivan Rosenberg from the Institute of Organic Chemistry and Biochemistry AS CR, v.v.i. for helpful discussions. This work was supported by the Academy of Sciences of the Czech Republic (Grant KAN200670701), by the Czech National Science Foundation (Grant 202/09/0193), and by the Ministry of Education, Youth and Sports (Grant OC09058).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.011.

References

Agrawal, S., Mayrand, S.H., Zamecnik, P.C., Pederson, T., 1990. Proceedings of the National Academy of Sciences of the United States of America 87 (4), 1401–1405.
Baker, B.F., Condon, T.P., Koller, E., McKay, R.A., Siwkowski, A.M., Vickers, T.A., Monia, B.P., 2001. Methods 23 (2), 191–198.
Bennett, C.F., Swayze, E.E., 2010. Annual Review of Pharmacology and Toxicology 50, 259–293.
Crooke, S.T., Lemonidis, K.M., Neilson, L., Griffey, R., Lesnik, E.A., Monia, B.P., 1995. Biochemical Journal 312, 599–608.
Dean, N.M., Bennett, C.F., 2003. Oncogene 22 (56), 9087–9096.
Eckstein, F., 2000. Antisense and Nucleic Acid Drug Development 10 (2), 117–121.
Fang, S., Lee, H.J., Wark, A.W., Kim, H.M., Corn, R.M., 2005. Analytical Chemistry 77 (20), 6528–6534.
Ghosh, M.K., Ghosh, K., Dahl, O., Cohen, J.S., 1993. Nucleic Acids Research 21 (24), 5761–5766.
Haruki, M., Noguchi, E., Kanaya, S., Crouch, R.J., 1997. Journal of Biological Chemistry 272 (35), 22015–22022.
Hogrefe, H.H., Hogrefe, R.I., Walder, R.Y., Walder, J.A., 1990. Journal of Biological Chemistry 265 (10), 5561–5566.
Homola, J., 2006. Surface Plasmon Resonance Based Sensors. Springer-Verlag, Germany, Berlin.
Juliano, R., Alam, M.R., Dixit, V., Kang, H., 2008. Nucleic Acids Research 36 (12), 4158–4171.
Juliano, R., Bauman, J., Kang, H., Ming, X., 2009. Molecular Pharmaceutics 6 (3), 686–695.
Kanaya, S., 2001. Ribonucleases, Pt A 341, 377–394.
Kanaya, S., Katsuda, C., Kimura, S., Nakai, T., Kitakuni, E., Nakamura, H., Katayanagi, K., Morikawa, K., Ikehara, M., 1991. Journal of Biological Chemistry 266 (10), 6038–6044.
Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T., Morikawa, K., 1990. Nature 347 (6290), 306–309.

King, K.C., Budihas, S.R., Le Grice, S.F.J., Parniak, M.A., Crouch, R.J., Gaidamakov, S.A., Isaaq, H.J., Wamiru, A., McMahon, J.B., Beutler, J.A., 2004. Analytical Biochemistry 331 (2), 296–302.
Lee, Y.I., Hong, Y.B., Kim, Y.H., Rho, H.M., Jung, G.H., 1997. Biochemical and Biophysical Research Communications 233 (2), 401–407.
Levicky, R., Horgan, A., 2005. Trends in Biotechnology 23 (3), 143–149.
Li, J., Wartell, R.M., 1998. Biochemistry 37 (15), 5154–5161.
Nowotny, M., Gaidamakov, S.A., Crouch, R.J., Yang, W., 2005. Cell 121 (7), 1005–1016.
Parniak, M.A., Min, K.L., Budihas, S.R., Le Grice, S.F.J., Beutler, J.A., 2003. Analytical Biochemistry 322 (1), 33–39.
Peterson, A.W., Heaton, R.J., Georgiadis, R.M., 2001. Nucleic Acids Research 29 (24), 5163–5168.
Rizzo, J., Gifford, L.K., Zhang, X., Gewirtz, A.M., Lu, P., 2002. Molecular and Cellular Probes 16 (4), 277–283.
Rychetsky, P., Hostomska, Z., Hostomsky, Z., Strop, P., 1996. Analytical Biochemistry 239 (1), 113–115.
Schultz, S.J., Champoux, J.J., 2008. Virus Research 134 (1–2), 86–103.
Vaisocherová, H., Snášel, J., Špringer, T., Šípová, H., Rosenberg, I., Štěpánek, J., Homola, J., 2009. Analytical and Bioanalytical Chemistry 393 (4), 1165–1172.
Vaisocherová, H., Zítová, A., Lachmanová, M., Štěpánek, J., Králíková, S., Liboška, R., Rejman, D., Rosenberg, I., Homola, J., 2006. Biopolymers 82 (4), 394–398.
Veal, G.J., Agrawal, S., Byrn, R.A., 1998. Nucleic Acids Research 26 (24), 5670–5675.
Wintersberger, U., 1990. Pharmacology and Therapeutics 48 (2), 259–280.
Zamaratski, E., Pradeepkumar, P.I., Chattopadhyaya, J., 2001. Journal of Biochemical and Biophysical Methods 48 (3), 189–208.