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Research Article

QCM- Based Biosensor for the Detection of Homocysteine

Fatma Ayhan*

Muğla Sıtkı Koçman University, Faculty of Science, Department of Chemistry, Biochemistry Division, Biochemistry & Biomaterials Research Laboratory (BIOMATREG), Muğla, Turkey (ORCID: 0000-0003-2220-4496), fayhan@mu.edu.tr

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Abstract

High plasma homocysteine levels can cause an increased risk of cardiovascular, cerebrovascular, and peripheral arterial diseases. Besides this, Alzheimer's disease and other dementias, osteoporosis, diabetes and renal disease due to folate and B-vitamin deficiency, various drugs or pre-existing atherosclerotic diseases may be the result of high homocysteine levels. The presented research work aimedto perform the detection of Homocysteine (Hcy) by using Quartz Crystal Microbalance (QCM) biosensor. The temperature controlled QCM system was a home-made designed and constructed equipment which can use silver electrod quartz crystal. The modification of silver electrod quartz crystals surfaces was achieved by the surface cleaning process with sodium hydroxide, acetone and methanol in a consecutive manner. Then self-assembled monolayer of cysteamine and chemical coupling of glutaraldehyde (GA) to free end of monolayer was achieved to create the new functional surface in order to complete the formation of spacer arm/ligand. Homocysteine specific recognizing ligand, anti-Homocysteine antibody was immobilized to glutaraldehyde coupled surfaces. The change in resonance frequency values were measured for each modification step. The optimization of dilution ratio of the antibody solution was performed to modified surfaces. The least dilution ratio of antibody, 1/10000 v/v, was determined as optimum antibody ratio. The detection of homocystein was analysed at a detection limit of 0.1 µM and the linear ranges of calibration curves were estimated as 0.1-2.0 µM and 10-50 µM. Homocysteine values indicated good linearities (R^2=0.9813 and 0.9875, respectively). The relative standart deviation (RSD %) for precision was calculated as less than 10%. In conclusion, it was found that the detection of homocysteine can be done both in nano- and micro-molar concentration levels. Additionally, designed biosensor showed desired stability and reproducibility. Finally, a new method different from the present methods for the use in the analysis of Hcy was proposed and developed which detects homocysteine by designed QCM technique with a rapid, cheeper and less pretreatment processes. Additionally, homocysteine detection was performed in nano- and micro- molar concentration values.

Keywords: Homocysteine, anti-Homocysteine Antibody, Quartz Crystal Microbalance (QCM), Surface Modification.

Homosistein Tayini Amaçlı QCM-Temelli Biyosensör

Öz

Yüksek plazma homosistein düzeyleri, kardiyovasküler, serebrovasküler ve peripheral arterial hastalık risklerini arttırabilmektedir. Bunun yanında doğumsal bozukluklar gibi diğer çeşitli patolojiler, Alzheimer hastalığına ve diğer gerilikler, osteoporoz, şeker ve böbrek hastalıkları da yüksek homosistein değerlerinin sonucu olabilmektedir. Sunulan araştırma çalışmasının amacı Kuartz Kristal Mikroterazi (QCM) biosensörü kullanarak Homosisteinin (Hsis) tayinini gerçekleştirmektir. Sıcaklık kontrollü QCM sistemi, yerli olarak tasarlanmış ve üretilmiş bir cihaz olup gümüş elektrodlu kuartz kristal kullanılmıştır. Gümüş elektrod kristal yüzeylerinin modifikasyonu sodium hidroksit, aseton ve methanolün ardışık olarak uygulanarak yapılan yüzey yıkama işlemi ile sağlanmıştır. Daha sonra sisteamin ile kendiliğinden tek tabaka oluşumu ile uzatma kolu/ligand oluşumu gerçekleştirilmesi amacıyla tek tabakanın serbest ucuna glutaraldehitin kimyasal olarak bağlanması sağlanarak yeni bir fonksiyonel yüzey elde edilmiştir. Glutaraldehit bağlı yüzeylere homosisteine özgü ligand olan anti-Homosistein antikoru immobilize edilmiştir. Her modifikasyon basamağı için frekans değerlerinin değişimi ölçülmüştür. Antikor çözeltisinin seyrelme oranı modifiye yüzeyler kullanılarak optimize edilmiştir. En düşük antikor derişimi olan 1/10000 v/v oranı optimize antikor oranı olarak belirlenmiştir. Homosisteinin en düşük tayin sınırı 0.1 µM olarak tespit edilmiştir ve kalibrasyon eğrilerinin doğrusal aralıkları 0.1-2.0 µM ve 10-50 µM olarak bulunmuştur. Belirtilen doğrusal aralıklarda Hsis değerleri oldukça yüksek doğrusallık (sırası ile R^2=0.9813 and 0.9875) göstermiştir. Bağıl standart sapmanın duyarlılığı % 10'dan

