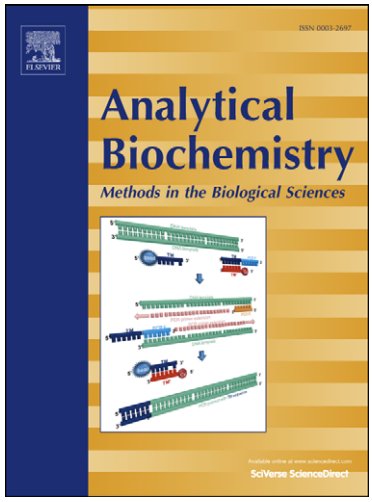

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Small organic molecules detection based on aptamer modified gold nanoparticles enhanced quartz crystal microbalance with dissipation biosensor

Short title: Amplified detection of adenosine with AuNPs-QCM
Subject Category: Biosensor

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Abstract:

Small molecules are difficult to detect by conventional quartz crystal microbalance with dissipation (QCM-D) technique directly because the changes in the frequency resulting from the binding processes of small biomolecules are often small. In the present study, an aptamer based gold nanoparticles enhanced sensing strategy for detection of small molecules was developed. The QCM crystal was first modified with a layer of thiolated linker DNA which can be partly base paired with the detection part containing the adenosine aptamer sequence. In the presence of adenosine, the aptamer bound with adenosine and folded to the complex structure which precluded the reporter part carrying AuNPs to combine with random coiled detection part. Therefore, the lower concentration of adenosine, the more AuNPs combined to the crystal. The resulting aptasensor showed a linear response to the increase of the adenosine concentration in the range of 0-2 μM with a linear correlation of r=0.99148 and a detection limit of 65 nM. Moreover, the aptasensor exhibited several excellent characterisitics, such as high sensitivity, selectivity, good stability, and reproducibility.

Key words:

QCM-D, gold nanoparticles, adenosine, aptamer, detection

1. Introduction

Aptamers are oligonucleotides (DNA or RNA) selected from SELEX (systemic evolution of ligands by exponential enrichment) [1,2]. They have high affinity and specificity in binding targets [3]. Compared with antigen-antibody, they have small sizes, chemical simplicities, simpler synthesis, easier storage, specific binding abilities and simple modification for further immobilization procedure which make them an ideal recognition element for biosensor applications and have already been applied to detection methods such as fluorescence [4], visual identification based on gold nanoparticles [5,6], instruments for signal readout like electrochemical amperometric [7] , potentiometric [8], impedance

transducer [9], SPR [10] and QCM [11] etc.

QCM-D (quartz crystal microbalance-dissipation) is a powerful tool for in situ real-time characterization of solid/liquid interfaces [12]. This technology has been widely used for the study of interactions of biological molecules because it provides rapid, highly sensitive information on the viscoelastic properties of the mass bound by simultaneous measurements of the frequency and dissipation factors. Therefore, much work concentrates on the exploration of mechanism and kinetics of large biomolecules binding processes in real time [13,14]. Although reports on detection of DNA, protein and inorganic ions based on QCM-D have been reported, detection of small organic molecules using QCM is rarely explored [15-17].

Adenosine which is an endogenous purine nucleoside has attracted much attention due to its important role. It can not only modulate neuronal activity and regulate several aspects of adipose tissue function, but also slow down the metabolic activity of the brain [18]. Adenosine has also been shown to be crucial in immune system [19,20]. Therefore, the detection of adenosine is in great need.

Many methods have been developed to improve the performance of QCM-D based biosensors such as amplification using liposomes [21], antibodies [22], DNA self-assembling [23] and nanoparticles [24]. Nanoparticles have a relatively large mass compared to targets and are used as an effective amplifiers in QCM-D sensor construction. Among them, gold nanoparticles (AuNPs) have been extensively used through thiol modified DNA coupling. Utilizing DNA-capped AuNPs, AuNPs can act as a mass enhancer to extend the limits of detection. A nanoparticle-amplified QCM-D DNA sensor using the sandwich hybridization of two specific probes has been demonstrated [25]. Recently, the detections of thrombin and mercury ion utilizing this method have also been developed [11,15].

Since the discovery of aptamer for adenosine [26], different reporter elements were combined with

this aptamer. Previous studies of aptamer-linked gold nanoparticles system using adenosine aptamer as a model showed that the construction of sandwich structure were highly dependent on aptamer overhang and DNA spacers on AuNPs and they suggested that to reach the optimal detection effect, neither aptamer overhang nor DNA spacers were needed [27]. Since this discovery, substantial research efforts are directed to the development of colorimetric [28], electrochemical [29] and fluorescent [4] aptasensors utilizing this sensing principle. However, the detection of adenosine using QCM-D as a platform has never been attempted.

