Acta Biochimica Iranica 1(3): 139-144, 2023

Original Article

Development of enzyme-linked immunosorbent assay (ELISA) based on covalent immobilization of antibody on plate for measurement of digoxin

Hamidesadat Enayati [1], Javad Mohammadnejad [1*], Alireza Nikfarjam [2]

[1] *Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran.*

[2] *MEMS and NEMS Laboratory, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran*

Article info:
Received: 5 September 2023
Revised: 25 September 2023
Accepted: 29 September 2023

[*] **Corresponding Author:**

Javad Mohammadnejad.
Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran. P.O. Box 14115-143, Tehran, Iran.
Email: Mohamadnejad@ut.ac.ir

ABSTRACT

Objectives: The aim of this study was to design an enzyme immunoassay based on a modified ELISA with high sensitivity for detecting digoxin.

Methods: The first step in development involved the conjugation of digoxin to the HRP (Horse Radish Peroxidase) enzyme via sodium metaperiodate oxidation. Surface modification, and thus assay modification, was achieved by the covalent immobilization of an anti-digoxin monoclonal antibody on a functional plate using 3-ATPES (3-aminopropyltriethoxysilane).

Results: The developed ELISA demonstrated superior sensitivity (0.026µg/ml) and lower variability in measurements repeated throughout a day, compared to a conventional ELISA (0.051µg/ml). This assay detected the exact amount of digoxin. The sensitivity and specificity of the modified ELISA surpassed other methods, and measurements were performed within a few hours.

Conclusion: An efficient ELISA kit was produced, characterized by its affordability, ease of learning, and absence of a hand-washing step.

Keywords: ELISA - Surface modification - covalent bonding

Use your device to scan and read the article online

Citation: Enayati H, Mohammadnejad J, Nikfarjam A. Development of enzyme-linked immunosorbent assay (ELISA) based on covalent immobilization of antibody on plate for measurement of digoxin. Acta Biochimica Iranica. 2023;1(3):139-144.

https://doi.org/10.18502/abi.v1i3.14550

Introduction

Digoxin is a cardiac glycoside that has been in use since the early twentieth century to improve symptoms and reduce hospitalization for heart failure patients by reducing the left ventricular ejection fraction (LVEF). It has also been used to control supraventricular arrhythmia in patients with atrial fibrillation (1-3), and its overall effect is to boost muscle strength in patients with heart failure. Digoxin has a narrow therapeutic index, and thus, without proper dosing and monitoring, toxicity can easily occur (4). If kidney function is normal, the myocardial concentration of digoxin in serum is relatively constant. The heart and serum digoxin distribution ratio is approximately 29 to 1. Therefore, it is possible to monitor digoxin therapy by measuring the levels of the drug in serum. A practical and sensitive method for the quantitative measurement of digoxin in serum is enzyme immunoassay. In this method, a patient's serum specimen or control sample is first added to the microplate wells. After a washing step,

digoxin conjugated enzyme is added, and the reactants are mixed. Digoxin conjugated enzyme and digoxin in a patient's serum react competitively for a limited number of antibodies stabilized on a palate surface. After incubation, the antibody linked to digoxin conjugated enzyme is released via aspiration. The activity of the enzyme present in the wells is measured by reaction with a suitable substrate and producing color. Using multiple control samples with specified concentrations of digoxin, a graph of activity and concentration can be drawn. Surface modification can enhance the efficiency, sensitivity, and reproducibility of the assay and also decrease the assay time. Strategies that allow the retention of the functional conformation of the antigen-binding sites of antibody molecules are essential and can be achieved by site-specific and orientated presentation of the antibodies on the surface used for immobilization (5, 6). In the current study, covalent stabilization is used to capture antibodies on the surface. In covalent immobilization, antibody molecules react chemically with the functional groups of the surface via free reactive groups such as amine or carboxyl groups. Covalent strategies are generally categorized on the basis of the chemical reaction approaches used. The importance of covalently immobilized antibody systems for achieving high sensitivity assays has already been demonstrated with various diagnostic platforms (7-10). Improved analyst sensitivity may be attributed at least in part to reduced protein losses due to antibody leaching and antibody exchange, which could increase the antibody surface coverage (7, 8).