* Corresponding Author: Prof. Dr. Fatma AYHAN, Muğla Sıtkı Koçman University, Faculty of Science, Department of Chemistry, Biochemistry Division, Biochemistry & Biomaterials Research Laboratory (BIOMATREG), Muğla, Turkey (ORCID: 0000-0003-2220-4496), fayhan@mu.edu.tr

küçük hesaplanmıştır. Sonuç olarak homosistein tayininin hem nano hem mikro molar derişim değerlerinde yapılabileceği bulunmuştur. Ek olarak tasarlanan biyosensör istenen kararlılık ve tekrarlanabilirlik göstermiştir. En son olarak Homosistein analizinde kullanıma yönelik yeni bir yöntem önerilerek geliştirilmiş ve bu hızlı, ucuz ve daha az ön işlemi olan QCM tekniği ile homosistein tayini başarı ile yapılabilmektedir.

Anahtar Kelimeler: Homosistein, anti-Homosistein antikoru, Kuartz Kristal Mikroterazi (QCM), Yüzey Modifikasyonu.

1. Introduction

Homocysteine (Hcy) is metabolised in the liver from the methionine, essential amino acid. (McCully, 2007; Kumar et. al., 2017). If metabolic pathways of sulfur-containing amino acids are defected due to inborn error, it is called homocystinuria. The concentration level of homocysteine in serum increase to higher than normal range (>10 μmol/L) (Kumar et. al., 2017; Jiang et. al., 2018) or cytotoxic values (>12 μmol/L) (Hankey and Eikelboom, 1999; Abraham and Cho, 2010; Hoffman, 2011).

Genetic alterations, vitamin deficiencies, and several other environmental factors such as increased intake of Met, certain medications, disease state, pregnancy, lactation and altered cellular export mechanisms may lead increased Hcy levels (Kumar et. al., 2017; Jiang et. al., 2018; Hasan et. al., 2019). The total homocysteine (tHCY) level in serum indicates the deficiencies of vitamin B12 (cobalamin), vitamin B6 (pyridoxine), and folate (Jiang et. al., 2018). Increased circulating levels of Hcy or hyperhomocysteinemia is known as risk factor of many health problems like coronary, cerebral, and peripheral atherosclerosis deseases. For example, blood Hcy level is also a well known risk factor for cardiovacular diseases (Abraham and Cho, 2010; Kumar et. al., 2017; Smith et. al., 2018). The high total homocysteine (tHcy) level was also accociated with vascular dementia. (Clarke, 2007; Nilsson et. al., 2013). Recently, it was reported that the Alzheimer's disease was connected by low red blood cell folate, serum folate, and serum B12 value levels (Smith et. al., 2018). The same defiencies besides genetic polymorphisms involves the transfer of one-carbon groups were reported as increased overall risk of cancer (Özkan et. al., 2007; Zhang et. al., 2015; Hasan et. al., 2019).

In recent years, various measurement methods were developed to determine the amount of tHcy in serum/plasma. The measurement with the high pressure liquid chromatography (HPLC) method was applied by using flourescence, electrochemical, mass spectrometry, and post_column derivatization detection (Refsum et. al., 1985; Refsum et. al., 1989; Ueland et. al., 1993; Ubbink, 2000; Alam et. al., 2019). HPLC method with fluorescence detection comes forward due to its principal and the most prefered procedure after certain modifications or derivatizations. There are other reports for the determination of Hcy based on electrochemical aptasensor (Kim et. al.,2017), enzyme-linked and flourescence polarization immunoassays (Frantzen et. al., 1998; Tewari et. al., 2006), fluorescence probe method (Kang et. al., 2017), Immunonephelometric Method (Zappacosta et. al., 2006) Electrochemical methods (Madasamy et. al., 2015) and colorimetric (Wang et. al., 2016).

The quartz crystal microbalance (QCM) method is based on the piezolectric effect and known as simple, cost effective, and high-resolution mass sensing technique with wide detection range including nanogram levels (Bunde et. al., 1998; Marx, 2003). The resonant frequency change of a QCM is on the order of MHz and the frequency change of 5 MHz has a corresponding thickness of ~ 330 μm. The mathematical relationship of a frequency change on the crystal surface for adsorbed mass was demonstrated by Sauerbrey in 1959 (Dixon, 2008). QCM based researches on the measurement of most organophosphore and carbamat pestiside (Karousas et. al., 2002), antigen/antibody interaction (Liu et. al., 2004), blood coagulation density and immune complement activation on artificial surfaces (Andersson et. al., 2005), the human metastatic breast cancer cells, MDA MB 231 cells (Bakhshpour et. al., 2019), histidine (Sönmezler et. al., 2018), and immunosensor for the detection of *Salmonella Typhimurium* (Fulgione et. al., 2017) were investigated.

