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

(First received 13 August 2020 and in final form 16 December 2020)

(DOI: 10.31590/ejosat.777852)

ATIF/REFERENCE: Ayhan, F. (2020). QCM- Based Biosensor for the Detection of Homocysteine. European Journal of Science and Technology, (20), 835-843.

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-existent atherosclerotic diseases may be the result of high homocysteine levels. The presented research work aimed to 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 electrode quartz crystal. The modification of silver electrode 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 homocysteine 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 linealities (R²=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, cheaper 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 arttırmaktedir. Bunun yanında douğal veziküller gibi diğer çeşitli patolojiler, Alzheimer hastalığına ve diğer gerilikler, osteoporoz, şeker ve böbrek hastalıkları da yüksek homocysteinenin sonucu olabilmektedir. Sunulan araştırma çalışmasının amacı Kuarz Kristal Mikroterazi (QCM) biosensörü kullanarak Homosistein (Hsis) tayini gerçekleştirmektir. Sıçrailik kontrollü QCM sistemi, yerel olarak tasarlanmış ve üretilmiş bir cihaz olup günümüz elektrodlu kuarz kristal kullanılmaktır. Gümsü elektrod kristal yüzeyinin modifikasyonu sodium hidroksit, aseton ve methanolun arıباك olarak uygulanarak yapılan yüzey yıkama işlemi ile sağlanmıştır. Daha sonra sistemim en kendiliğiniндег teh tabaka oluşturmaya ve uzuanna kolu/ligand oluşturulmuş gerçekleştirmesini amaçlamak 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 ölçülmiştir. Antikör çözeltisinin seyrelme orani modifiye yüzeyler kullanılarak optimize edilmiştir. En düşük antikor derişi olan 1/10000 v/v orami optimize antikor orami olarak belirlenmiştir. Homosisteinin en düşük tayin mimi 0.1 µM olarak tasar edilmiştir ve kalibrasyon eğrilerinin dogrusal aralıkları 0.1-2.0 µM ve 10-50 µM olarak bulunmuştur. Belirtilen doğrusal aralıklarda Hsis değerleri oldukça yüksek doğruluklu (sirası ile R²=0.9813 and 0.9875) göstermiştir. Bağlı standart sapmanın duyarlılığı % 10’dan
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 affected 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 diseases. For example, blood Hcy level is also a well known risk factor for cardiovascular diseases (Abraham and Cho, 2010; Kumar et al., 2017; Smith et al., 2018). The high total homocysteine (tHcy) level was also associated 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 deficiencies 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 fluorescence, electrochemical, mass spectrometry, and post-column derivatization detection (Refsum et al., 1985; Refsum et al., 1989; Ueland et al., 1993; Ubbsnik, 2000; Alam et al., 2019). HPLC method with fluorescence detection comes forward due to its principal and the most preferred 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 fluorescence 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 piezoelectric 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 carbamet 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 modified silver electrode surface and the optimization 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), acetone (CH3COCH3), methanole (CH3OH), cysteamine (C3H7NS), NaHPO4-NaH2PO4, glutaraldehyde (GA), tetraborate, HCl were purchased from Merek KGaA (Darmstadt, Germany). Quartz crystals with silver electrode (10 MHz, AT cut, MEC Quartz Limited Honkong & Mainland China) were purchased from Ozdsan 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 electrode quartz crystals

The surfaces of silver electrodes 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, cysteamine and glutaraldehyde immobilizations were given in details below (Figure 1).
The designed QCM system can measure the frequency of two electrodes and gives the frequency change of electrodes comparatively in order to compare frequency difference of two electrodes and diminish the errors may come from the device. So, the frequency difference of two untreated crystals were measured at first. Then the first procedure was applied to one of the electrode while other was kept untreated and again frequency change was measured. In the second step, the same process was applied to untreated electrode but second step was done to other electrode and followed by frequency 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 electrode quartz crystals were cleaned by immersing consecutively to 0.5 M NaOH solution, acetone, and methanol for 30 min. The electrode 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 (NH₂) 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 electrodes were rekorded. The bifunctional reagent, glutaraldehyde was used as spacer arm through Schiff’s base reaction with free amine group end of cysteamine. Silver electrode 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 electrode 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 +4°C until use within one week and all the experiments were performed in sterile environmental conditions to avoid contamination in specific molecule containing solutions. The schematic 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 electrode 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 +4°C 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 electrode quartz crystals were waited in 3 mL Hcy solution for 30 min on magnetically stirrer at room temperature. Then, silver electrode 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 electrode Surfaces Modification steps and Immobilization of the specific diagnostic molecule.](image-url)
3. Results and Discussion