Materials and Methods

The assay was designed in two main stages: the conjugation of digoxin to the HRP enzyme and the modification of antibodies and surface for the covalent binding of the anti-digoxin monoclonal antibody to the ELISA plate surface.

Digoxin Conjugation to HRP Enzyme

The conjugation of digoxin to the HRP enzyme by sodium metaperiodate oxidation was done as follows: 5.3 mg of digoxin was added to 360 µl of absolute ethanol, and the suspension was stirred on a magnetic stirrer for 10 min. 450 ml of freshly prepared sodium metaperiodate (0.07 M) was slowly added to the digoxin while it was being mixed. After about an hour, 170 µl of ethylene glycol (0.05 M) was added and stirred for 10 minutes on a shaker. 3 mg of HRP enzyme was dissolved in 500 ml of PBS (phosphate buffered saline 1M, pH 7.4) and was placed on a magnetic stirrer until completely dissolved. The sodium metaperiodate solution was slowly added to the HRP solution while stirring, at room temperature for 1 h. 2.1 mg of sodium borohydride was dissolved in 400 µl of deionized water (DIW) and added to the solution and placed at room temperature for about 4h. The pH of the solution was brought to 6 by formic acid. By ammonium hydroxide (0.1 M), the pH was raised to about 9.0. The resulting solution was centrifuged for 30 min at 4°C with a rotational speed of 3000 rpm.

Developing an ELISA Procedure to Ensure Digoxin Binding to HRP

100 µl of anti-digoxin monoclonal antibody (3.145 µg/ml) was coated in 6 wells, and also 100 µl of BSA (10 µg/ml) was added in 2 wells as a control, at 4°C overnight. The wells were washed by PBST and then blocked with 100 ml of blocking solution (BSA 100 µg/ml) at room temperature. After 1 h, the wells were washed by PBST to remove all extra blocking solution. Then, 100 µl of digoxin-HRP in 3 concentrations of 1, 0.1, and 0.01 µg/ml were added to each well, respectively. 2 wells were coated by BSA, and then 100 µl of Digoxin-HRP (1 µg/ml) was added and placed in room temperature for 1 h. After washing the wells by PBST, 100 ml of TMB substrate was added to each well, and the plate was incubated for about 10 minutes at 37 °C. The enzyme reaction was terminated, and the color development was measured at 450 nm.

Modification of antibodies and surface for covalent binding of anti-digoxin monoclonal antibody to the ELISA plate surface

In the next step, for the covalent binding of the anti-digoxin monoclonal antibody to the plate, the surface

Table 1: Comparative analysis of the modified ELISA procedure and the commercially used conventional ELISA procedure in terms of timings of various assay steps.

Immunoassay Steps and Parameters	Conventional ELISA (passively adsorbed Ab)	Modified ELISA (covalently captured Ab)
Immobilization of antibody	Overnight at RT	2 hrs 30 min
Binding of HRP	2 hrs at RT	1 hr at 37° C
TMB substrate assay	20 min at RT	20 min at 37° C
Total immunoassay duration	~ 20 hrs	~ 6 hrs
Sensitivity	0.053µg/ml	0.026µg/ml
Immobilization strategy applicable to many polymeric surfaces	Not possible	Possible

modification of polystyrene plates was performed using the scheme shown in Fig. 1.

ELISA plate modification

Each well of the 96-well plate was treated with 100 µL of absolute ethanol for 5 min at 37 °C and washed five times with 300 μL of DIW. Subsequently, each well was treated with 100 μL of 1.0% (w/v) KOH at 37 °C for 10 min, followed by five washings with 300 μL of DIW. The KOH-treated wells were then functionalized with amino groups by incubation with 100 μL of 2% (v/v) APTES per well at 80°C for 1 h inside an incubator, in order to achieve maximum salinization. The reaction mechanism following the KOH activation and APTES-based surface fictionalization involves mild oxidation followed by a hydrolytic APTES polymerization on the oxidized surface through its alkoxy groups. The plate was equilibrated to room temperature for 20 min. The amine-functionalized plate was subsequently washed five times with 300 μL of DIW in order to remove excess unbound 3-APTES from the surface.

