Simultaneous Detection of L-lactate and D-glucose Using DNA Aptamers in Human Blood Serum

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Abstract: L-lactate is a key metabolite indicative of physiological state, glycolysis pathways, and various diseases such as sepsis, heart attack, lactate acidosis, and cancer. Detection of lactate has been relying on a few enzymes that need other substrates. In this work, DNA aptamers for L-lactate were obtained using a library-immobilization selection method and the highest affinity aptamer reached a K_d of 0.43 mM as determined using isothermal titration calorimetry. The aptamers showed up to 50-fold selectivity for L-lactate over D-lactate and had no measurable response to other closely related analogs such as pyruvate and 3-hydroxybutyrate. A fluorescent biosensor based on the strand displacement method showed a limit of detection of 0.55 mM. Simultaneous detection of L-lactate and D-glucose in the same serum solution was achieved. This work has broadened the scope of aptamers to very simple metabolites and provided a useful probe for the continuous and multiplexed monitoring.

With limited oxygen, anaerobic glycolysis occurs to convert D-glucose to L-lactate. L-glucose is not naturally occurring, while D-lactate is produced by some bacterial strains.[1] In this paper, unless otherwise stated, glucose refers to D-glucose and lactate refers to L-lactate. Lactate in blood normally ranges from 0.5 to 2.2 mM. Lactate can spike to over 20 mM during an intense physical exercise.[2] Athletes are keen to monitor lactate to stay in the aerobic metabolic state. While lactate can be quickly converted to pyruvate, a high level of lactate is an indication of a variety of physiological and pathological conditions such as sepsis and heart attack, acidosis and cancer.[3] Measurement of lactate is also important in the food industry to monitor fermentation, bacterial contamination and wine production.[4-5] In addition, lactate has been proposed for estimating water contamination.[6-7]

The detection of lactate has been traditionally carried out using enzymes namely lactate oxidase (LOx) and lactate dehydrogenase (LDH).[4, 8-10] The formal produces H_2O_2 as a co-product, which is the same as glucose oxidase (GOx) and many other enzymes, making it a potential source of interference especially during multiplexed detection with a high spatial resolution. LDH requires NAD^+ as an electron acceptor. The reliability and sensitivity of such enzyme-based biosensors is affected by enzyme activity, denaturation, and the concentration of oxygen or NAD^+, making calibration difficult.

Due to their metabolic connections, for various important applications, it is highly desirable to monitor glucose and lactate at the same time, such as during exercise,[2] diabetic lactic acidosis,[11] and cancer.[12] Various biosensors have been reported to detect lactate and glucose at the same time, all based on these enzymes.[13-18]

Aptamers are single-stranded nucleic acids and they have been shown to bind to a diverse range of target molecules.[19-24] Unlike enzymes, aptamers rely solely on binding for target recognition and are independent of other reactants such as oxygen or NAD^+.[25] Thus, aptamers are ideal for continuous monitoring. Lactate and glucose are both low epitope molecules and it has long been perceived to be difficult to obtain aptamers for them.[26] Fortunately, these two molecules are present in mM concentrations in blood, and thus aptamers in this affinity range are sufficient. An aptamer for glucose was reported by the Stojanovic group with a K_d of 10 mM.[27-28] Isolation of aptamers for lactate appeared to be even more challenging since it has only three carbons and is a negatively charged molecule. Thus, no aptamers for lactate are known to date. In this work, we first performed an aptamer selection experiment to isolate DNA aptamers for lactate. We then designed fluorescent biosensors for the detection of lactate. Finally, simultaneous detection of both lactate and glucose in a serum solution was achieved using the two sensors labeled with different fluorophores.

We used the library immobilization method by hybridizing our DNA library containing a 30-nt random region (N_{30}) to a biotinylated DNA attached to streptavidin beads. After incubation with L-lactate, the binding sequences were collected and amplified by PCR.[27, 29-34] Our aptamer selection was performed for a total of 20 rounds, during which the lactate concentration was gradually reduced from 1 M to 0.5 mM. The round 20 library was deep sequenced. We aligned the 10 most abundant sequences in round 20 and found that they can be divided in three families. Family 1 represented by Lac201 has two conserved regions that switched side compared to Family 2 represented by Lac204. This has been commonly observed in small molecule binding aptamers such as the aptamers for theophylline and the DNA aptamer for uric acid.[35-36] This indicates the two orientations of lactate for the same way of binding. Family 3 represented by Lac202 has a different set of conserved sequences, which suggests a different way of binding.