In this paper, a reusable aptamer based AuNPs enhanced QCM-D biosensor with high sensitivity and selectivity was developed. The QCM crystal was first modified with a layer of thiolated linker DNA which can be partly base paired with the detection part containing the adenosine aptamer sequence. In the presence of adenosine, the aptamer bound with adenosine and folded to the complex structure which precluded the reporter part carrying AuNPs to combine with random coiled detection part. In the absence of adenosine, the aptamer immobilized onto the chip surface through hybridization with capture DNA was in random coil conformation and is free to hybridize with amplification element containing AuNPs. Therefore, lower concentration of adenosine will cause larger frequency change. With the aid of AuNPs, the detection limit can be effectively decreased. Furthermore, because the detection part was immobilized on the crystal surface through hybridization, the sensor can be reused after some simple treatments. As far as we know, this is the first time adenosine has been detected by QCM-D technique which will expand the application of QCM-D biosensor.

2. Experimental

2.1 Chemicals

Trisodium citrates, hydrogen tetrachloroaurate (□) ($HAuCl_4$) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). 6-mercaptohexal-1-ol (MCH), tris (2-carboxyethyl) phosphine (TCEP), adenosine, uridine, cytidine and guanosine were purchased from Sigma and used as received. All oligonucleotides designed according to the literature [27] in the present study were purchased from Sangon Biotechnology Inc. (Shanghai, China), and sequences of all oligonucleotides are listed as follows.

Linker DNA:

5'-ACT-CAT-CTG-TGA-**AGA-GAA**-CCT-GGG-GGA-GTA-TTG-CGG-AGG-AAG-GT-3'

Capture DNA: 3'-HS-$(CH_2)_3$-**TGA-GTA-GAC-ACT**-5'

Reporter DNA: 3'-**TCT-CTT-GGA-CCC**-$(CH_2)_6$-SH-5'

Linker DNA is a long sequence containing complementary sequences to both capture DNA (underlined) and reporter DNA (bold), as well as adenosine aptamer (normal). Both the Capture DNA and Reporter DNA are thiolated DNAs. All DNA solutions were prepared by dissolving DNA in enzyme-free water as stocks. Different concentrations of adenosine and 1 mM uridine, cytidine, and 2 μM guanosine were prepared in the buffer containing 300 mM NaCl, 25 mM tris (hydroxymethyl) aminomethane (Tris) acetate, pH 8.2 (hybridization buffer).

2.2 Synthesis and Functionalization of Gold nanoparticles with reporter probe DNA

Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ according to the literature [5]. Briefly, 10 ml of 38.8 mM sodium citrate was immediately added to 100 ml of 1.0 mM $HAuCl_4$ refluxing solution under stirring, and the mixture was kept boiling for another 15 min. The solution color turned to a wine red, indicating the formation of AuNPs. The solution was cooled to room temperature with continuous stirring. The size of the AuNPs were verified by scanning electron

micrograph (SEM) using a microscope.

The process of probe DNA labeling was performed as follows [5]. Appropriate amount of thiolated DNA (3 nmol) was activated with 10 mM acetate buffer (pH 5.2) and TCEP with a concentration 100-fold more than thiolated DNA was added for 1 h and then freshly prepared 1 ml AuNPs of 10.2 nM was added and the mixture was shaken gently overnight. Over the course of 16 h, the DNA-AuNP conjugates were aged in salts (0.1 M NaCl, 10 mM acetate buffer) for 24 h. Excess reagents were removed by centrifuging at 15000 rpm for 30 min. The red precipitate was washed, recentrifuged, and dispersed in 1 ml of hybridization buffer.

2.3 QCM-D measurements

A Q-sense chamber (Q-sense E4) was used for real-time simultaneous measurement of frequency and dissipation changes. Au-coated 5MHz AT-cut quartz crystals were used as the reaction carriers. The Sauerbrey sensitivity of these crystals is 1 Hz= 17.7 ng/cm^2. The frequency and dissipation responses were recorded at 5, 15, 25, 35, 45, 55 and 65 MHz, corresponding to the overtones, n=1, 3, 5, 7, 9, 11 and 13, respectively. For clarity, only the normalized frequency shift ($\Delta f_{normalized} = (\Delta f_n/n)$) and the dissipation shift, ΔD, for the third overtone are presented. During the measurements, the crystals were mounted in a thermal static liquid chamber set at 25^{o}C to provide a rapid, non-perturbing exchange of the liquid (non-flowing liquid) over one side of the sensor.

The QCM-D crystals were first rinsed with solution of 5:1:1 mixture of milli-Q water, ammonia (25%) and hydrogen peroxide (30%) heated to 75 ^{o}C for 5 minutes. Then the crystals were cleaned with milli-Q water and ethanol, and dried with a gentle flow of nitrogen gas in order to remove grease and other pollutants from the crystal surface. Prior to the immobilization of crystal surface, capture stock solution (100 μM) prepared by immobilization buffer (20mM Tris-HCl, 0.1M NaCl, 5mM $MgCl_2$,

pH=7.2) was reduced in 10 mM TCEP for 1h to prevent any oxidation by-products of mercapto-modified DNA. The resulting solution was then diluted with immobilization buffer (IB buffer) to the desired concentration.