Anti-digoxin monoclonal antibody binding to amine functionalized plate

5 µl of monoclonal digoxin antibody (629 µg/ml) was mixed with 995 µl of PBS 1X. This mixture was then combined with a premixed solution of 1-ethyl-3-[3 dimethylaminopropyl] carbodiimide hydrochloride (EDC) (4 mg/mL) and N-hydroxysulfosuccinimide (SNHS) (11mg/mL) at a ratio of 99:1 for 15 min at 37°C. The result was EDC cross-linked anti-digoxin antibody.

Development of ELISA to compare the modified and unmodified plate

To evaluate and compare the modified and unmodified plate (covalent and non-covalent binding of the monoclonal antibody to the surface), an ELISA test was performed. 100 μl of the resulting EDC cross-linked anti-digoxin antibody solution in four varying concentrations from 0.3µg/mL to 3.1µg/mL (prepared in 0.1 M PBS, pH 7.4) were added to each of the functionalized wells (100 μL). Additionally, four different dilutions of unmodified anti-digoxin antibody were added to six typical wells and incubated for 1 h at 37°C. The wells were then washed five times with 300µL of PBS 1M. The wells were blocked with skimmed milk (100 μl) and were incubated for 30 min at 37°C, and subsequently washed five times with 300 µL of PBS. 100µl of HRP-Digoxin conjugation (1µg/mL) was added and incubated for 1 h at 37°C, followed by five PBS washes. The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was subsequently added, and the plate was incubated at 37°C. The reaction was stopped after 10 min by the addition of 100 μL of 1N H_2SO_4. The absorbance was recorded at a primary wavelength of 450 nm with a reference wavelength of 540 nm. This assay was repeated several times, and average data were recorded (Fig. 2 and 3). The control for this study was 0.1 M PBS, pH 7.4, and the absorbance of the control was subtracted from all the assay values.

Results and discussion

As shown in Fig. 2, in the case of surface modification (covalent binding of the antibody to the plate) at a concentration of 1.3 µg/ml of the monoclonal antibody, the absorption was about 2.1. In contrast, in the case of unmodified (non-covalent binding of the antibody to the plate), the absorption was about 1.1. This difference can also be observed at a concentration of 0.62 µg/ml of the monoclonal antibody. As observed in Fig. 3, in the case of surface modification (covalent binding of the antibody to the plate), at a concentration of 1.5 µg/ml of the monoclonal antibody, the absorption was 1.05. However, in the unmodified case, the absorption was observed to be about 0.63. This difference was also observed at a concentration of 0.3 µg/ml of the monoclonal antibody.

In this study, a modified ELISA was developed to detect digoxin in serum. The anti-digoxin antibody was immobilized on aminopropyltriethoxysilane-mediated amine-functionalized plates using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and N-hydroxysulfosuccinimide-based heterobifunctional cross-linking. The analytical sensitivity was determined using the following formula:

[Average absorbance of blank + 3 (SDblank)]

The analytical sensitivity of the developed assay was 0.026µg/mL, compared to 0.053µg/mL for the conventional assay. Therefore, the new approach has considerably more advantages compared to the conventional ELISA. Conventional ELISA procedures have been followed for decades for the detection of

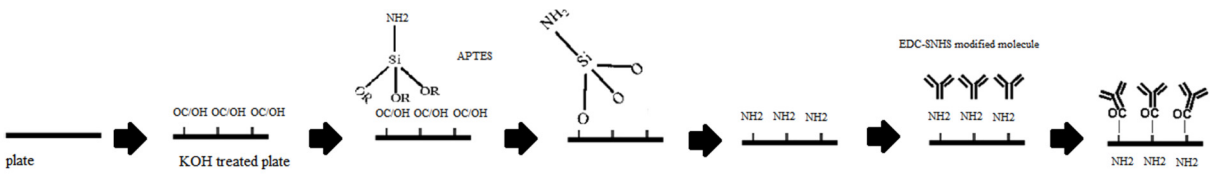

Figure 1: Schematic of the ELISA plate surface modification.