Therefore, the detection and quantifying of Hcy for use in academic studies and/or clinic applications is very important. In current study, QCM based detection of Hcy by measuring resonant frequency change was developed. The silver electrode surfaces of quartz crystals were modified with several steps. The Hcy specific ligand (homocysteine antibody) was immobilized on modifed silver electrode surface and the optimization of of antibody amount was performed to investigate the convenient working concentration. The calibration range of Hcy biosensing was determined and the detection limit were estimated. So, a new Hcy biosensing method is crucial for high sensitivity and also to lessen the time, expenditure, and pretreatment processes.

2. Material and Method

2.1. Materials

Anti-Homosistein antibody (ab 6482) was purchased from Abcam, Sodium hydroxyde (NaOH), asetone (CH_3COCH_3), methanole (CH_3OH), cysteamine (C_2H_7NS), Na_2HPO_4-NaH_2PO_4, glutaraldehyde (GA), tetraborate, HCl were perchased from Merck KGaA (Darmstadt, Germany). Quartz crystals with silver electrod (10 MHz, AT cut, MEC Quartz Limited Honkong & Mainland China) were purchased from Özdisan Limited Company (Turkey). QCM measurements on silver electrode surfaces were performed with a home-made temperature controlled quartz crystal microbalance system designed and constructed by our group (Kocum et. al., 2010). Ultrapure water was used throughout the research.

2.2. Surface modifications of silver electrod quartz crystals

The surfaces of silver electrods were modified in order to achieve a chemical modification or immobilization process. The pretreatment steps were composed of cleaning, activation, and immobilization of functional grups. The experimental procedures of surface activation, cysteanime and glutaraldehyde immobilizations were given in details below (Figure 1).

The designed QCM system can measure the frequency of two electrods and gives the frequency change of electrods comperatively in order to compare frequency difference of two electrods and diminish the errors may come from the device. So, the frequency defference of two untreated crystals were measured at first. Then the first procedure was applied to one of the electrod while other was kept untreated and again frequency change was measured. In the second step, the same process was applied to untreated electrod but second step was done to other electrod and followed by freguency change measurement. These stages were applied till the final experimental step was reached.

Surface modification steps were performed as previously reported (Ayhan et. al., 2007; Erdamar et. al., 2008). Briefly, silver electrod quartz crystals were cleaned by immersing consecutively to 0.5 M NaOH solution, acetone, and methanol for 30 min. The electrod surfaces were washed with deionized water to desorb the physically bounded molecules. The frequency changes was recorded in each surface cleaning stage after dried at 37°C for 30 min. The silver electrode surfaces was functionalized with cysteamine molecule to perform self-assembled monolayer, which has a tiol (SH) and an amine (NH_2) end group. The immobilization of functional molecule was carried out with 18 mM cysteamine concentration in 0.1 M phosphate buffer (pH=7) conditions for 2 hr in dark. The frequency of the rinsed and dryed electrods were rekorded. The bifunctional reagent, glutaraldehyde was used as spacer arm through Schiff's base reaction with free amine group end of cysteamine. Silver electrod quartz crystals were soaked to glutaraldehyde solution whose concentration was adjusted to 0.66 M in sodium tetraborate/HCl buffer (pH: 8.2) and kept in dark for 2 h. The frequency changes were measured after washing and drying processes were completed as mentioned before. So, spacer arm bounded silver electrode quartz crystal surface was attained.

2.3. Immobilization of the specific diagnostic molecule

In this stage, Hcy specific molecule, anti-Hcy antibody was immobilized to spacer arm bounded silver electrod surfaces. Anti-Hcy antibody solutions were prepared by diluting in 0.1 M PBS (phosphate buffered saline), pH 7.4. The concentration of stok antibody solution was adjusted as 1/2000 dilution. The immobilization of anti-Hcy antibody was carried out in 3 mL antibody solution for 30 min at room temperature with slowly stirring. The unbounded antibody was washed in ultra pure water and dried. Five different dilution ratios were selected as antibody concentrations in order to estimate the linear range of antigen-antibody coupling. All stok solutions were kept at +4ºC until use within one week and all the experiments were performed in sterile environmental conditions to avoid contamination in specific molecule containing solutions. The shematic presentation of the proposed reaction sequence was given in Fig. 1.

