Aptapaper

Using Aptamer-Based Proximity-dependent Ligation and Nucleic Acid Sequence Based Amplification for Diagnostic Protein Detection

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Contributions:

Conceptualization: N.E., C.C,; Methodology: A.N., N.E, C.C,; Software: A.N.; Formal Analysis: N.E., A.N.; Investigation: A.R., N.E, C.C., A.H., A.G., Z.B., D.L., C.K., R.P.,V.V., I.K., A.N., R.S.; Resources: R.S.; Writing-Original Draft: A.R., A.N., E.R., C.C.; Writing- Review and Editing: A.R., V.V., AG, A.N.; Visualization: V.V.; Supervision: A.R., N.E, C.C., R.S; Project Administration: R.S, C.C, D.L., A.H.; Funding Acquisition: V.V., A.G., A.N., R.S., A.R, C.C., N.E.,

Abstract

Using aptamer-based proximity-dependent ligation (PDL), nucleic acid sequence based amplification (NASBA), and cell-free epression, we attempted to design a proof of concept for a paper-based diagnostic device that produces a colorimetric output in the presence of a target protein biomarker. This system, which could be performed outside the lab with minimal training, could provide an inexpensive alternative to enzyme-linked immunosorbent assays (ELISA). The reaction scheme begins with two single-stranded oligonucleotide probes which selectively bind to independent sites on a protein biomarker via aptamer domains. The selective binding of the oligonucleotide probes to the target protein promotes the ligation of the two probes into one continuous ssDNA strand by T4 DNA ligase. The oligonucleotide formed by the ligation of the probes is amplified exponentially via a modified NASBA reaction to increase the signal, enabling lower detection limits for protein biomarkers. After amplification, the alpha fragment of LacZ is expressed through a cell-free expression reaction. The alpha fragment can then undergo alpha complementation with the LacZ omega fragment present in the reaction mixture, forming functional LacZ capable of cleaving XGAL, forming a colorimetric output. The system is designed to operate as a one-pot reaction under isothermal conditions, and would be freeze dried onto paper for use as a point-of-care device. By using aptamer-based PDL as the foundation for the detection scheme, this system could potentially attain the extremely low detection limits characteristic of PCR-coupled PDL in an inexpensive, easily portable system. As proof of concept, we attempted to demonstrate that components of this setup could be viable with Thrombin as the target protein. While we did not test the nucleic acid sequence based amplification in this context yet, our probably positive result from the PDL assay is an indication that this system has potential to meet our design goals.

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Competing Interests:

The authors have declared that no competing interests exist.

Ethics Statement:

N/A

Data Availability:

All data are fully available without restriction.

Full Submission:

Introduction:

Accurate, rapid, and affordable disease diagnosis in regions with little health care infrastructure remains a major challenge in the world. We set out to solve this major global issue through the use of aptamer-based technology. Aptamers are DNA or RNA segments that bind with high specificity and affinity to particular protein targets (Pardee et.al. 2016). It has been previously demonstrated that aptamer recognition of biomarkers can be used as a diagnostic tool through the use of a toehold riboswitch (Green et.al. 2014), and that such detection systems can be flash frozen onto paper for later use (Pardee et.al. 2014). Our previous research showed that a major obstacle to the efficacy of these systems for real-world diagnostics is a lack of signal amplification. While this can be easily overcome in a lab setting, it is impractical in the poor infrastructure areas where this paper-based technology would be most appealing. To combat this, we turned to proximity dependent ligation, followed by NASBA. Proximity dependent ligation involves two single stranded DNA probes, and an ssDNA “bridge” segment. The two probes are each designed with a target protein specific aptamer on one end, and half of a reporter gene sequence on the

other. The bridge segment is complementary to the non-aptamer ends of the two probes. When the two probes find the target protein, the aptamers bind to it. This increases the effective concentrations of their non-aptamer ends, allowing the bridge segment to anneal to the two free ends of the probes, connecting them. This complex is ligated, and the reporter gene is now whole and can be transcribed. The key feature of this system is that the whole reporter gene is only transcribed in the presence of the target protein, retaining the diagnostic capability of the aptamer riboswitch (Fredriksson et.al 2002). However, unlike the riboswitch, proximity dependent ligation can serve as the launching point for NASBA, which allows PCR-like amplification at a single temperature in a one-pot reaction (Compton 1991). This whole system can be flash frozen onto paper and used as a disease diagnostic device without the need of laboratory infrastructure.