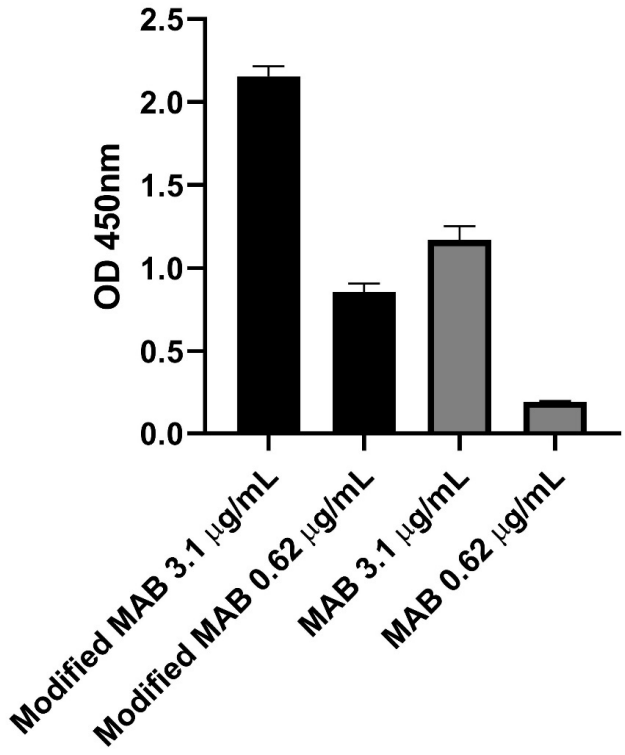

Figure 2: The average of optical density of HRP bound digoxin at a primary wavelength of 450 nm for modified and unmodified monoclonal antibody in two varying concentrations (0.62 and 3.1 µg/ml).

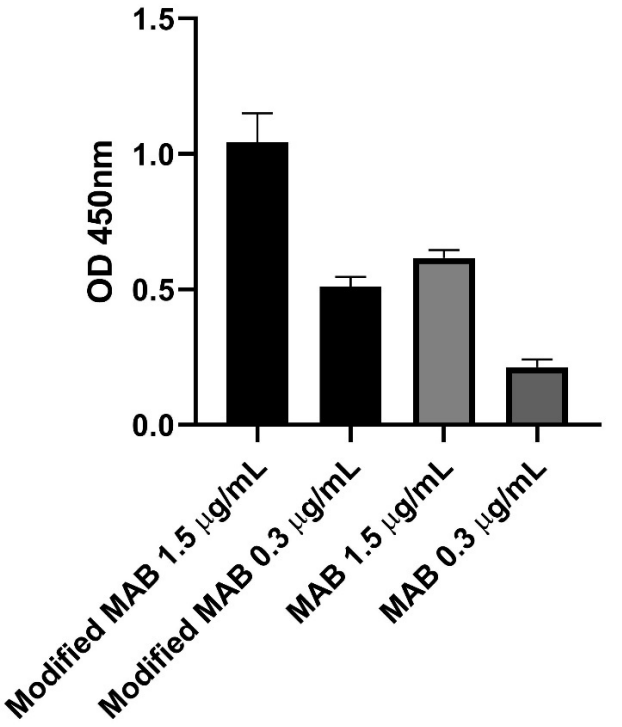

Figure 3: The average of optical density of HRP bound digoxin at a primary wavelength of 450 nm for modified and unmodified monoclonal antibody in two varying concentrations (0.3 and 1.5 µg/ml).