Three possible secondary structures of Lac201 were predicted by Mfold (Figure S1).[37] We reason that the one shown in Figure 1B was the correct folding since it can satisfy the sequence alignment within this family. The green region covaried to form a short 3-base-pair stem and the two conserved regions (shown in blue and red) are on the two sides of this short stem. The folded structure of Lac204 has the conserved nucleotides switched sides and thus is considered to have the same binding mechanism as Lac201 (Figure 1C). Family 3 appeared 8 times in the top 70 sequences, and seven of them have the exact 29-nt marked in purple in Figure 1A (Figure S2). Based on Mfold, the predicted bulged two-way junction structure of Lac202 is shown in Figure 1D.

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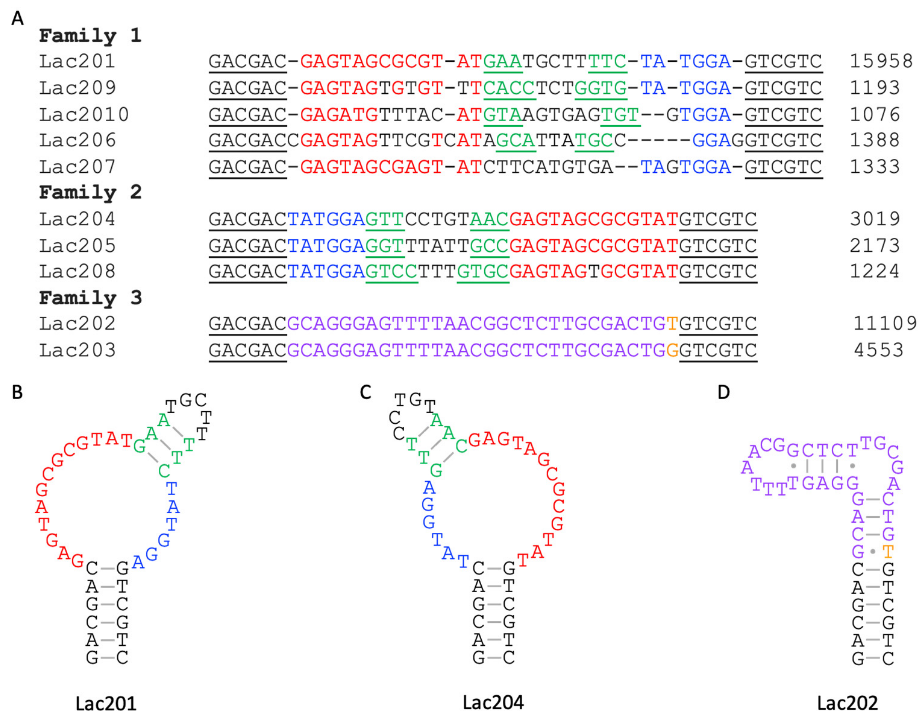

Figure 1. (A) Sequence alignment of the first 10 most abundant sequences in the round 20 library and their copy number out of a total of obtained 51,152 sequences. The predicted secondary structures of (B) Lac201, (C) Lac204, and (D) Lac202 aptamers.

To test aptamer binding to lactate, we first performed isothermal titration calorimetry (ITC). As shown in Figure 2, when lactate was titrated into Lac201 aptamer, an exothermic profile was observed with a fitted K_d of 0.43 mM, which is near the physiological level of lactate. Thus, this aptamer should be useful to measure lactate concentration fluctuation. Considering the simple structure of lactate, this is a quite reasonable binding affinity. For comparison, the glucose aptamer has a K_d of 10 mM.[27-28] By comparing the structure of glucose and lactate, the carboxylate in lactate like promoted binding via metal-mediated interactions. The ΔH of the reaction was 0.80 kcal/mol, and the ΔS was 13.1cal/mol K ($T\Delta S$ = 3.8 kcal/mol at 20°C). Thus, both enthalpy and entropy contributed to the binding, and the entropy contribution even dominated. A positive entropy change is advantageous for the use of this aptamer at higher temperature (e.g. body temperature).