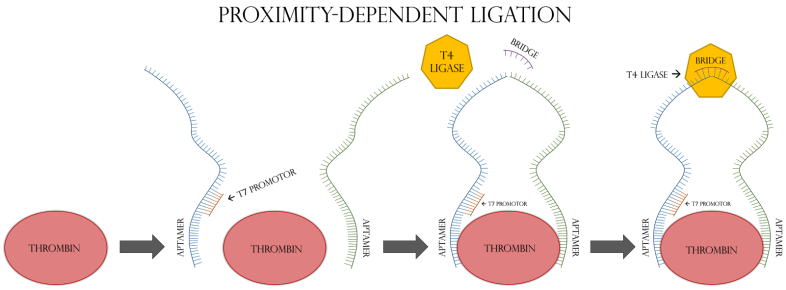


Figure 1: Details the process of proximity dependent ligation utilized to detect the protein biomarker, Thrombin.

Comments

mbecich: Did you make these graphics yourself? If so, please make the fonts more readable. If not, please attribute the images.

renberg: We did make these ourselves for our poster. They were crisper there, but got a bit pixelated when we made it smaller for this. We will make a cleaner version for the final manuscript. Thanks for the feedback!

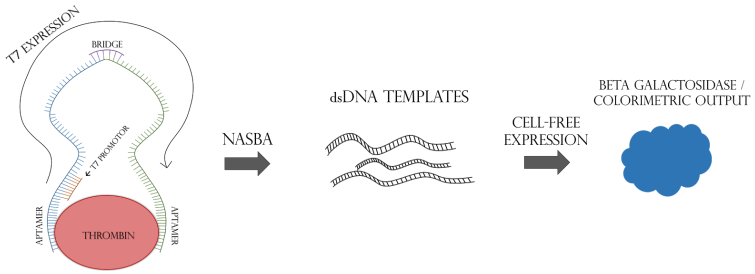


Figure 2: Details the downstream processes of PLA that allow for the cell-free expression of beta-galactosidase as out colorimetric indicator of Tuberculosis.

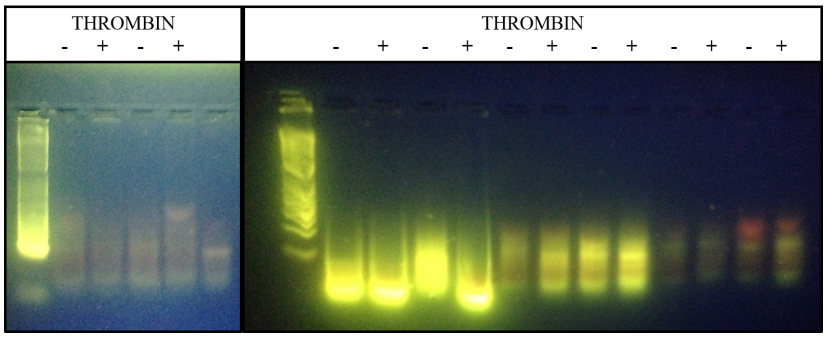


Figure 3: (a) The fourth band shows the size probe 1 and 2 ligated , (b) Proximity ligation assay at different conditions

Comments

MathieuBorel: Lack of details... It would perhaps be interesting to merge the 3 figures, and build a more detailed caption. Figure 3 is very dense in informations, and it's hard to get to the point.

renberg: We will add more detail to the 3rd figure's caption, and try to clarify the important points. We may also combine the first two figures, but it doesn't really make sense to combine with the 3rd since they are not super related.

mbecich: Agree with Mathieu, could you please make this figure clearer to guide your eyes?

renberg: How would you recommend doing this? Boxes highlighting sections? Arrows?

mbecich: Yes! Either/or would work well (more than just +/-)

Methods:

Materials. Cell free expression kits, Gibson Assembly kits and T4 ligase were purchased from New England Biolabs. RNAse inhibitor was purchased from Roche and thrombin was purchased from Haematologic technologies Inc.