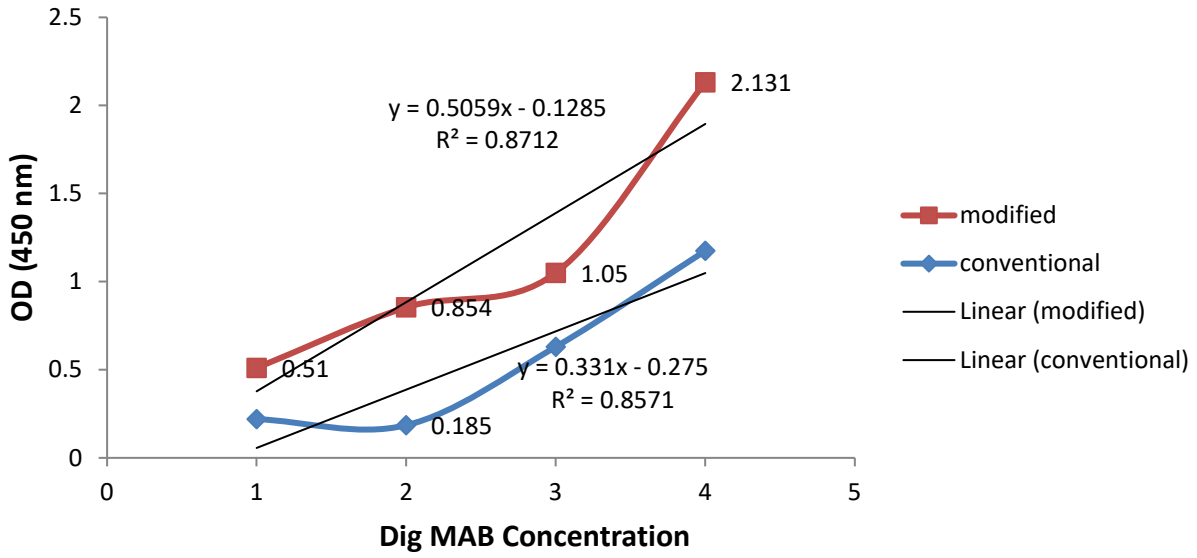

Figure 4: Analytical comparison of developed ELISA and conventional ELISA

analysts of importance in industrial, healthcare, and academic research. However, improvements on existing ELISA technologies are continuously attempted by many groups (11, 12). The developed ELISA in this study has comparatively better (0.026 µg/ml) and lower variability in the measure repeated during a day. Digoxin was taken as the model assay system to demonstrate the utility of the developed ELISA procedure since all the assay components were commercially available in kit form. This is essential to do robust and highly precise comparison of the developed ELISA with the commercially existing conventional ELISA procedures, as the same assay components were used under the same conditions. This approach of immobilizing antibody on chemically modified solid supports has potential applications in many other assays and formats.

In this study, the conventional ELISA plate with 96 wells made of PS (polystyrene) was used for chemical modification and stabilization of the antibody. The most important factor that may be attributed to the enhanced sensitivity of the modified assay is the covalent immobilization of the anti-digoxin capture antibody, since the covalently cross-linked antibodies should not leach out during the assay procedure in comparison to the use of passively adsorbed antibodies, where leaching may occur more easily. The orientation of antibody molecules in the developed ELISA with covalent bound is another reason for higher sensitivity and antigen capture efficiency compared to the conventional ELISA. All assays were performed under the same conditions to decrease variability. A standard EDC-based cross-linking strategy was employed to immobilize the anti-digoxin antibody. The developed ELISA procedure decreased the overall assay duration. Thus, the reported procedure is a rapid ELISA format with the additional benefit of being generic in nature. Hence, using the reported surface modification technique, it is possible to develop rapid and high sensitivity assays.

It was demonstrated that the covalently cross-linked monoclonal antibodies captured significantly higher amounts of digoxin. This has improved the detection limit and the overall assay sensitivity of the developed ELISA procedure in comparison to the conventional ELISA. However, the commercially available ELISA kits have different capture and detection antibody combinations, which may be a factor in their reduced sensitivities (13). The sensitivity and specificity of any immunoassay are dependent on the antigen capture efficiency of an antibody, which is subsequently governed by the nature of the antibody.