3. Validation of the method: estimation of calibration curves, quantification and detection limits

Antibody coupled silver electrod crystals were treated with antigen, Hcy in order to investigate the detection limit and linear concentration range of Hcy. The linearity of method was tested in the 0,1-50 µM range. All the Hcy standart solutions were prepared from 50 µmol/L stok standart Hcy solution in 0.1 M PBS, pH 7.4, stored at +4ºC and usage was adjusted to complete within one week. Fresh 0.1 M PBS at pH 7.4 was prepared before each experimental study. Anti-Hcy antibody immobilized silver electrod quart crystals were waited in 3 mL Hcy solution for 30 min on magnetically stirrer at room temperature. Then, silver electrod crystals were washed in PBS and ultra pure water and frequency changes were recorded after drying process. The experimental results were evaluated to obtain linear regression equations, experimental linear ranges, determination coefficients (r^2), limits of detection and quantification. It will be favourable work outcome if the detection and quantification levels cover the concentration level of the animal models. All the frequency changes were recorded after readings were stabilized. The results were reported as the average of at least three experimental results.

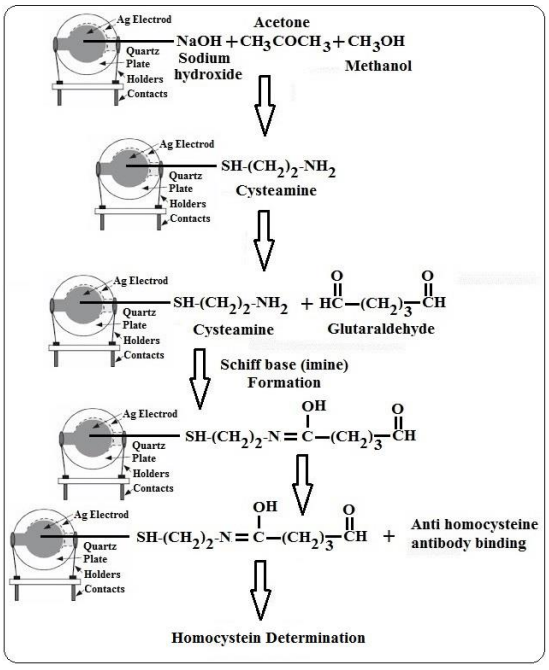


Figure 1. *Schematic Representation of the proposed Silver Piezo Crystal electrod Surfaces Modification steps and Immobilization of the specific diagnostic molecule.*

3. Results and Discussion

3.1. QCM experiments

Silver electrod quartz crystals are sensitive to temperature and humidity changes which is regarded as their disadvantage. So, the estimation of temperature depandance of silver electrods was performed in the first stage of the research study. QCM system have a chamber that can also be adjusted to control the temperature (Figure 2).

The frequency change of untreated electrods of 10 MHz for 20-37°C temperature range were given in Figure 3. Higher temperatures were examined due to denaturation probability of biological molecules when utilize them. Almost linear change in frequency values were observed in this range but low temperatures resulted with high frequency variation compared to higher temperature values for blank crystals.

Figure 2. *QCM system a) general view of system with frequency counter on right, b) mounted silver electrode quartz crystals, c) temperature controller on crystal mounted chamber (red).*

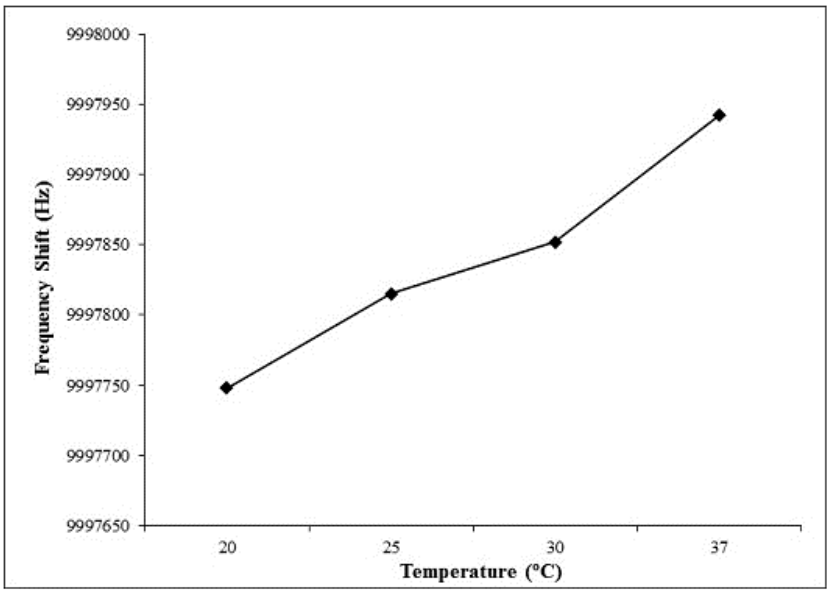


Figure 3. *The relation betweet temperature and frequency of silver electrod quartz crystals.*