Plasmid Cloning. The LacZ gene from the biobrick(BBa_K564012) was subcloned into the PET28a plasmid, a bacterial cell free expression vector. Overhang Chain Polymerase reaction was performed in the LacZ sequence adding EcoRI and Xbal upstream and; HindII and PstI downstream. The PCR output was then digested with EcoRI and HindIII and ligated into PET28a. This plasmid was used to create a delta M-15 mutant plasmid, which has the amino-acid residues 11-41 deleted, leaving only the LacZ omega fragment. This deletion was performed using the Q5 site direct mutagenesis kit from NEB.

Comments

mbecich: Watch the spacing typos, capitalization of the restriction enzyme, and extra bold titlehead. You may consider introducing this section with a brief sentence on the goal you set out to achieve with cloning, rather than jumping into the steps.

renberg: Thanks for the heads up on the typos, we will fix those. Intro sentence is a good idea too. Thanks for all the feedback!

Proximity dependent ligation assay. Incubated 200ng of the two probes and connector with thrombin at an equimolar concentration with 2uls of T4 DNA ligase for the proximity ligation to occur. Heated samples samples at 70C to heat inactivate ligase and break connector-probe interactions, then immediately loaded in a 1.75% agarose.

Comments

MathieuBorel: Mind the first sentence which is not clear. May be sould you give concentrations insted of a mass for the DNA probes amouts ? How much time did you heat the samples ? 1.75 % agarose "gel". Which buffer did you use, which voltage ? How did you reveal your DNA (BET, CYBR green...)

renberg: Thanks for these questions, we will add more detail about our methods and try to explain the assay more clearly.

Cell line. The DH5a chemically competent cells were used for plasmid proliferation purposes.

Comments

MathieuBorel: Did you produce them or bought them ?

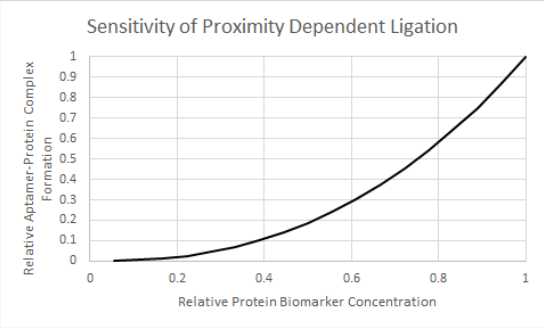
renberg: Bought them. We will specify brand.

mbecich: Perhaps retitle this model organism and expand on the DH5-alpha? Otherwise, this may not be worthy of its own section.

emernic: That is a good point. Perhaps we should just remove this section and specify the cell-type used for cloning elsewhere in the methods section.

Mathematical Modeling. A mathematical model was constructed in order to test the sensitivity of the proposed proximity dependent ligation assay (PLA) in the detection of biomarkers. An enzyme kinetics-based model was constructed using the Wolfram Mathematica (10.4) symbolic algorithm program. The model aimed to quantify the concentration of aptamer-protein complexes at chemical equilibria (and thereby, elucidate the rate of protein recognition by the aptamers) for different concentrations of the initial reactants. The initial reactants characterized in the mathematical model include the concentrations of aptamer 1, aptamer 2, the connector oligonucleotide, and the target protein. Intermediate complexes formed prior to the final aptamer-protein complex were also considered in the mathematical model.

The mathematical model was able to conclude the high sensitivity with which the proximity dependent ligation assay is able to detect protein biomarkers. At low concentrations of the protein, little to no formation of the final aptamer-protein complex was seen, suggesting the low probability of the assay in producing false positive results. The concentration of the aptamer-protein complex was also seen to increase at an exponential rate with a gradual increase in the initial protein concentration, implicating the high sensitivity of the assay in the detection of biomarkers at relatively high protein concentrations.



The graph depicts the general pattern seen for the formation of the aptamer-protein complex when the protein biomarker concentration was modified. The observable pattern was also limited to specific values and ranges of protein concentrations, and the mathematical model produced insensible aptamer-protein complex concentration and gibbs free energy values for many of the tested protein concentrations. Our simplified mathematical model may not have been able to capture the sophisticated interactions that take place at the microscopic level. A model that captures the microscopic interactions of the aptamers and biomarkers should be able to quantitatively, rather than qualitatively, discern the mechanisms of the assay.