For detailed experiment, 1 μM capture probe and 5 μM MCH were first introduced to produce the first layer of the sensor surface. The thiolated capture probe was self-assembled onto the gold crystal surface through thoil-gold bonding. MCH was used to block all the uncovered gold surfaces as well as to eliminate all the physical adsorption DNA [30]. The immobilization of capture DNA was performed at 25 ^{o}C with a circulation mode at a flow rate of 20 μl min^{-1}.

After thoroughly flushed with immobilization buffer, hybridization of Linker DNA onto the modified capture DNA was conducted. 0.01 μM linker DNA was first incubated with different concentrations of adenosine for 30 min. Then the mixed solution was injected into the chamber for hybridization, and Reporter-AuNPs solution was injected afterwards for signal amplification. For all the on-line hybridization steps, a lower flow rate of 10 μl min^{-1} was adopted.

2.4 Characterization of the sensing surface

The morphology of the chip surface with capture, capture/linker and capture/linker/reporter probe structures were observed by using an atomic force microscopy (AFM) (Veeco Inc., America) in tapping mode. All the structures of the unmodified chip, the capture modified, capture/linker DNA after incubation with excessive adenosine and reporter probe successively, and capture/linker DNA after incubation with reporter probe chips were washed with deionized water and then dried with nitrogen gas. After that, AFM images of these four chips were taken at room temperature in air. A Si cantilever with an oscillation frequency of 125 kHz and a spring constant of 14 N/m (SI-DF20, Seiko Inc., Tokyo, Japan) was used for AFM images.

3. Results and Discussion

3.1 Adenosine-Responsive Sensing System and Experimental Principle

The system contained a thiolated aptamer DNA sequence as capture probe, linker DNA as detection probe, AuNP-linked DNA as reporter probe, and adenosine as the analyte, respectively. The linker DNA contained an adenosine aptamer fragment (normal font) and an extension (underlined and bold). The underlined part of the linker hybridized to the thiolated DNA immobilized on the gold chip, whereas the bold part and a small fraction of the normal part of the linker hybridized to the reporter probe functionalized with AuNPs. The fabrication of the sensing design is shown in Scheme 1. The surface of a gold chip was first modified with a 3'-thiol-modified DNA strand (capture sequence) via sulfur-gold affinity. Subsequently, the linker DNA partly combined with adenosine was flushed through the chip surface. Since an aptamer can bind tightly and specifically to a variety of small molecules to form a tertiary complex with a binding constant greater than that of an ordinary DNA duplex, when the amplification part of the 12-base segment close to the 5' end of the linker DNA was introduced into the system, if the linker has already recognized adenosine, it will be unable to hybridize with the amplification part and vice versa. Therefore, when the adenosine concentration is low, few linker DNAs are combined with adenosine and large amounts of AuNPs can combine with linker DNAs which can cause a significant amplification.

3.2 Characterization of the sandwich structure by AFM

Since adenosine is a kind of organic molecule with relatively small molecular weight, AuNPs were employed to amplify the small signal involved in the adenosine-binding event into a measurable QCM-D signal. In order to characterize the AuNPs amplification effects, AFM images of the bare chip,

chip modified with capture DNA and chip in the absence and presence of adenosine after AuNPs amplification were compared. From the topographic AFM images in Fig. 1a, the chip was fairly bare and smooth with no significant peaks or "islands". However, after the modification of capture DNA, the surface height was raised and several "islands" can be seen (Fig. 1b). This trend is more obvious in the capture/linker DNA modified chip after incubation with excessive adenosine and reporter probe successively (Fig. 1c). The sensing surface after modification by capture/linker DNA/reporter probe shows denser "islands" (Fig. 1d). The heights in Fig. 1c and 1d are higher than that in Fig. 1b, but the height change is not so obvious in Fig. 1c and 1d indicating that reporter probe has been attached to both surfaces. The height (~17 nm) of these "islands" in Fig. 1d is a little lower than the sum of the length of capture/linker DNA/reporter probe (~11 nm) and diameter of AuNPs (~13 nm) which may due to the flexible carbon chains in DNA ends and the dry condition when conducting AFM. The agreement of these data clearly indicates the formation of capture/linker DNA/reporter probe sandwich on chip surface.

3.3 Optimization of the sensing surface

The immobilization of capture DNA onto gold chip is the first step in modifying the sensing surface. Therefore, it is important that proper amount of DNA has been immobilized onto the chip surface. Under the same condition, the immobilization of pure capture DNA first and then MCH (MCH afterwards treatment) gives only 2 Hz frequency change while the simultaneous immobilization of DNA and MCH (MCH simultaneous treatment) leads up to 12 Hz frequency response which is 6-fold higher than subsequent MCH treatment method. The immobilization of capture DNA without MCH

simultaneous treatment will result in much more physical adsorption instead of mercapto-gold affinity interaction and this kind of physical adsorption will take up much room which further hinders the interaction of mercapto-modified capture DNA to covalently combine onto the chip surface. Therefore, after the treatment of MCH, all the physical adsorption capture DNAs are flushed and only little amount of capture is immobilized. We further find out that this MCH afterwards treatment will cause much more vacant room for AuNP deposition instead of duplex formation for signal amplification which is difficult for regeneration. However, the immobilization by MCH simultaneous treatment shows a good regeneration and the frequency can bounce back to the point after capture DNA immobilization.