3.2. Cleaning and activation of silver piezo crystal surface

Surface modifications, cleaning and activation steps were tested for different temperatures since the silver electrods were found to be sensitive to temperature changes. First, the electrod surface needs to be cleaned or activated in order to make modifications. Therefore, the activation of electrod surface was carried out with a basic and an organic compound, NaOH and acetone, respectively, and then methanol cleaning steps. The biggest frequency change was estimated for methanol cleansing compound greatly due to formation of (–OH) groups on electrod surface. Then, silver surfaces were treated with 18 mM cysteamine and 0.66 M GA and frequencies were determined after washing with water and dried (Ayhan, 2007; Ayhan, 2014). Cysteamine and glutaraldehyde modifications were also performed at 20, 25, 30, and 37°C. The frequency responses of silver electrods with temperature were schematized in Figure 4. The experimental processing temperature seems to affect the frequency response. The frequency change of surface cleaning, cysteamine immobilization and glutaraldehyde modification tend to increase up to 30°C but show a remarkable decrease at 37°C. Figure 5 depicts the final frequency alteration of cleaning, activation, cysteamine and GA immobilization steps at four temperatures.

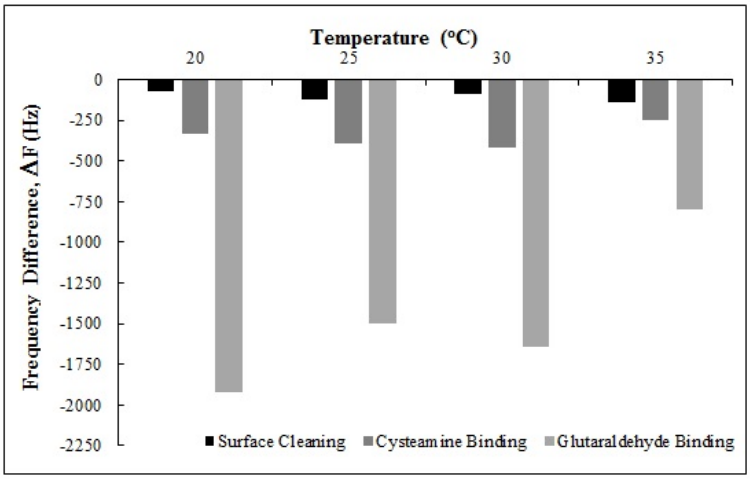


Figure 4. *Frequency change for surface modification reactions at various temperatures.*

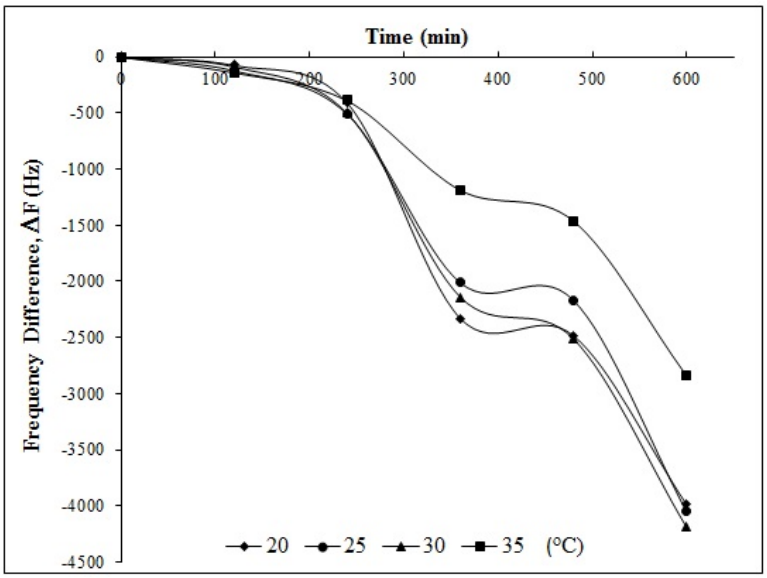


Figure 5. *The effect of Temperature on Frequency Change of Crystals During the Process Steps.*

