Evaluation of RNA Structures by Using a Probe-immobilized 27 MHz Quartz Crystal Microbalance

Tomomitsu Ozeki, Hiroyuki Furusawa, and Yoshio Okahata*
Department of Biomolecular Engineering and Frontier Collaborative Research Center, Tokyo Institute of Technology, B-53 4259 Nagatsuta, Midori-ku, Yokohama 226-8501

(Received October 19, 2005; CL-051323; E-mail: yokahata@bio.titech.ac.jp)

Binding behaviors of RNAs to a complementary ssDNA-immobilized quartz crystal microbalance (QCM) were consistent with the predicted secondary structure of RNA including a loop, stem, bulge, tail, and single strand domains.

RNAs play important roles in various functions such as catalysis, RNA splicing, regulation of transcription, and transport of proteins across membranes. The secondary and/or tertiary structures of RNAs are strongly related to the functions such as the stem loop structure of prokaryotic mRNA in the transcription process,[1] a hairpin loop structure of eukaryotic mRNA in splicing,[2] the cloverleaf structure of tRNA in the translation process,[3] and the hammerhead structure of ribozymes.[4] In the case of antisense targeting, the main subject is to know where is the suitable target site of mRNA for the specific binding of the probe DNA.[5] For predictions of secondary and/or tertiary RNA structures, RNase-mapping method,[6] enzyme mapping method,[7] and computational method[8] have been studied. Since the reliability of computational method is thought to be about 70%[9] and it is time consuming for enzyme assays, the simple experimental method to evaluate the secondary and/or tertiary RNA structures has been still expected.

In this communication, we report the binding kinetics of a simple or complex RNAs predicted to include secondary structures such as a stem, loop, bulge, and single strand to the probe ssDNA immobilized on a 27-MHz quartz-crystal microbalance (QCM), in order to evaluate experimentally effects of RNA secondary structures on hybridizations in the buffer solution. A QCM is known to provide a very sensitive mass measuring device in aqueous solution and its resonance frequency decreases linearly upon the increase of mass on the QCM electrode at the nanogram level.[10–12] QCMs have been applied to detect quantitatively the DNA–DNA hybridization,[10] the DNA–protein interaction,[11] and the enzyme reaction on DNAs.[12]

A schematic illustration of an experimental setup is shown in Figure 1. Affinix Q4 was used as a QCM instrument (Initium Co., Ltd, Tokyo, http://www.initium2000.com) having four 500 μL cells equipped with a 27-MHz QCM plate (8.7-mm diameter of a quartz plate and an area of 4.9 mm^2 of Au electrode) at the bottom of the cell and the stirring bar with the temperature-controlling system.[10–12] The 27-MHz QCM was calibrated to change frequency by 1 Hz, responding to the mass increase of 0.62 ng cm^{-2} on the electrode. Biotinylated ssDNA probes were commercially available and immobilized on an avidin-immobilized QCM plate according to previous papers.[10–12] Immobilization amount on the Au electrode (4.9 mm^2) was controlled to be about 15% surface coverage (90 ng cm^{-2}, 15 pmol cm^{-2}) to avoid a steric hindrance of RNA bindings. RNAs (50-mer and 276-mer) were synthesized by standard *in vitro* transcription

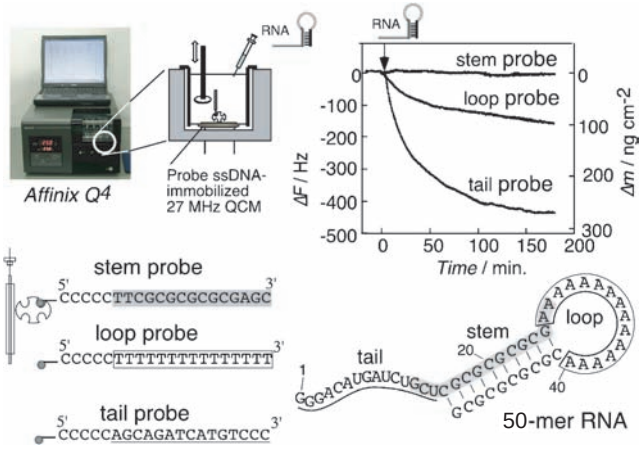

Figure 1. A schematic illustration of a 27-MHz QCM instrument (Affinix Q4) and typical binding behaviors of the 50-mer RNA predicted to include a stem, loop, and tail domains to a complementary stem, loop, and tail probe ssDNA (15-mer complementary) on the QCM ([RNA] = 25 nM, 10 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA, 20 °C). The secondary RNA structure is predicted by the computer method (mfold).

reactions[13] that were conducted in a total volume of 100 μL containing 10 pmol template DNA, 0.5 mM each of the four ribonucleoside 5′-triphosphates (NTPs), and 50 units T7 RNA polymerase by incubation at 37 °C for 1 h. For purification, the resulting RNA products were applied to denatured 15% PAGE and isolated from the gel by elution. Recovered RNAs were denatured with heating (95 °C, 1 min) and subsequently annealing with cooling quickly before use to prevent self-dimerization of RNA. RNase free water was used for all reactions. The stable secondary RNA structures of stem, loop, and tail parts were predicted by the mfold computational program.[14]

Typical binding behaviors of the excess amount (25 nM) of the 50-mer RNA to the 15-mer probe on the QCM plate, in which DNA sequences are complementary to the respective RNA sequences, are shown in Figure 1. The 50-mer RNA hardly bound to the stem probe and relatively bound to the loop probe. The RNA, however, bound to a large extent to the tail probe. Since 90 ng (15 pmol) cm^{-2} of the probe ssDNA was immobilized on the QCM, the maximum binding amount of 270 ng (15 pmol) cm^{-2} of the 50-mer RNA indicates a 1:1 binding of the RNA to the tail probe on the QCM.