3.4 Performance of AuNPs amplified QCM-D biosensor

The detection of adenosine was firstly studied by monitoring the QCM-D responses induced from adenosine binding with immobilized adenosine aptamer. As shown in Fig. 2a, there is no evident change of frequency even when adenosine concentration is as high as 1 mM. However, when using the proposed design, a concentration of below 10 μM can be detected (Fig. 3 inset). Along with the decrease of adenosine concentration, the AuNPs-enhanced frequency change gradually increased. In addition, the frequency response slightly decreased while the adenosine concentration further increased to 10 μM (Fig. 3 Inset). This result demonstrated that most of linker DNA was in the form of single stranded structures and few free sites were left for Reporter-AuNPs binding, which was in accordance with the previously discussed sensing mechanism. The AuNPs and DNA linker chains act perfectly as mass enhancers for frequency signals amplification and the decrease of detection limit can be achieved. A plot of the frequency change versus adenosine concentration gave a linear response (Fig. 3) with the equation Y=A+BX=-13.43445X+111.0672 (X was the concentration of adenosine, 10^{-6} M; Y was the

frequency change) with a coefficient r=0.99148. The detection limit was 65 nM estimated using three times the signal-to-noise ratio of blank solution. The detection limit obtained from the present study is a little lower than methods conducted by electrochemical impedance spectroscopy and chronocoulometry [31,32] and is more than 10-fold lower than the method using label-free and reagentless sensor [7,33], 50-fold lower than the method utilizing DNAzyme and fluorescence [34], more than 3 orders of magnitude lower than the detection limit reported using a colorimetric method based on AuNP aggregation [35]. It is reported that extracellular adenosine levels would be increased due to ATP release during cellular stress such as inflammation and hypoxia [36,37] and the rapid conversion of ATP to adenosine [38]. When this occurs, the extracellular adenosine level is increased approximately 4-fold [39]. Since the adenosine concentration in brain and normal human blood was estimated to be 50-200 nM [40], the adenosine concentration will be increased to 200-800 nM under abnormal conditions. Therefore, the designed sensor can be used as an alarm in case of abnormal conditions.

The selectivity was further investigated by comparing the AuNPs-enhanced frequency change in response to adenosine with that of various analogue molecules possibly interfering with adenosine detection. The effect of uridine and cytidine which belong to the nulceosides family and might interfere with the response of the aptasensor was studied. As shown in Fig. 4, after the addition of 2 μM adenosine, cytidine and uridine, guanosine under the same experimental conditions, the adenosine induced a very large response while cytidine, uridine and guanosine did not produce any significant changes compared with non-sample experiment. The mixture of 2 μM adenosine, 2 μM cytidine, 2 μM uridine and 2 μM guanosine was also tested. The frequency change was almost the same with pure 2 μM adenosine showing less than 6% difference. These results demonstrated that the developed strategy

had a sufficient specificity to detect adenosine.

3.5 Regeneration of AuNPs amplified QCM-D biosensor

The QCM-D chip after the binding of AuNPs amplification element was regenerated by rinsing the surface with a high alkalinity and ionic strength buffer (1M NaOH and 1M NaCl), and then washing buffer solution. This resulted in a complete release of all the DNA binding events including the binding between linker and detection aptamer as well as the binding between detection element and amplification part. The results from four cycles of reusing shows that the interaction between those DNA molecules are reversible and the sensing surface can be reusable, as shown in Fig. 5.

Conclusion

A sensitive and selective AuNPs enhanced, QCM-D based sensing strategy for adenosine detection was constructed. The design relies on the structure-switching properties of aptamers upon binding to their target molecules and signal enhancement of nanotechnology. This work is the first one in reporting the detection of adenosine molecule using AuNPs amplified QCM-D based sensing approach. This sensing platform can be reusable and shows a good repeatability after even four times reusing and surely expand the application of QCM-D as sensors.

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Figure legends

Scheme 1. A schematic representation of adenosine detection by aptamer using AuNPs as amplification element.

Figure 1. AFM topographic images of the unmodified chip (a), the capture DNA modified chip (b), the capture/linker DNA modified chip after incubation with excessive adenosine and reporter probe successively (c) and capture/linker DNA modified chip after incubation with reporter probe (d) in tapping mode. The image size is 2 μm × 2 μm

Figure 2. On-line AuNPs enhanced frequency changes to different concentrations of adenosine incubated with 0.05 μM linker DNA. (a) detection of 1 mM adenosine without AuNPs amplification (b) blank (c) detection of 0.2μM adenosine with AuNPs amplification (d) detection of 1 μM adenosine with AuNPs amplification (e) detection of 2 μM adenosine with AuNPs amplification (f) detection of 5 μM adenosine with AuNPs amplification (g) detection of 10 μM adenosine with AuNPs amplification

Figure 3. The linear relationship between frequency response and adenosine concentration in the range of 0-2000 nM. Inset: Frequency response of the QCM-D biosensor to different concentrations of adenosine from 200 nM to 10 μM. The error bars represent the standard deviation of three measurements.