Comments

mbecich: You should be more explicit about how your mathematical model guided you. It is fine to admit that it only had qualitative accuracy, but I think more information on the parameters are necessary to establish your claim because enzyme-kinetics are very well studied. What makes this modeling especially informative for your purposes? Feel free to reach out to me if you would like some advice on how to frame this; I just completed a Systems Biology course at Stanford and modeling can be an incredibly powerful tool for research, but there is no need to force it.

emernic: Thank you for your feedback and your offer! We actually completely redid and expanded this section in our next draft.

Results:

Successfully deleted the alpha fragment in Lacz+pet28 plasmid, leaving only the omega fragment. This plasmid is constitutively expresses the omega fragment which complements the alpha fragment produced in the proximity-dependent ligation forming functional LacZ.

Comments

MathieuBorel: Proof for this should be given in a figure.

emernic: Good point. We will include an image showing successful in vivo alpha complementation.

Proximity dependent ligation allows the system to only be activated in the presence of its target protein. Figure 3(a,b) show the proximity dependent ligation assays. The probes ligated should be about 300 base pairs long, the samples were stained with SYBR Gold dye for staining.Samples in Figure 3a were left incubating at 35C for 9 hours and heated to 70C to deactivate ligase and melt the connector-probe interactions. The first three lanes have the different sizes of connectors in ascending base pair length (going from left to right), no thrombin was added to the first three lines. The next three lanes are identical except they also contain thrombin. The last lane is probe 1 alone without any other reaction components added. The bands observed for probe 1 alone seem to suggest that two common conformations for probe 1, an unfolded conformation which is observed to run at

approximately the position of the 100bp band on the ladder, and a folded conformation which runs farther down on the gel. The bands for the ligation reactions do not seem to show significant ligation activity, because there is no clear high molecular weight band; however, there may be a small amount of ligation occurring in lane 5, the reaction with the 3C connector and thrombin added, evidenced by the faint molecular weight band in that lane.

Comments

MathieuBorel: This piece of information is interesting, but perharps sould it be reported in the method section and in the figure 3 caption ?
emernic: Yes, this section can get convoluted and difficult to follow. We are redoing it in our new draft. Thank you.

Figure 3b shows the assay at more conditions Lane 2 is a 30 minute reaction at room temperature without thrombin. Lane 3 is a 30 minute reaction at room temperature with thrombin added. Lanes 4 and 5 are the same as lanes 3 and 4 but run at 37 degrees C. Lane 6 is a 4 hour reaction at room temperature without thrombin. Lane 7 is a 4 hour reaction at room temperature with thrombin added. Lanes 8 and 9 are the same as lanes 6 and 7 but run at 37 degrees C. Lane 10 is a 19 hour reaction at room temperature without thrombin. Lane 11 is a 19 hour reaction at room temperature with thrombin added. Lanes 12 and 13 are the same as lanes 10 and 11 but run at 37 degrees C. Although there is no band that seems to be the right size(300base pairs) the faint red bands show a higher molecular weight than the rest, these samples were incubated at 37C for 19 hours, more experiments are needed to find the optimal incubation time and temperature for the PDL.

Comments

MathieuBorel: Great piece of information :-) Did you try to analyse more in depth what was included in these bands ? Do you have more clues of the reason it's not migrating at the expected size ?
emernic: Due to time restrictions we were not able to do further characterization of these bands. I think it is an interesting result too.