After confirming binding, we then designed a biosensor for the detection of lactate. We extended the Lac201 and Lac202 aptamers by 5-nt on the 5′-end and labeled them with a FAM fluorophore. They were then hybridized with a 12-mer quencher-labeled DNA resulting in quenched fluorescence (Figure

3A). Upon the addition of lactate, aptamer binding would release the quencher-labeled strand to produce enhanced fluorescence.[38] These two sensors were then respectively incubated with different concentrations of lactate and indeed lactate-dependent fluorescence enhancement was observed (Figure 3B, D). Both sensors had an instantaneous fluorescence increase and the fluorescence immediately reached a stable value. Both sensors reached around 5-fold saturated fluorescence increase compared to the background. We then plotted the fluorescence enhancement as a function of lactate concentration (Figure 3C, E). Lac201 has a dynamic range up to 20 mM and a limit of detection (LOD) of 0.55 mM, which nicely covers the physiological range of lactate. For five commercial lactate biosensors based on LOx, none could cover the full range from 1 to 23 mM.[39] Therefore, our aptamer sensor has a potential advantage in the detection range.

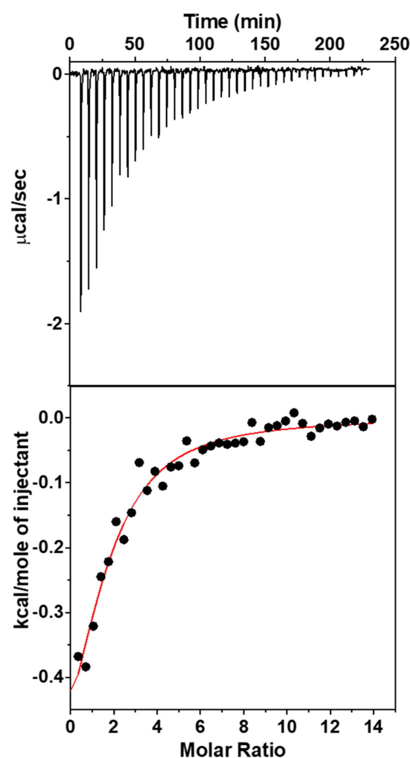

Figure 2. ITC thermogram and integrated heat of titrating 20 mM L-lactate to 300 μM Lac-201 at 20°C in the SELEX buffer.

Although the apparent K_d of Lac201 from this sensor data was 13.5 mM, its actual K_d was calculated to be 0.39 mM after considering the competing effect of the quencher-labeled strand (Figure S3).[38, 40] This is quite close to the 0.43 mM obtained from ITC. We then tested the response to D-lactate, which also showed an instantaneous response (Figure S4). At 100 mM level, the response was still far

from saturation (Figure 3C, blue dots) and an accurate K_d cannot be fitted. The response to 20 mM D-lactate was similar to 2 mM L-lactate, and thus the selectivity was around 10-fold.