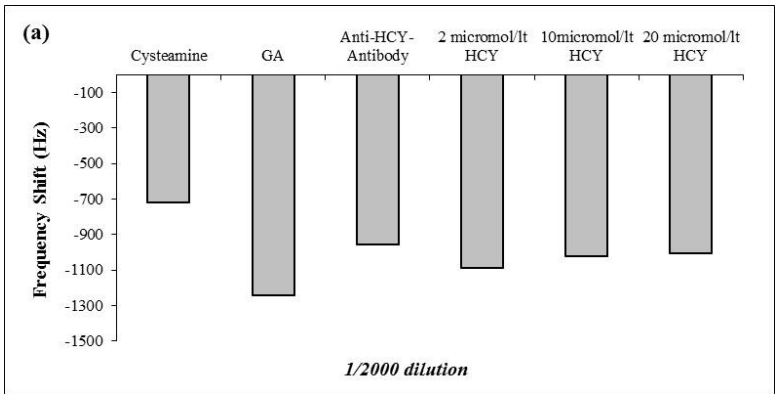
3.3. The immobilization of biosensing molecule, anti-Hcy antibody

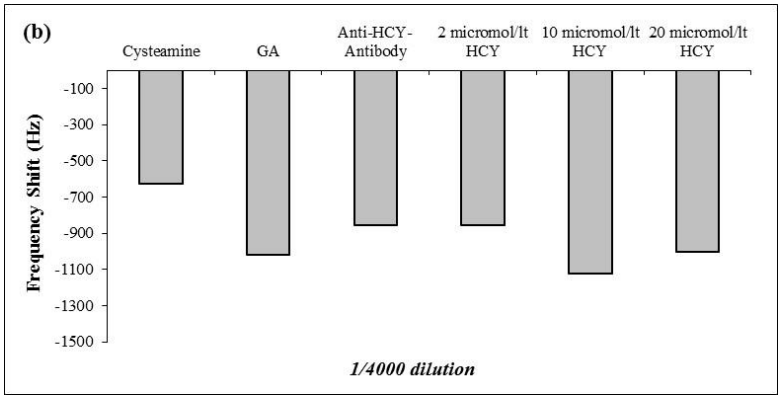
Anti-Hcy antibody was coupled to aldehyde group created on silver electrod in previous step. Starting dilution was done according to producer as 1/2000. The dilution was optimized by testing five concentrations from 1/2000 to 1/10000 v/v dilution range. The electrod was immersed to pH 7.4 PBS buffer and ultra pure water after treatment time was ended and dried at 35ºC. QCM reading was recorded as monitor indicator stabilized. Hcy solutions of 2, 10, 20, 35, and 50 µM concentrations was analysed for each anti-Hcy antibody dilution. Frequency decrease values for 1/2000 v/v anti-Hcy antibody dilution after Cys immobilization step were given in Figure 6a. It can be seen that higher Hcy amount than surface bounded anti-Hcy antibody was bounded to silver electrod. The same phenomena can be said for greated Hcy concentrations. Therefore, dilution was increased to 1/4000 v/v and again biosensing freguencies were given in Figure 6b. Anti-Hcy antibody biosensing frequencies show fluctuation for Hcy concentrations bigger than 2 µM. A nonlinear response

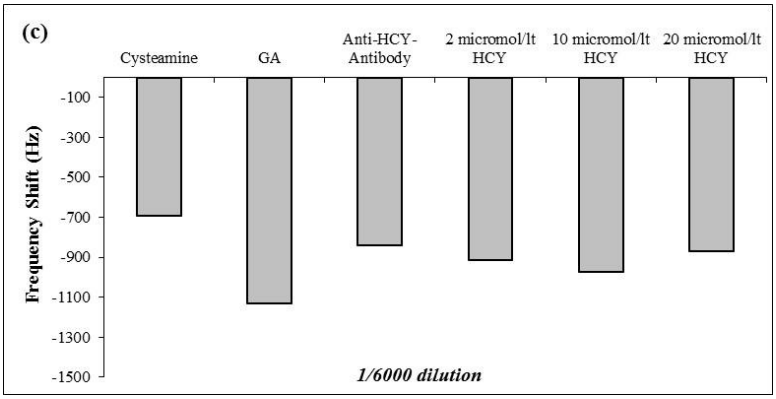
can be seen also for 1/6000 and 1/8000 v/v dilutions (Figure 6c. and 6d.).