3.1. QCM experiments

Silver electrode quartz crystals are sensitive to temperature and humidity changes which is regarded as their disadvantage. So, the estimation of temperature dependence of silver electrodes 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 electrodes 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 between temperature and frequency of silver electrode 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 electrodes were found to be sensitive to temperature changes. First, the electrode surface needs to be cleaned or activated in order to make modifications. Therefore, the activation of electrode 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 electrode 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 electrodes 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.

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

Anti-Hcy antibody was coupled to aldehyde group created on silver electrode 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 electrode was immersed to pH 7.4 PBS buffer and ultra pure water after treatment time was ended and dried at 35°C. 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 electrode. The same phenomena can be said for greatered Hcy concentrations. Therefore, dilution was increased to 1/4000 v/v and again biosensing frequencies were given in Figure 6b. Anti-Hcy antibody biosensing frequencies show fluctuation for Hcy concentrations bigger than 2 μM. A nonlinear re
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 sense 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).
3.4. Calibration curves

Hcy were tested from 0.1 $\mu$M to 50 $\mu$M 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 $\mu$M - 2 $\mu$M concentration range showed a regression coefficient of $r^2=0.9813$ (Figure 7a). The higher Hcy concentration range from 10 $\mu$M-50 $\mu$M 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 standard deviation (RSD) value of 7.17 % was found. The level of RSD may come from random errors of the method stages especially sensitivity of silver electrod connection ends/pins.

The second calibration range will provide Hcy detection in healthy 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-
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.

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 QCM technique with a rapid, cheaper 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.

5. Acknowledge

This work was supported by the Scientific and Technological Research Council of Turkey (Grant No:105S052) for financial assistance. Special thanks to Prof. Dr. Hakan Ayhan and Prof. Dr. İ. Cengiz Koçum for their scientific support.

References

Abraham, JM, Cho, L, (2010) The homocysteine hypothesis: Still relevant to the prevention and treatment of cardiovascular disease? (Review). Cleveland Clinic Journal Of Medicine, 77:911-18.

Alam, S. F., Kumar, S., Ganguly, P., (2019), Measurement of homocysteine: a historical Perspective. J. Clin. Biochem. Nutr. November, 65, 3, 171-177.

Andersson, M, Andersson, J, Sellborn, A, Berglin, M, Nilsson, B, Elwing, H, (2005). Quartz crystal microbalance-with dissipation monitoring (QCM-D) for real time measurements of blood coagulation density and immune complement activation on artificial surfaces. Biosensors and Bioelectronics, 21: 79-86.

Ayhan, F, Gülsu, A, Ayhan, H, (2007) Quartz Crystal Piezo Electrode Surface Modification for Gold Nano-particles Immobilization. Hacettepe J. Biol. & Chem., 35: 203-208.

Ayhan, F., Kaya, G., Ayhan, H, (2014). Homocysteine-BSA-affinity based biosensor design. Turkish Journal of Biochemistry, 9(3):383–396.

Bakhshpour, M., Kevser Psikin, A., Yavuz, H., Denizli, A., (2019). Quartz crystal microbalance biosensor for label-free MDA MB 231 cancer cell detection via notch-4 receptor. Talanta, 204 840–845.

Beitollahi, H., Zaimbashi, R., Mahani, M, T., Tajik, S., (2020). A label-free aptasensor for highly sensitive detection of homocysteine based on gold nanoparticles. Bioelectrochemistry 134, 107497.

Bunde, RL, Jarvi, J, Rosentetter, JJ, (1998) Piezo electric quartz crystal biosensor, Talanta, 46: 1223-36.
Clarke, R., (2007). Homocysteine, B vitamins, and the risk of dementia. The American Journal of Clinical Nutrition, 85:329–30.

Dixon, M. C., (2008). Quartz Crystal Microbalance with Dissipation Monitoring: Enabling Real-Time Characterization of Biological Materials and Their Interactions. Journal of Biomolecular Techniques 19:151–158.

Erdamar, A, Ayhan, F, Koçum, İC, Ayhan H, (2007). Urease Immobilized Piezoelectric Quartz Crystal for Urea Conversion. Hacettepe J. Biol. & Chem., 36: 173-180.