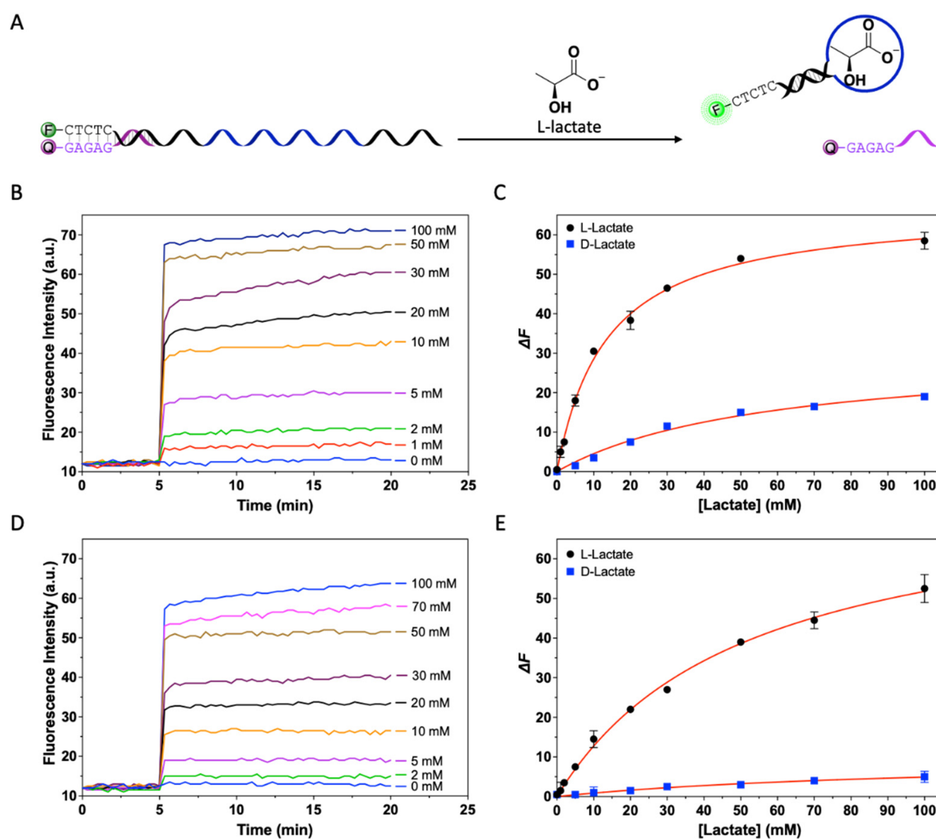

Figure 3. (A) Scheme of the fluorescent biosensor for lactate detection. Kinetics of sensor signaling using the (B) Lac201 and (D) Lac202 aptamers. Lactate was added at 5 min. Change of fluorescence as a function of L-lactate and D-lactate concentration using the (C) Lac201 and (E) Lac202 aptamers.

We then fitted the sensor response of Lac202, which has an even broader range up to 100 mM, and its LOD was calculated to be 1.2 mM. The real K_d was determined to be 1.92 mM. Its selectivity for L-lactate over D-lactate was even better. 100 mM D-lactate response was similar to 2 mM of L-lactate (around 50-fold selectivity). The difference in the chiral selectivity of these two aptamers also indicated their different ways of binding to lactate. The chiral selectivity of aptamers is well documented.[41-42] The chiral selectivity is analytically useful since most instrumentation method can hardly distinguish L- and D-lactate.[1] Since D-lactate exists in blood only in patients with some rare diseases,[43] its interference is not a concern for the detection of L-lactate.

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After confirming binding and sensitivity of the sensors, we then tested the selectivity against some related compounds, especially those might be present in blood in high concentrations (Figure 4). Since Lac201 has a more sensitive response, we first focused on it. Pyruvate is an important analyte since it is the product of lactate oxidation. 3-Hydroxybutyrate differs from lactate only by a single methylene group and it is a main keto body.[44] None of them showed any response. Acetate being a carboxylic acid, also showed no response. Among the two sugars and four amino acids, only cysteine and serine showed a slight response at 5 mM level. Note that other than lactate and glucose, all the other tested molecules are only in μM concentration (the physiological range of these molecules supplied below their structures in Figure 4),[45] yet they showed no response even at 1 mM. Even better selectivity was achieved for the Lac202 sensor (Figure S5). Overall, for the analysis of lactate in blood or serum, the sensitivity and selectivity of Lac201 are sufficient.

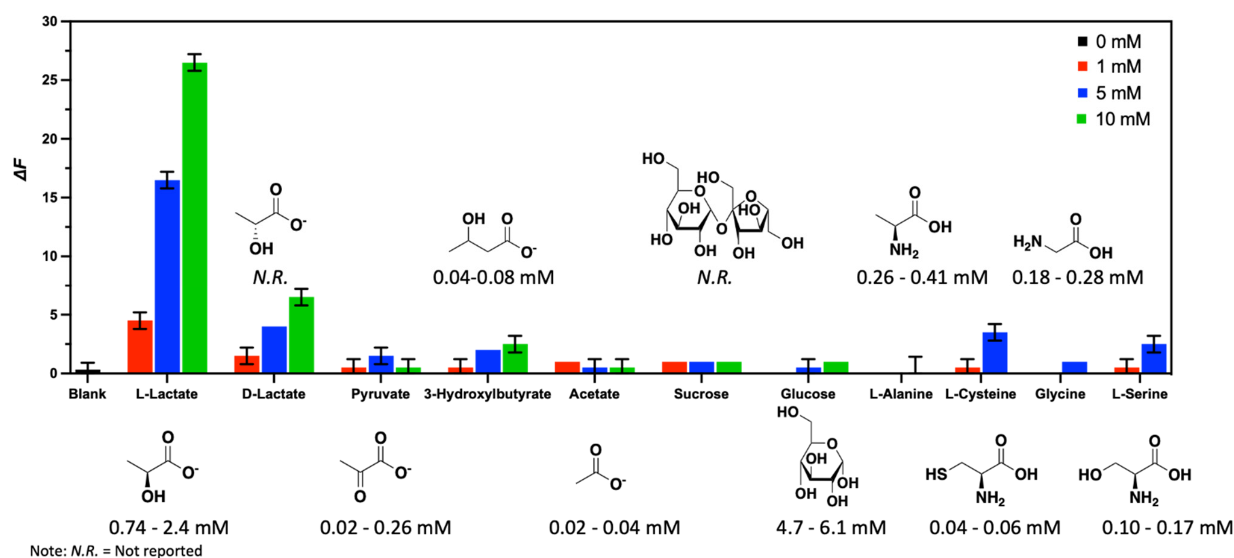

Figure 4. Selectivity of the Lac201 sensor against some common biomolecules in serum. The structure and normal physiological concentration range of these analytes (values from ref.[45]) are also shown.