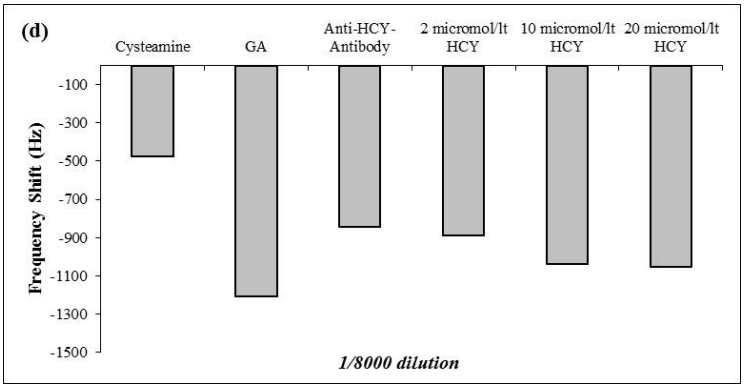
When antibody dilution rate of 1/10000 was used, it can be said that almost one-to-one correspondence was achieved for 2 and 10 µM Hcy (Figure 6e). Therefore 1/10000 v/v dilution for anti-Hcy antibody was decided to apply in the following experiments since this dilution sence more precise and less amount of antibody can be used.

Cysteamine was also successfully used as self-assembled molecule in surface modification applications in order to immobilize anti-human serum albumin for the detection of human serum albumin (Liu et. al., 2019).









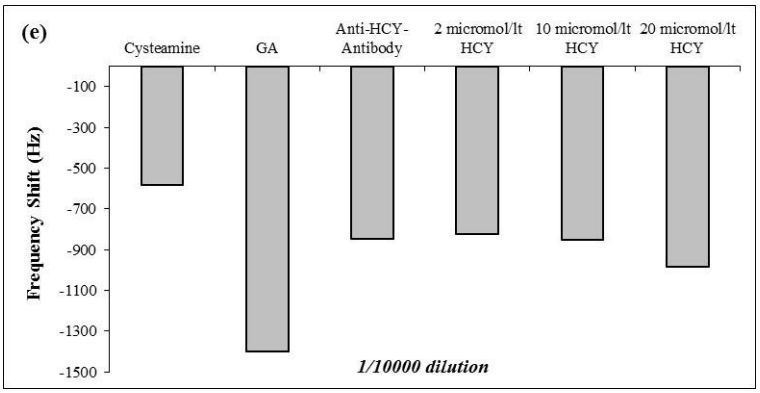


Figure 6. *The frequency changes of various Anti-Hcy antibody dilutions silver electrod quartz crystals. Hcy dilution ratios: a) 1/2000; b) 1/4000; c) 1/6000; d) 1/8000; e) 1/10000 v/v.*

3.4. Calibration curves

Hcy were tested from 0.1 µM to 50 µM concentration range in order to constitute calibration curve. Anti-Hcy antibody dilution was kept at 1/10000 ratio as decided in the previous section. Two calibration curves were obtained using four replicates. The linear responses for the 0.1 µM - 2 µM concentration range showed a regression coefficient of r^2=0.9813 (Figure 7a). The higher Hcy concentration range from 10 µM-50 µM also resulted with a linear behaviour which regression coefficient was calculated as 0.9875 (Figure 7b). Precision tests were performed by the repeated frequency change readings of the same sample and relative standart deviation (RSD) value of 7.17 % was found. The level of RSD may come from random errors of the method stages aspecially sensitivity of silver electrod connection ends/pins.