Forgacsova, A., Galba, J., Mojzisova, J., Mikus, P., Piestansky, J., (2019). Ultra-high performance hydrophilic interaction liquid chromatography – Triple quadrupole tandem mass spectrometry method for determination of cysteine, homocysteine, cysteinyl-glycine and glutathione in rat plasma. Journal of Pharmaceutical and Biomedical Analysis, 164, 442–451.

Franzen, F., Faaren A.L., Alffheim, I., Nordhei, A.K., (1998). Enzyme conversion immunoassay for determining total homocysteine in plasma or serum. Clin Chem, 44: 311–316.

Fuigione, A., Cimafonte, M., Ventura, B. D., Iannaccone, M., Ambrosino, C., Capuano, F., Proroga, Y. R., Velotta, R., Capparelli, R., (2018). QCM-based immunosensor for rapid detection of Salmonella Typhimurium in food. Scientific Reports, 8:16137.

Hankey GJ, Eikelboom JW, (1999). Homocysteine and vascular disease. Lancet, 354:407–13.

Hasan, T., Arora, R., Bansal, A. K., Bhattacharya, R., Sharma, G. S., Singh, L. R., (2019). Disturbed homocysteine metabolism is associated with cancer. Experimental & Molecular Medicine 51:21.

Hoffman, M, (2011) Hypothesis: Hyperhomocysteinemia is an indicator of oxidant stresses. Medical Hypotheses 77: 1088–1093.

Jiang, H., Li, C., Wei, B., Wang, Q., Zhong, J., Lu, J. (2018). Serum homocysteine levels in acne patients. J Cosmet Dermatol. 17:523–528.

Kang, M.F., Qiao, H.X., Meng, Y.L., Xin, Z.H., Ge, L.P., Dai, M.Y., Xu, J.J., Zhang, C.H., (2017) Selective detection of cysteine over homocysteine and glutathione by a simple and effective probe. Anal. Methods 9: 1707-1709.

Karousos, N.G., Aouabdi, S., Way, A.S., Reddy SM, (2002). Quartz Crystal Microbalance with Dissipation Monitoring: Enabling Real-Time Characterization of Biological Materials and Their Interactions. Journal of Biomolecular Techniques 19:151–158.

Kocum, C., Erdamar, A, Ayhan, H., (2010) Design Of Temperature Controlled Quartz Crystal Microbalance System Instrumentation Science & Technology, 38:39-51.

Kumar, A., Palfrey, H. A., Pathak, R., Kadowitz, P. J., Gettys, T. W., Murthy, S. N. (2017). The metabolism and significance of homocysteine in nutrition and health. Nutrition & Metabolism 14:78.

Ligkianni, V., Janel, N., Ledru, A., Beaune, P., (2006) Thiol compounds metabolism in mice, rats and humans: Comparative study and potential explanation of rodent's protection against vascular diseases, Clinical Chimica Acta, 469: 189–96.

Kim, H.J., Lee, K.S., Jeon, Y.J., (2017). Electrochemiluminescent chemodosimeter based on iridium (III) complex for point-of-care detection of homocysteine levels Biosens. Bioelectron. 91, 497-505.

Kocum, C., Erdamar, A, Ayhan, H., (2010) Design Of Temperature Controlled Quartz Crystal Microbalance System Instrumentation Science & Technology, 38:39-51.

Kumar, A., Palfrey, H. A., Pathak, R., Kadowitz, P. J., Gettys, T. W., Murthy, S. N. (2017). The metabolism and significance of homocysteine in nutrition and health. Nutrition & Metabolism 14:78.

Ligkianni, V., Janel, N., Ledru, A., Beaune, P., (2006) Thiol compounds metabolism in mice, rats and humans: Comparative study and potential explanation of rodent's protection against vascular diseases, Clinical Chimica Acta, 472: 140-6.

Liu, Y., Zhang, W., Yu, X., Zhang, H., Zhao, R., Shangguan, D., Li, Y., Shen, B., Liu, G., (2004). Quartz crystal biosensor for real-time kinetic analysis of interaction between human TNF-α and monoclonal antibodies, Sensors and Actuators: B, 99: 416–24.

Liu, Y., Wang, Q., Li, M., (2019). Aldehyde group functionalized iridium(III) complexes for the selective sensing of homocysteine. Journal of Organometalic Chemistry, 898, 120874.