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Since the detection of lactate in blood serum is critical for its application, we then tested the Lac201 sensor in 10% human serum (Figure S6). The response was very similar to that in buffer and we still obtained a detection range of up to 20 mM and a limit of detection of 1.4 mM. Therefore, serum proteins had little effect on the performance of the sensor.

Finally, we tested if we can achieve simultaneous detection of both lactate and glucose. The glucose aptamer was labeled with a Cy5 fluorophore and the same quencher-labeled DNA was used. Although our glucose sensor had a slightly different design compared to the literature reported one,[27] it responded to glucose as expected (Figure S7). We mixed the two sensors in the same serum solution and then added lactate and glucose (Figure 5A). When lactate was added, only the FAM channel fluorescence increased, whereas when glucose was added, only the Cy5 channel fluorescence increased (Figure 5B). Both sensors had instantaneous responses. Figure 5C shows a photograph of the sensor response to lactate and glucose. Therefore, these two aptamers can detect both lactate and glucose in the same sample without cross activities.

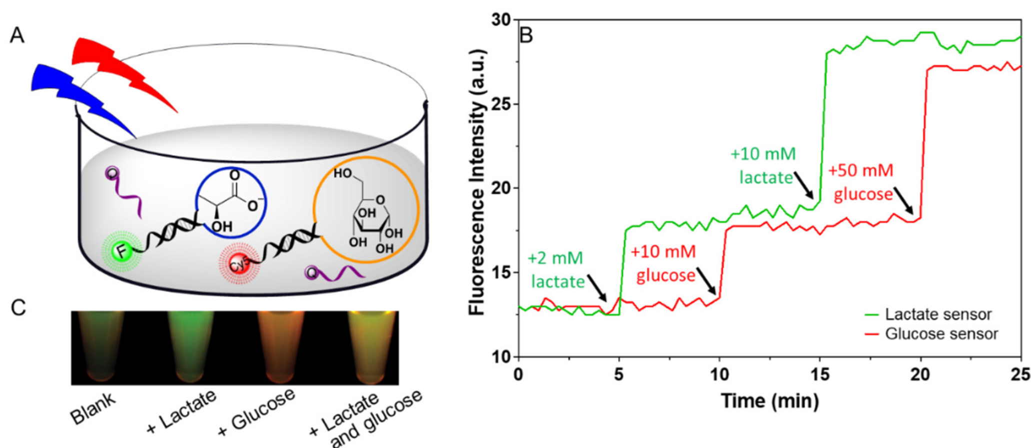

Figure 5. (A) A scheme showing simultaneous detection of glucose and lactate by mixing the two aptamer-based biosensors with different fluorophores. (B) Time-dependent responses of the two sensors in the same 10% human serum sample. (C) A photograph of the sensor when adding lactate and glucose.

The fact that high quality aptamers can be obtained for molecules as simple as lactate is very encouraging for the aptamer field. It will stimulate the isolation of aptamers for other

important metabolites that traditional can only be detected by enzymes. This work reinforces the feasibility of the next generation biosensors for metabolites by replacing enzymes with aptamers. Given the excellent selectivity of the sensors, multiplexed detection of even more related analytes, especially in a wearable biosensor format is a viable and attractive future direction of the field.[25, 46]

In summary, we reported the first DNA aptamers that can bind L-lactate with a K_d around 0.4 mM and it can nicely cover the physiological concentration range of L-lactate. Since it does not require other substrates and can directly sense the concentration change by its fast binding, this aptamer will find applications in biomedical diagnosis, food industry and environmental monitoring. In addition, this work indicates the range of analytes that aptamers can bind. Even for a very simple and low-epitope molecule like L-lactate, high quality aptamers can still be obtained. This will motivate researchers to explore aptamer-based analytical methods for other simple yet important metabolites. Finally, multiplex detection of glucose and lactate is a nice demonstration of aptamer sensors, showing a good potential for future in vivo continuous monitoring of multiple analytes.

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Keywords: lactate • biosensors • aptamers • glucose • fluorescence

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