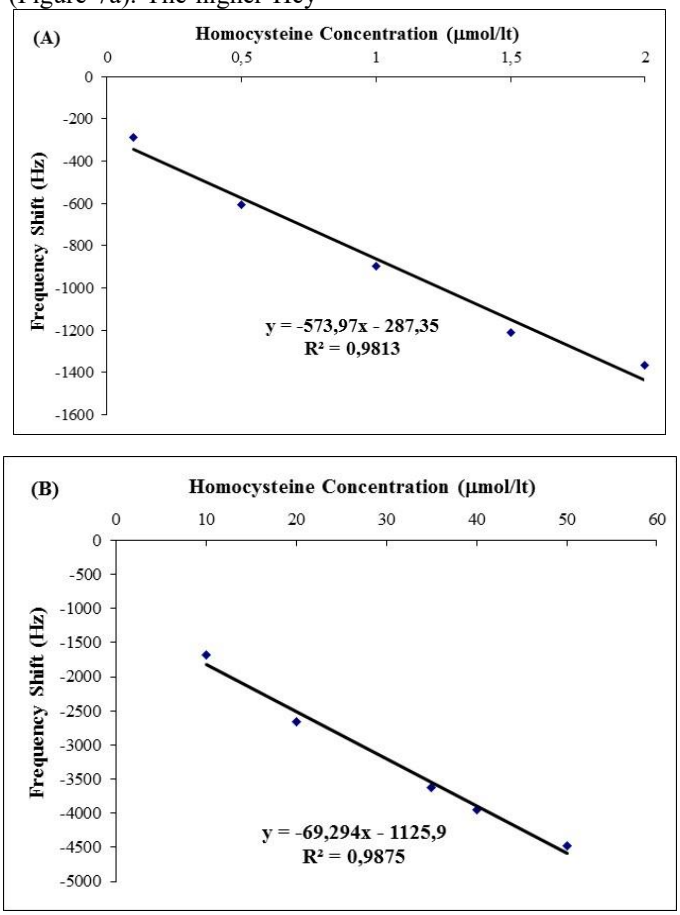


Figure 7. *Calibration curves for a) 0,1-2,0 µM and b) 10-50 µM Hcy concentration ranges.*

Hence, 1/10000 dilution rate of anti-Hcy antibody was successfully immobilized to silver electrod piezo crystal surfaces. The tested Hcy detection range resulted with two linearized range regarded as calibration curves. One was in the concentration range of 0.1-2.0 µM while other range was estimated as 10-50 µM Hcy concentration. The calibration curve of low Hcy levels can be advantageous for animal experiments in case less amounts of biological fluids have to be analysed (Likogianni et. al., 2006). The second calibration range will provide Hcy detection in healty people and patients (Özkan et. al., 2007).

A brief summary of the different detection methods for Hcy was shown in Table 1. The table gives the limit of detection of Hcy previously reported in the literature accessed by us. HPLC-Fluorescence method showed a sensitive detection after labeling of sulfur containing amino acids with 4-

(*N*,*N*dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBDF). An aptasensor was created by grafting of Hcy-Binding-Aptamer (HBA) onto the surface of Au nanoparticles/Glassy Carbon Electrode (Au/GCE). Two methods report the limit of detection as 0.13 μM and 0.01 μM, respectively, In our study, we revealed a sensitive method by using the advantages of specific recognizing ligand and QCM technique for biomolecule sensing as 0.1 μM Hcy detection limit.

Table 1. *The Comparison of Various Detection Methods of Homocystein*

Detection system	**Limit of detection**	**Referennces**
Capillary electrophoresis	12 μmol/L	Zinellu et al., 2010
Electrochemical Assay	Moderate (16–30 μM), medium (30–100 μM) and severe (>100 μM)	Madasamy et. al., 2015
Enzyme-Linked ımmunoassay	8 to 27 mM.	Frantzen et. al., 1998
Chemiluminescent	4.9–62 μmol/Hcy	Tewari et. al., 2006
Fluorescence probe	10^{-8} to 10^{-2} M (naked eye)	Xia et al., 2018
Iridium(III) complex	0.7 μM	Liu et al., 2019
Immunonephelometric Method	0.5 μM	Zappacosta et. al., 2006
UHPLC–MS/MS	0.18 μM	Forgacsova et al, 2019
HPLC-Flourescence	0.13 μM	Wada et al., 2013
QCM biosensor	0.1 μM	This study
Apta sensor	0.01 μM	Beitollahi et al., 2020

In consequence, the deficiency of vitamin B6, B12, and folic acid, renal failure, and genetic variations in enzymes of Hcy metabolism may cause high plasma Hcy concentration levels. That's why undesirable Hcy levels other than normal or cytotoxic values increase cardiovascular, serebrovascular, and peripheral arterial diseases risks. Therefore, blood Hcy concentrations have to be known in order to reveal the disease hazards. In the research, we have performed the detection of Hcy with a different approach and method compared to other methods. The calibration curves can be used in high Hcy concentrations either for blood Hcy measurement or in low Hcy levels for small living tissues or organisms. The proposed method allows the detection of Hcy concentrations in nano amounts like 100 nM.

QCM technique with a rapid, cheeper and less pretreatment processes. In future works, other researches can be conducted with other kinds of biological thiol, human serum or urine samples to analyse Hcy in order to validate the method more precisely.

4. Conclusions and Recommendations

The proposed QCM method was optimized for the Hcy detection based on the specific antibody-antigen interaction. The biosensor can detect two ranges of Hcy concentrations (0.1-2 μmol/L and 10-50 μmol/L) with correlation coefficients of 0.9813 and 0.9875. The detection limit of the biosensor is 0.1 μmol/L.. The analysis of homocysteine was realized in two linear ranges, nano- and micro- molar concentration values with high sensitivity. Finally, a new method different from the present methods was proposed and developed which detects homocysteine by designed

5. Acknowledge

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