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Insulin-binding aptamer-conjugated graphene oxide for insulin detection[†]

Ying Pu^{‡,a,b}, Zhi Zhu^{‡,b}, Da Han^{‡,b}, Huixia Liu^a, Jun Liu^{a,b}, Jie Liao^a, Kejing Zhang^{a,b}, and Weihong Tan^b

^aXiangya Hospital, Central South University, P.O. Box 190, Changsha, Hunan, 410008, China.

^bDepartment of Chemistry and Department of Physiology and Functional Genomics, University of Florida, Gainesville, Florida, 32611-7200, USA.

Abstract

This paper describes a simple and sensitive aptamer/graphene oxide (GO) based assay for insulin detection. GO can protect DNA from nuclease cleavage, but aptamers can be detached from the GO surface by specific target binding. This exposes the aptamers to enzymatic cleavage and releases the target for a new cycle. Cycling of targets leads to significant signal amplification and low LOD.

Insulin is a hormone that is central to the regulation of carbohydrate and fat metabolism in the body. It plays a very important role in the control of blood glucose concentration. The detection of insulin is especially significant in clinical work, because the level of insulin is the most critical indicator of the endocrinal function of beta cells and serves as a valuable basis for the diagnosis of diabetes mellitus, insulinoma, insulin resistant syndrome, *etc.* In particular, diabetes mellitus is one of the most commonly occurring chronic diseases, and it affects over 40 million people in China. Obesity, western dietary habits, sedentary lifestyles, and aging will continue to drive a dramatic growth of diabetes for decades. A rapid and reliable laboratory test for detection of insulin levels in serum can be helpful to diagnose diabetes in its early stages.^{1,2}

The current detection methods for insulin are mainly based on radioimmunoassay (RIA),^{3,4} enzyme-linked immunosorbent assay (ELISA)⁵ and chemiluminescence immunoassay (CLIA).⁶ RIA is now the routine method for quantitative insulin detection. However, the reagent is radioactive and the entire process is tedious and time-consuming with expensive and sophisticated instruments involved. The other methods also require complicated equipment and their specificity is not sufficient.

Aptamers are single-stranded nucleic acids or peptide molecules with unique secondary structures that can bind specifically to their targets. Aptamers are usually selected by

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Correspondence to: Huixia Liu; Weihong Tan.

[‡]Co-first authors.

repeated rounds of *in vitro* selection termed SELEX (systematic evolution of ligands by exponential enrichment).^{7,8} Aptamers can be selected for a broad range of targets, including small molecules, proteins, nucleic acids, cells, tissues and organisms.^{9–12} The binding ability of aptamers is as good as that of antibodies, but the synthesis, maintenance and delivery of aptamers are much easier, making aptamers promising molecular receptors for bioanalytical applications.^{13–15}

Another development in clinical diagnostics and treatment is the increasing use of nanomaterials for sensitive, selective, rapid, and cost-effective analysis devices. Among these, carbon nanomaterials are especially attractive, because of their rich chemical, optical and mechanical properties. For example, carbon nanotubes and single-stranded DNA(ssDNA) assemblies have been used for homogeneous detection of biomolecules.^{16–18} Graphene oxide (GO) has also been utilized by Lu *et al.*¹⁹ for sensitive and selective detection of DNA and proteins. There are several advantages of using GOs as the substrate to build biosensors. First, water-soluble GOs can be regarded as graphene covalently decorated with oxygen-containing functional groups, which allow GOs to interact with a wide range of molecules *via* covalent, non-covalent and/or ionic interactions. Furthermore, GOs interact differently with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). ssDNA adsorbs stably on the GO surface due to π -stacking interactions, but dsDNA does not, and this difference is essential for sensing design. Second, GOs have been reported to be super-quenchers to a wide range of fluorophores, *via* fluorescence resonance energy transfer or non-radiative dipole–dipole coupling. Third, due to the steric-hindrance effects, GO can protect biomolecules from enzymatic digestion or degradation in biological environments. Fourth, GOs, as nanocarriers, have ultra-high surface areas for loading, including multiple molecules for multiplex sensing. Inspired by the significance of GO, we designed an insulin sensor by conjugating the insulin binding aptamer (IBA)²⁰ with GO for sensitive and selective insulin detection. Fig. 1 shows a schematic representation of our proposed two sensing mechanisms.

As the first step, the interaction of aptamer, GO and target insulin were investigated, as shown in Fig. 1A. The insulin aptamer is labeled with fluorophore on 3' end and is incubated with a GOs solution. Due to the strong π – π interaction between single-stranded aptamer and GOs, the IBA/GO complex is formed, bringing the fluorophore to the surface of GO and quenching the fluorescence. Upon the introduction of target insulin, the IBA can bind with insulin and undergo a conformational change from ssDNA to dsDNA, thereby weakening its interaction and releasing it from the GO surface. As a result, the separation of the fluorophore from the GO surface restores the fluorescence signal.