Madasamy, T., Santschi, C., Martin, O. J., (2015). A miniaturized electrochemical assay for homocysteine using screen-printed electrodes with cytochrome c anchored gold nanoparticles. Analyst, 140: 6071–6078.

Marx, KA, (2003) Quartz Crystal Microbalance: A Useful Tool for Studying Thin Polymer Films and Complex Biomolecular Systems at the Solution-Surface Interface, Biomacromolecules, 4 : 1099 - 1120.

McCully, K. S. (2007). Homocysteine, vitamins, and vascular disease prevention. Am J Clin Nutr; 86:1563S–8S.

Nilsson, K., Gustafson, L., Hultberg, B., (2013). Elevated Plasma Homocysteine Level in Vascular Dementia Reflects the Vascular Disease Process. Dementia and Geriatric Cognitive Disorders Extra. 3:16–24.

Özkan, Y., Akaydin, S., Firat, H., Çalışkan-Can, E., Arıç, S., Şimşek, B., (2007). Usefulness of Homocysteine as a Cancer Marker: Total Thiol Compounds and Folate Levels in Untreated Lung Cancer Patients. Anticancer Research 27: 1185-1190.

Refsum, H, Helland, S, Ueland, M, (1985) Radioenzymatic determination of homocysteine in plasma and urine. Clin Chem 31:624-628.

Refsum, H, Ueland, M, Svardal, AM, (1989) Fully Automated Fluorescence Assay for Determining Total Homocysteine in Plasma, Clinical Chemistry, 35: 1921–7.

Smith, A. D., Refsum, H., Bottiglieri, T., Fenech, M., Hooshmand, B., McCaddon, A., Miller, J. W., Rosenberg, I., H., Obeid, R., (2018). Homocysteine and Dementia: An International Consensus Statement. Journal of Alzheimer’s Disease. 62, 561–570.

Sönmezler, M., Özgür, E., Yavuz, H., Denizli, A., (2019), Quartz crystal microbalance-based histidine sensor. Artificial Cells, Nanomedicine, and Biotechnology, 47 (1) 221–227.

Tewari, P.C., Zhang, B., Bluestein, B.I., (2018). Serum homocysteine chemosensor based on photochromic diarylethene compounds. Dyes and Pigments, 155, 442-448.

Ubbink, JB, (2000) Assay Methods for the Measurement of Total Homocyst(e)ine in Plasma. Seminars in Thrombosis and Hemostatis, 26:233-41.

Ueland, PM, Refsum, H, (2017) Total Homocysteine in Plasma or Serum: Methods and Clinical Applications (Review), Clinical Chemistry, 39: 1764-79.

Wada, M., Hirose, M., Kuroki, M., Ikeda, R., Sekitan, Y., Takamura, N., Kuroda, N., (2013). Simultaneous determination of homocysteine, methionine and cysteine in maternal plasma after delivery by HPLC-fluorescence detection with DBD-F as a label. Biomedical Chromatography, 27, (6) 708-713.

Wang, J., Ma, L., Liu, G., Ding, H., Pu, S., (2016). Cysteine and homocysteine chemosensor based on photochromic diarylethene with fluorine. Tetrahedron, 72, 8479-8485.

Xia, Y., Zhang, H., Zhu, X., Zhang, G., Yang, X., Li, F., Zhang, X., Fang, M., Yu, J., Zhou, H., (2018). A highly selective two-photon fluorescent chemosensor for tracking homocysteine via situ reaction. Dyes and Pigments, 155, 159–163.

Zappacosta, B., Persichilli, S., Minucci, A., et al. (2006). Evaluation of a new enzymatic method for homocysteine measurement. Clin Biochem; 39: 62–66.

Zhang, D., Wen, X., Wu, W., Guo, Y., Cui, W., (2015). Elevated Homocysteine Level and Folate Deficiency Associated with Increased Overall Risk of Carcinogenesis: Meta-Analysis of 83 Case-Control Studies Involving 35,758 Individuals. PLOS ONE 18, 1-16.

Zinelu, A., Sotgia, S., Scania, B., Pisana, E., Sanna, M., Sati, S., Deiana, L., Sengupta, S., Carru, C., (2010). Determination of homocysteine thiolactone, reduced homocysteine, homocystine, homocysteine–cysteine mixed disulfide, cysteine and cystine in a reaction mixture by overimposed pressure/voltage capillary electrophoresis. Talanta, 82, 1281–1288.