Figure 4. QCM-D frequency changes for different nucleosides in hybridization buffer. (a) blank; (b) adenosine; (c) guanosine; (d) uridine; (e) cytidine; (f) mixture of 4 nucleotides in 2 μM. The error bars

represent the standard deviation of three measurements.

Figure 5. The frequency response of aptamer based AuNP amplified biosensor against the number of amplification/regeneration cycles.

Figure 1

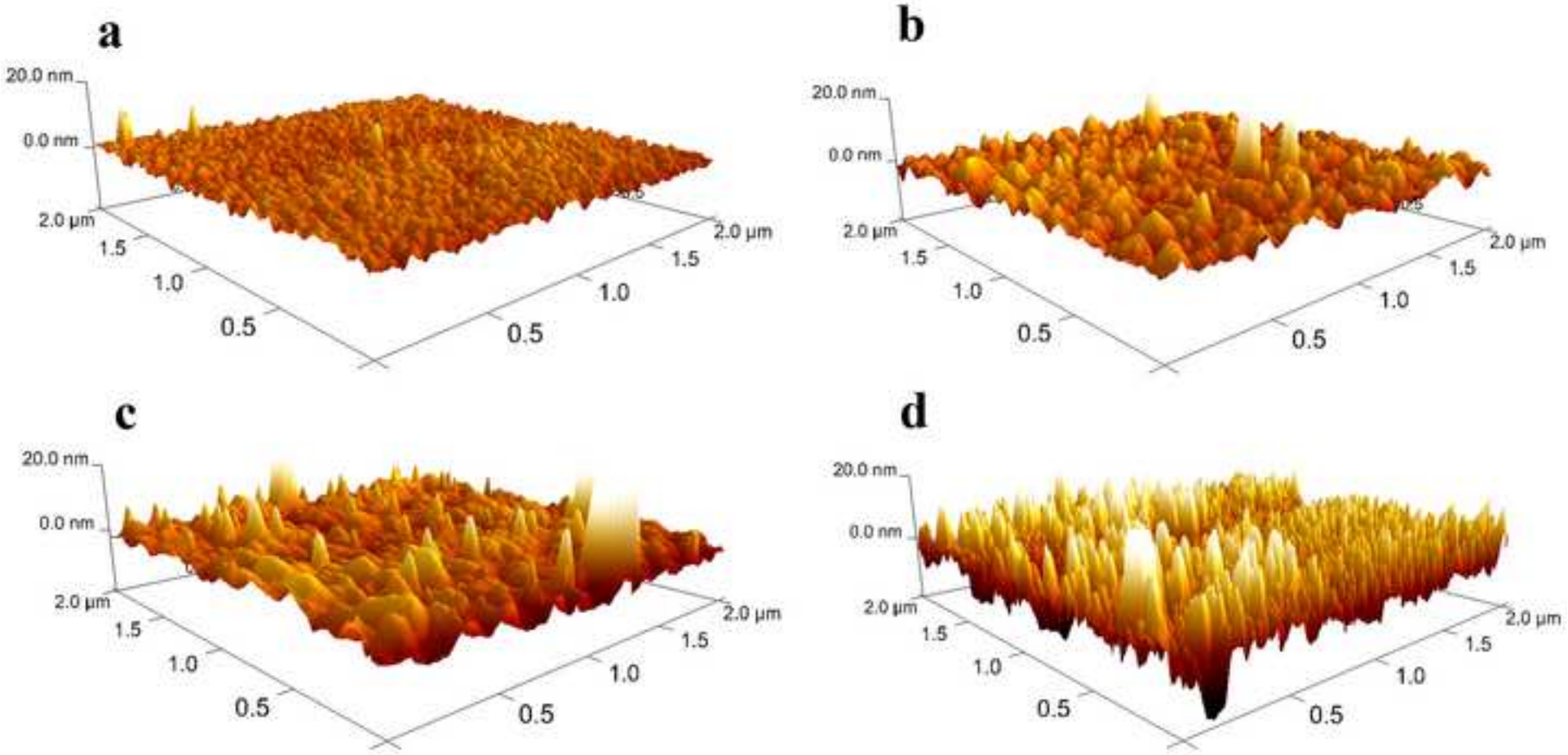


Figure 2

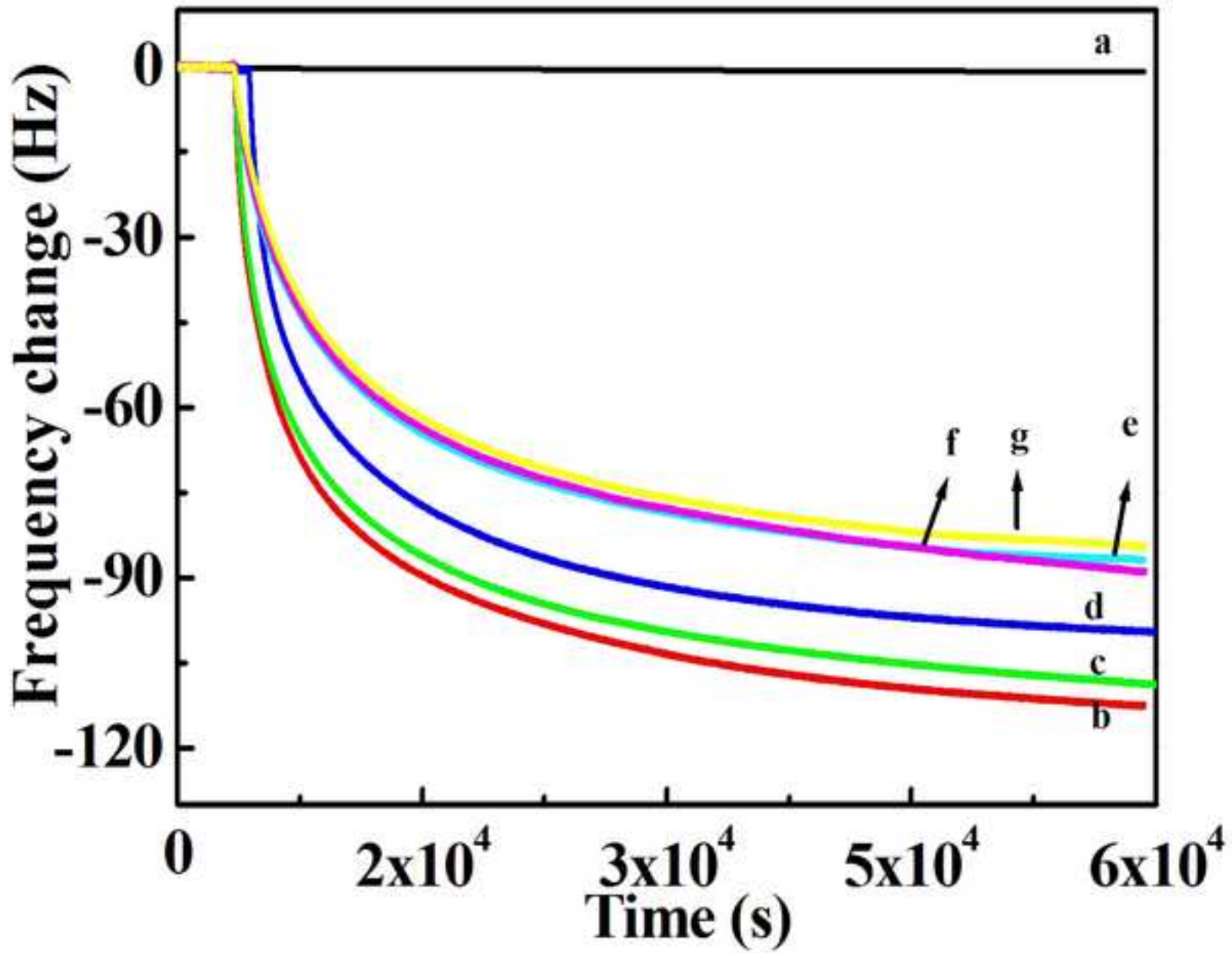