To realize this design, the insulin aptamer, adapted from Yoshida *et al.*'s work,²⁰ was synthesized with fluorescein conjugated on the 3' end. When incubated with GO solution, a dramatic fluorescence signal decrease was observed, indicating strong adsorption of ssDNA on GO and high fluorescence quenching efficiency. Subsequently, different concentrations of insulin, ranging from 5 nM to 50 μ M, were added to the IBA/GO complex solution. Referring to Fig. 2A, the fluorescence signal became distinguishable from background after addition of 500 nM insulin. The calibration curve in Fig. 2B was plotted with fluorescence intensity at 516 nm *versus* the concentration of insulin. A linear relationship was observed in

the 500 nM to 5 μ M range. The results demonstrate that GOs are efficient quenchers for dye-labeled aptamers, and that target molecules, insulin in this case, can bind with aptamer and remove it from GO surface to restore fluorescence. However, in this experimental mode, one target binds and releases only one aptamer and accompanying signaling molecule. Due to the intrinsic property of this insulin binding aptamer, the K_d is relatively high and the limit of detection (LOD) is 500 nM. Further optimization may lower this a little, but the LOD would still be in the hundred nM range. With no signal amplification, the working principle has been demonstrated, but with limited sensitivity.

In order to lower the LOD and improve the detection sensitivity with this high K_d aptamer, a signal amplification mechanism is needed. Inspired by recent work of Lu *et al.*,¹⁹ as well as the ability of GOs to protect surface-bound DNA from enzymatic digestion, the second sensing scheme was proposed, as illustrated in Fig. 1B. Deoxyribonuclease I (DNase I) is a nuclease that can cleave ssDNA, dsDNA or chromatin into fragments. In the absence of target, aptamers are attached to the GO surface and are protected from DNase I digestion. However, in the presence of target, the aptamer forms a stable, rigid structure with the target and is released from the GO. The free aptamer in solution is subsequently digested by DNaseI, thereby releasing the target. The released target then binds to another aptamer, removes it from the GO, and the cycle repeats. By digesting the aptamer and recycling the target, a significant amplification of the signal can be realized to give high sensitivity for target detection.

The amplification assay was prepared by mixing the fluorescein-labeled aptamer with GO to form the aptamer-GO complex. Then, target insulin and DNase I (25U) were simultaneously added, and the mixture was incubated at room temperature for another 2 h. Subsequent fluorescence measurements showed a dramatic increase in the final fluorescence intensity. Fig. 3A shows the fluorescence emission spectra of the fluorescein-labeled IBA/GO complex upon the addition of DNase I and insulin at concentrations from 5 nM to 5 μ M. The fluorescence intensity started to recover after addition of only 10 nM insulin, and Fig. 3B shows the calibration plot. The inset of Fig. 3B shows the results of three parallel experiments performed to evaluate the LOD of this method. Based on the equation $LOD = 3\sigma/S$ (σ is the standard deviation for the blank, S is the slope of calibration curve), the LOD of the amplification strategy was determined to be 5 nM. In contrast, the LOD was 500nM for the 1 : 1 binding strategy without amplification. Although the system has not yet been fully optimized, these results clearly demonstrate that the amplification strategy greatly enhances the sensitivity of the insulin detection, and the LOD was decreased by almost one-hundred fold.

The specificity of the amplified assay was also examined. As shown in Fig. 4, we challenged the system with several common proteins, such as streptavidin, avidin and bovine serum albumin (BSA), each at 5 μ M. Significantly higher fluorescence was observed with the target insulin compared to the control proteins. These results clearly demonstrate the high specificity of our amplified aptamer/GO assay for insulin detection.

In conclusion, we have demonstrated two sensing schemes based on the IBA/GO complex. In the 1 : 1 binding strategy, the interactions among the aptamer, GO and target insulin were

investigated, and the results proved that target-aptamer binding events can compete with that of aptamer-GO binding and release aptamer from the GO surface for sensing. Furthermore, the amplified strategy using DNase I enhanced the sensitivity and lowered the LOD, based on the ability of GO to protect aptamers from nuclease cleavage. The assay can be prepared by simply mixing the aptamer, GO, nuclease, and target insulin. The sensitivity of this assay was one-hundred fold higher than the traditional 1 : 1 binding assays and the LOD was lowered to 5 nM insulin. Thus, the proposed amplified assay based on the use of an IBA conjugated GO can be expected to provide a sensitive platform for the insulin detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Illustration of insulin binding aptamer (IBA)/GO sensing scheme. (A) 1 : 1 binding strategy that one target can release only one aptamer from the GO surface. (B) Amplification *via* DNase I digestion of the aptamer to release the target molecule for more reaction cycles.

Fig. 2. Fluorescence results of 1 : 1 binding strategy. (A) Fluorescence-emission spectra of the Fluorescein-labeled IBA/GO complex upon the addition of insulin at different concentrations. (B) Calibration curve for insulin detection. The inset shows the linear range of 500 nM to 5 μM.

Fig. 3. Fluorescence results for the amplified detection strategy. (A) Fluorescence-emission spectra of the Fluorescein-labeled IBA/GO complex upon the addition of insulin at different concentrations. (B) Calibration curve for insulin detection. The inset shows the linear range of 5 nM to 100 nM used to calculate the LOD.

Fig. 4.
Selectivity of the amplified IBA/GO assay for insulin over other control proteins:
streptavidin, avidin, BSA (each at 5 μ M).