Conclusion

In the study under discussion, a rapid ELISA procedure was developed for the highly sensitive detection of digoxin. This was based on the covalent immobilization of anti-digoxin antibody on a 3-APTES-functionalized plate. The developed ELISA demonstrated comparatively better analytical sensitivity (0.026 µg/ml) and lower variability in measurements repeated during a day, compared to conventional ELISA (0.051 µg/ml). This strategy can be employed for rapid assay development for specific biosensor/diagnostic applications.

Acknowledgment

The author thanks Dr. Alireza Nikfarjam for their unwavering consultation and cooperation.

Conflict of Interest

The authors have nothing to declare.

References

1. Cheng JW, Rybak I. Use of digoxin for heart failure and atrial fibrillation in elderly patients. Am J Geriatr Pharmacother. 2010;8(5):419-427. https://doi.org/10.1016/j.amjopharm.2010.10.001
2. Castiglione V, Aimo A, Vergaro G, Saccaro L, Passino C, Emdin M. Biomarkers for the diagnosis and management of heart failure. Heart Fail Rev. 2022:1-19. https://doi.org/10.1007/s10741-021-10105-w
3. Eichhorn EJ, Gheorghiade M. Digoxin-new perspective on an old drug. N Engl J Med. 2002;347(18):1394-1395. https://doi.org/10.1056/NEJMp020118
4. Rich MW, McSherry F, Williford WO, Yusuf S, DIG Group. Effect of age on mortality, hospitalizations and response to digoxin in patients with heart failure: the DIG study. J Am Coll Cardiol. 2001;38(3):806-813. https://doi.org/10.1016/S0735-1097(01)01442-5
5. Blažek D, Celer V. The production and application of single-chain antibody fragments. Folia Microbiol. 2003;48(5):687-698. https://doi.org/10.1007/BF02993480
6. Steinhauer C, Wingren C, Hager ACM, Borrebaeck CA. Single framework recombinant antibody fragments designed for protein chip applications. Biotechniques. 2002;33(S6):S38-S45. https://doi.org/10.2144/dec02steinhauer
7. Corso CD, Dickherber A, Hunt WD. An investigation of antibody immobilization methods employing organosilanes on planar ZnO surfaces for biosensor applications. Biosens Bioelectron. 2008;24(4):805-811. https://doi.org/10.1016/j.bios.2008.07.011
8. Das RD, Maji S, Das S, RoyChaudhuri C. Optimization of covalent antibody immobilization on macroporous silicon solid supports. Appl Surf Sci. 2010;256(20):5867-5875. https://doi.org/10.1016/j.apsusc.2010.03.066
9. Darain F, Gan KL, Tjin SC. Antibody immobilization on to polystyrene substrate-on-chip immunoassay for horse IgG based on fluorescence. Biomed Microdevices. 2009;11:653-661. https://doi.org/10.1007/s10544-008-9275-3
10. Dixit CK, Vashist SK, O'Neill FT, O'Reilly B, MacCraith BD, O'Kennedy R. Development of a high sensitivity rapid sandwich ELISA procedure and its comparison with the conventional approach. Anal Chem. 2010;82(16):7049-7052. https://doi.org/10.1021/ac101339q
11. Malakaneh M, Rasaee M, Rahbarizadeh F, Madani R, Forozandeh M, Khabiri K, et al. Characterization of a monoclonal antibody against neopterin using an enzyme-linked immunosorbent assay with penicillinase as label. Hybridoma. 2001;20(2):117-121. https://doi.org/10.1089/02724570152057616
12. Di Lorenzo D, Roggeri G, Iacobello C, Belloli S, Gion M, Za Va D, et al. Evaluation of a radioreceptor assay to assess exogenous estrogen activity in serum of patients with breast cancer. Int J Biol Markers. 1991;6(3):151-158. https://doi.org/10.1177/172460089100600303
13. Camara PD, Velletri K, Krupski M, Rosner M, Griffiths WC. Evaluation of the Boehringer Mannheim ES 300 immunoassay analyzer and comparison with enzyme immunoassay, fluorescence polarization immunoassay, and radioimmunoassay methods. Clin Biochem. 1992;25(4):251-254. https://doi.org/10.1016/0009-9120(92)80029-G