Figure 3

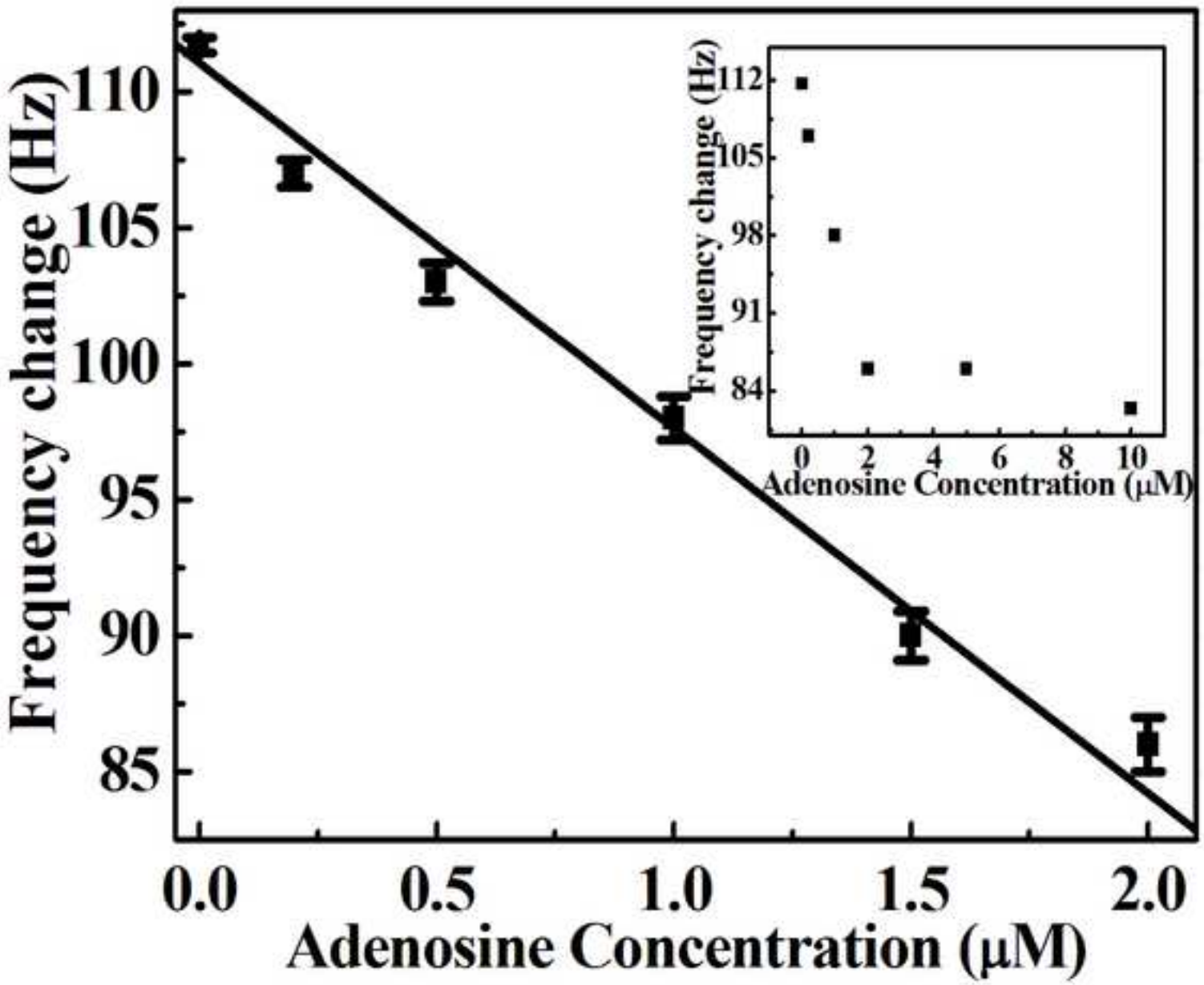


Figure 4

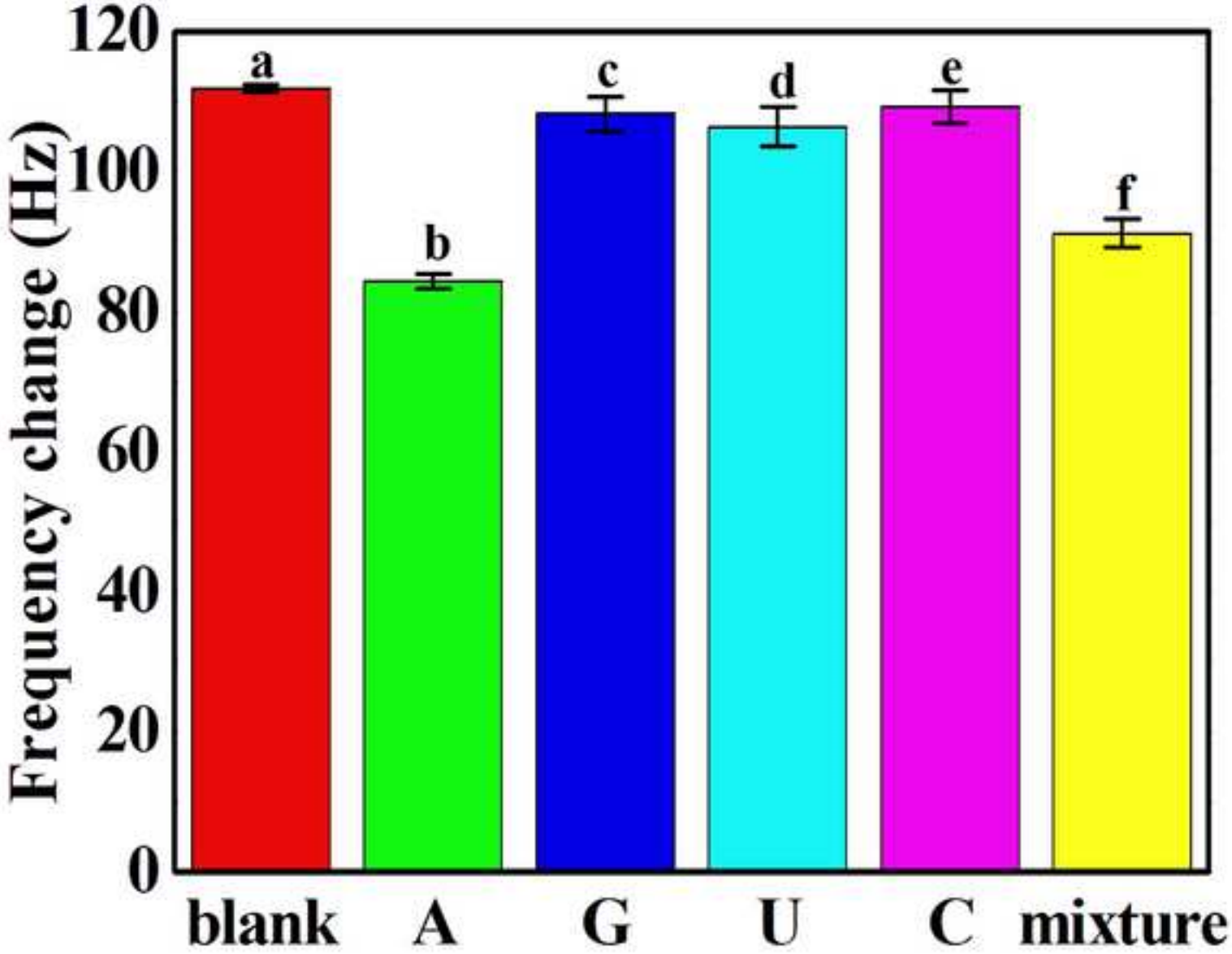
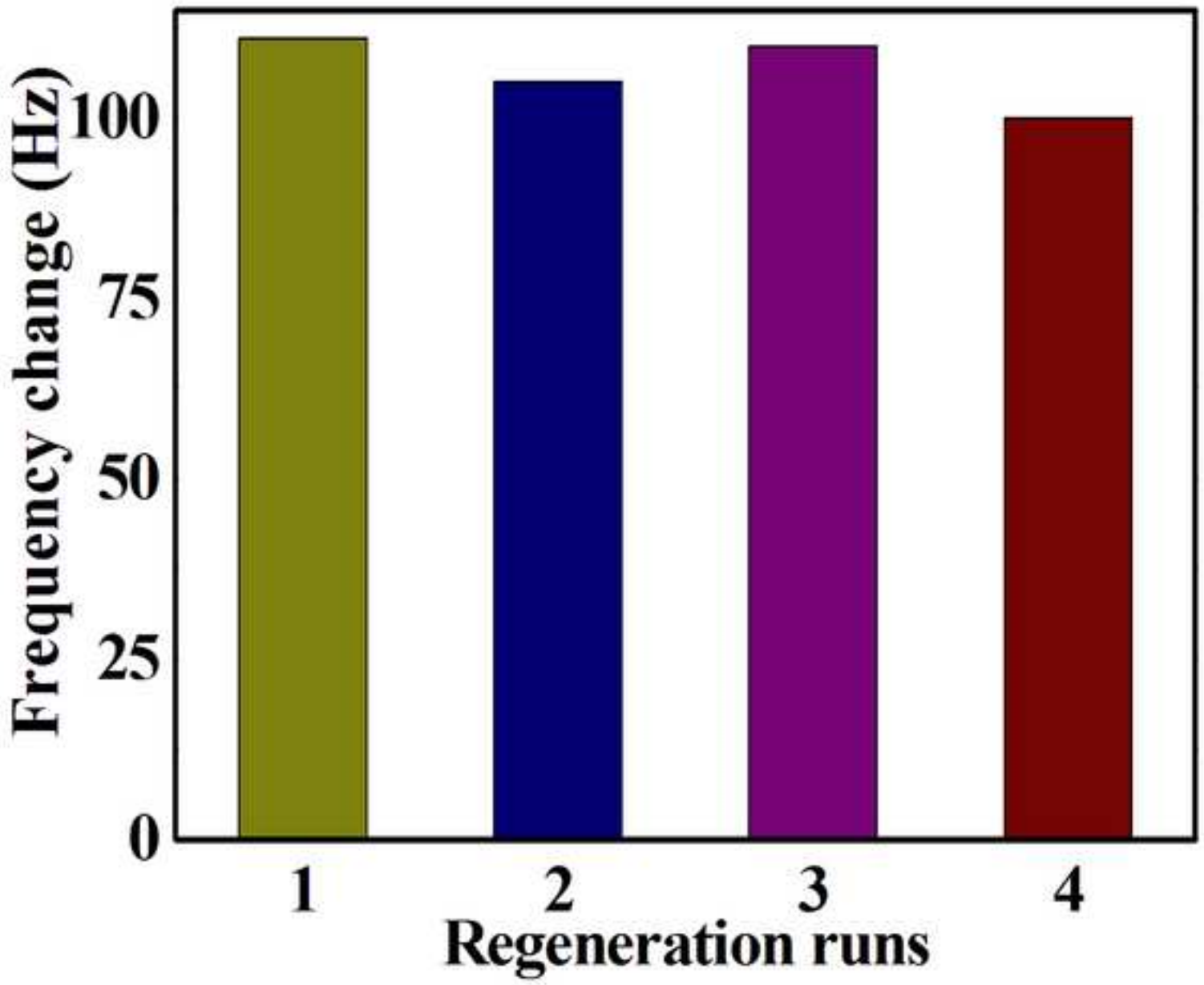


Figure 5



Scheme 1