Discussion/Conclusion:

We aimed to design proximity dependent ligation (PDL) probes and an amplification system for thrombin detection as a model which could be extended to detect biomarkers for diseases. Mutagenesis was used to create a lacZ ΔM15 mutant inserted into a protein expression plasmid (pET28a) with a lac-inducible T7 promoter. This lacZ ΔM15 mutant was designed to lack the α-fragment of LacZ. In theory, PDL—in the presence of thrombin—would initiate the expression of the missing LacZ α-fragment. This α-fragment would then combine to the lacZ ΔM15 mutant, creating functional LacZ that acts as the colorimetric output in our proposed diagnostic device. Unfortunately, although we tested the PDL reaction under a variety of experimental conditions, we were unable to successfully show significant ligation using our probes on an agarose gel using SYBR gold stain. Future steps include testing LacZ α-complementation using empty pBlueScript KS (+) vector and lacZ ΔM15 mutant in pet28a to express the two LacZ fragments. This could be done in vitro by using T3 and T7 polymerases to drive expression of the two fragments and measuring absorbance of the colored product. Complementation could also be tested in vivo by transforming both plasmids into BL21 E. coli on double antibiotic selection plates with IPTG and X-gal. The success of this project could allow for the creation of a broad variety of paper-based assays for protein biomarkers, creating far-reaching positive impacts on diagnostic testing including lowering cost of production and consumption, minimizing the time it takes for a complete diagnostic test, and increasing diagnostic accuracy. This would be a major improvement for healthcare systems around the globe.

Comments

MathieuBorel: Your work is promising, I would appreciate if you could write some sentences about futures experimentations to be done to fully validate this system.
emernic: Good point. We added this in our new draft. Thanks!
mbecich: Yes, definitely expand on this section! A lot of promising material here, but the end felt rushed.
emernic: Yes, we will add more discussion and analysis here. Thanks.

Acknowledgements:

Marc Ammerlaan, Pearl Tu, OptiMize, Dennis Drobeck, iGEM, Caymen Chemical, University of Michigan

Comments

mbecich: Perhaps detail each acknowledgment a little more clearly?
emernic: This is a good point. We should specify their contributions.

CITATIONS:

1.

Pardee, K., Green, A.A., Takahashi, M.K., Braff, D., Lambert, G., Lee, J.W., Ferrante, T., Ma, D., Donghia, N., Fan, M., et al. (2016). Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. Cell 165, 1255–1266.

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Green, A.A., Silver, P.A., Collins, J.J., and Yin, P. (2014). Toehold Switches: De-Novo-Designed Regulators of Gene Expression. Cell 159, 925–939.

3.

Pardee, K., Green, A.A., Ferrante, T., Cameron, D.E., DaleyKeyser, A., Yin, P., and Collins, J.J. (2014). Paper-Based Synthetic Gene Networks. Cell 159, 940–954.

4.

Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gústafsdóttir, S.M., Östman, A., and Landegren, U. (2002). Protein detection using proximity-dependent DNA ligation assays. Nature Biotechnology 20, 473–477.

5.

Compton, J. (1991). Nucleic acid sequence-based amplification. Nature 350, 91–92.

Comments

MathieuBorel: Your work lacks of some references, particularly with the modeling part.
emernic: Yes. This is a good point. We have actually completely expanded the modeling section in our new draft to be much more detailed.

PLOSFeedback: Hi @renberg (/user/renberg) Thanks for your submission to the PLOS iGEM Project. Our editorial team have reviewed your article and have the following feedback: This paper looks at designing a paper-based colorimetric sensor for protein biomarkers. Overall, this is a preliminary paper and the structure of the paper is a little unclear. The methods section needs more details regarding the source of reagents used, e.g., the catalog number and company name. However, there is good potential to develop this work further and expand on the topic. If we can provide any clarifications or comments please do let us know - collections@plos.org

> **emernic:** Thank you for your feedback. Since submitting this, we have made major revisions to increase the clarity of the paper and expanded on the modeling of the system to highlight and explain this section. Hopefully the changes we have made will be a significant improvement. Thanks again.

mbecich: Great work Michigan! I was leading an aptamers sub-project on my own iGEM team, and I remember reaching out to you via email early in the summer. I am glad to see that the work was a success! Overall, you distilled your research nicely into a paper. I think the language could be improved (I noticed times when it was too informal or was too "hand-wavy." Try to stick to the facts. I appreciated your honesty about deficiencies in the projects; perhaps at those times try focusing more on future directions and expand on what you tried (failed/succeeded with). Good luck!

> **emernic:** Thanks for your feedback. It is always fun to see another group working on a similar topic! Since submitting this paper we have greatly revised and improved the paper to hopefully be much more detailed and informational.