From the time-courses of frequency decreases (mass increases) of the 50-mer RNA binding to the probe ssDNA, binding rate constants (k_1), dissociation rate constants (k_{-1}), and binding constants (K_a) could be obtained according to the following Eqs 1–4.[10,11] When the RNA concentration in the solution was varied in the range of 2.5–100 nM, the k_1 and k_{-1}

$$[\text{DNA}] + [\text{RNA}] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{DNA–RNA}], \quad (1)$$

$$[\text{DNA–RNA}]_t = [\text{DNA–RNA}]_\infty \{1 - \exp(-t/\tau)\}, \quad (2)$$

$$\Delta m_t = \Delta m_\infty \{1 - \exp(-t/\tau)\}, \quad (3)$$

$$\tau^{-1} = k_1[\text{RNA}] + k_{-1}. \quad (4)$$

Table 1. Binding parameters of the 50-mer RNA to probe ssDNAs on the QCM[a]

Probe ssDNA	$k_1/10^4\,\text{M}^{-1}\,\text{s}^{-1}$	$k_{-1}/10^{-4}\,\text{s}^{-1}$	$K_a/10^7\,\text{M}^{-1}$
tail	2.3	2.7	8.3
loop	1.3	3.1	4.3
stem	—	—	No binding

[a]10 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA, and 20 °C.

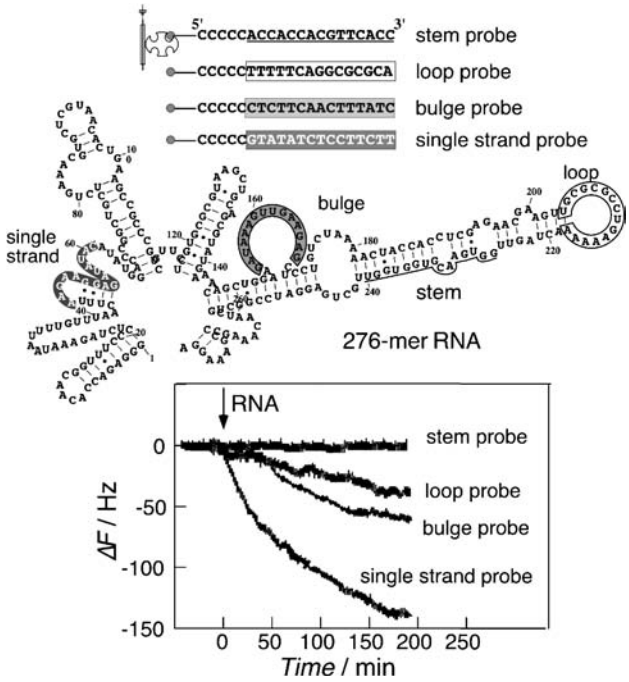

Figure 2. Binding behaiviors of the 276-mer RNA predicted to include loop, bulge, single strand, and stem domains to the complementary stem, loop, bulge, and single strand probe (15-mer complementary) on the 27-MHz QCM ([RNA] = 0.8 nM, 10 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA, 20 °C).

could be obtained from the slope and intercept of the linear correlation of Eq 4, and K_a could be calculated from k_1/k_{-1}. The obtained kinetic parameters are summarized in Table 1.

The K_a value ($8.3 \times 10^7\,\text{M}^{-1}$) for the tail probe (15-mer) to the tail part (15-mer shown as an underline) of the 50-mer RNA was consistent with that of the 15-mer DNA–DNA hybridization ($K_a = 9$–$10 \times 10^7\,\text{M}^{-1}$).[10] The K_a value for the loop part was decreased to about a half of that for the tail part. This is mainly due to the decrease of the binding rate constant (k_1) from $2.3 \times 10^4\,\text{M}^{-1}\,\text{s}^{-1}$ to $1.3 \times 10^4\,\text{M}^{-1}\,\text{s}^{-1}$, because the dissociation rate constant (k_{-1}) hardly changed. Thus, the binding rate is relatively decreased due to the distortion of the loop structure of the 50-mer RNA, but the dissociation rate is not affected. It is reasonable that the stem part of the RNA did not bind to the ssDNA.

In order to extend this method to the long and complex RNA, we chose the 276-mer mRNA encoding a peptide sequence of a part of bZIP DNA-binding domain[11] and measured binding behaviors to four different 15-mer ssDNA probes (stem, loop, bulge, and single strand) on the QCM, whose sequences are complementary to the domain sequence of the RNA. The secondary structure of the 276-mer RNA was also predicted to exist as a stable form by the computer method (mfold).[14] The result is shown in Figure 2.

The RNA did not bind to the stem probe and fairly bound to the single strand probe, as expected. The single strand domain of the RNA is calculated to have an unstable stem region. This is confirmed by the binding experiment that the single strand probe largely bound to the RNA. Bindings to the loop and bulge probes were restricted as expected. Thus, the relative order of bindings is reflecting the predicted secondary structures of the RNA. In contrast with the small 50-mer RNA, it is difficult to analyze quantitatively and kinetically the binding behavior of the large 276-mer RNA. Because the frequency changes may be affected by the viscoelasticity of the bound large RNA and the steric hindrance may not be avoided for the binding.

In summary, the highly sensitive 27-MHz QCM is useful to evaluate secondary and/or tertiary structures of RNAs from binding behaviors or kinetics to the probe in the buffer solution. The strategy described in this manuscript offers an alternative way to verify the in silico secondary structure of RNA.

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