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Flavonoids activity determination of ginkgo sample using electrochemical method

Determinación de la actividad de flavonoides de la muestra de ginkgo mediante un método electroquímico

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Abstract

The determination of flavonoids activity is very important for drug effect evaluation. In this work, we proposed an advanced hydrogel based electrochemical approach for the quick determination flavonoids activity in plant extract. Zinc ions and chitosan have been used as raw materials for hydrogel synthesis. Then, the presence of the hydroxy radicals can initiate the depolymerization process. The presence of the flavonoids can delay the depolymerization and consequently used for determining their activity. We used ginkgo leaf extract as an example, demonstrated the successful preparation of the hydrogel platform and showed potential application in flavonoids activity determination. we believe the proposed hydrogel sensing platform shows great potential in antioxidants screening application.

Keywords: Electrochemical sensing, flavonoids activity, ginkgo extracts, hydrogel, crosslinking.

Resumen

La determinación de la actividad de los flavonoides es muy importante para la evaluación del efecto del fármaco. En este trabajo, propusimos un enfoque electroquímico avanzado basado en hidrogel para la determinación rápida de la actividad de los flavonoides en el extracto de la planta. Los iones de zinc y el quitosano se han utilizado como materias primas para la síntesis de hidrogel. Entonces, la presencia de los radicales hidroxi puede iniciar el proceso de despolimerización. La presencia de los flavonoides puede retrasar la despolimerización y, en consecuencia, usarse para determinar su actividad. Utilizamos el extracto de hoja de ginkgo como ejemplo, demostramos la preparación exitosa de la plataforma de hidrogel y mostramos una aplicación potencial en la determinación de la actividad de los flavonoides. Creemos que la plataforma de detección de hidrogel propuesta muestra un gran potencial en la aplicación de detección de antioxidantes.

Palabras clave: Detección electroquímica, actividad de flavonoides, extractos de ginkgo, hidrogel, reticulación.

1 Introduction

Flavonoids are widely found in some plants and berries in nature, totaling about 4,000 species. The molecular structure of flavonoids is different, such as rutin, hesperidin, quercetin, green tea polyphenol, anthocyanin, anthocyanin. Taking flavonoids is a supplement to your health (Chen *et al*., 2018; Granato *et al*., 2018; Wang *et al*., 2018). Two decades ago, scientists discovered that ginkgo trees, known as living fossils, contained relatively high levels of flavonoids, mainly extracted from the leaves of ginkgo trees. Flavonoids are a powerful antioxidant that can effectively remove oxygen free radicals from the body. Anthocyanins, for example, inhibit the full stage spill of lipid peroxides (Jędrejek *et al*., 2017; Nam *et al*., 2017; Salla *et al*., 2016). This ability to block oxidation is more than 10 times greater than vitamin E. This antioxidant action can prevent cell degeneration, aging, and cancer.

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Flavonoids improve blood circulation and lower cholesterol. Flavonoids in some plants also contain a PAF anticoagulant factor, which can greatly reduce the incidence of cardiovascular and cerebrovascular diseases and improve the symptoms of cardiovascular and cerebrovascular diseases (AL-Ishaq *et al*., 2019; Grassi *et al*., 2015). Flavonoids, called anthocyanins, have been shown in animal studies to reduce blood sugar by 26% and tribasic fatty acids by 39%. It can also stabilize collagen, so it has a good effect on diabetic retinopathy and capillary embrittlement (Fu *et al*., 2020c; Haq *et al*., 2019; Karimi-Maleh *et al*., 2019b; Khodadadi *et al*., 2019; Shamsadin-Azad *et al*., 2019).

Ginkgo biloba extract is effective in treating cardiovascular and cerebrovascular diseases and nervous system diseases (Liu *et al*., 2015; Park *et al*., 2016; von Gunten *et al*., 2016; Wahby *et al*., 2017). Ginkgo biloba leaf mainly contains flavonoids and terpenolides. According to medical pharmacological studies, ginkgo biloba flavonoids have anti-inflammatory, anticyclic adenylate activity, antihistamine activity and other effects. With the rapid development of instrumental analysis technology, the methods for the determination of total flavonoids content are constantly updated, including spectrophotometry, near-infrared diffuse reflection spectroscopy and high performance liquid chromatography (Baghizadeh *et al*., 2015; Bijad *et al*., 2018, 2013; Eren *et al*., 2015; Gómez-Guerrero *et al*., 2019; Jamali *et al*., 2014; Oliveira *et al*., 2019; Tahernejad-Javazmi *et al*., 2018). These analytical methods are widely used in the measurement of flavonoids, but cannot be used to measure the activity of flavonoids. The activity of flavonoids can be considered as an electrochemical property in nature, so electrochemical methods have been widely used in the study of natural flavonoids (Li *et al*., 2017; Lindner *et al*., 2019; Ren *et al*., 2016). The main research directions can be summarized as electrochemical analysis, electrochemical evaluation of antioxidant activity, electrochemical study of interaction with DNA and electrode process dynamics (Cheraghi *et al*., 2017; Fu *et al*., 2020c, 2020b, 2020a; Karimi-Maleh *et al*., 2019a; Shahmiri *et al*., 2013; Xu *et al*., 2020).
Cyclic voltammetry is a simple and rapid electrochemical method used by many researchers to detect flavonoids. In addition, differential pulse voltammetry, oscillopolarography and flow injection ampere are also used. The selection of appropriate methods has great influence on the improvement of detection sensitivity. Volikakit *et al*. (Volikakis and Efstathiou, 2000) studied the feasibility of the detection of 12 kinds of flavonoids by the voltammetry method of adsorption reverse extraction in the flow injection system, and investigated the influence of pH, enrichment potential and time, the presence of other substances and other factors. There are two kinds of electrochemical methods to study the activity of natural flavonoids. The first is to measure electrochemical parameters (such as redox potential, etc.) for natural flavonoids(Alavi-Tabari *et al*., 2018; García-Amador *et al*., 2019; Karimi-Maleh *et al*., 2016; Karimi-Maleh and Arotiba, 2020; Miraki *et al*., 2019; Sánchez-Franco *et al*., 2019). The second is to measure the current signal for the reactive oxygen species and to obtain the weakening degree of the signal caused by the addition of natural flavonoids (Cao *et al*., 2015; Kyriakis *et al*., 2015; Xue *et al*., 2018; Zakaryan *et al*., 2017). By studying the interaction between drug molecules and DNA, we can have a deeper understanding of the pathogenesis of some diseases and the mechanism of drug action in human body. For example, Hsin *et al*. (Constantinou *et al*., 2005) found that genistein can reduce oxidative DNA damage induced by methylglyoxal and thus protect human monocytes. At present, the interaction between natural flavonoids and DNA has not been fully studied. For example, most scholars believe that quercetin has anti-cancer and anti-DNA oxidative damage effect, but some reports believe that quercetin is a carcinogen, which has a damaging effect on DNA. In this work, we proposed a simple hydrogel based electrochemical method for measuring the flavonoids activity. The metal ions crosslinked hydrogel has been prepared using a simple wet chemical method. The flavonoids activity of ginkgo extract has been successful measured using proposed hydrogel.

2 Materials and methods

2.1 Chemicals

Zn(CH3COO)2, CH_3COOH, $C_6H_8O_6$, uric acid, $K_4[Fe(CN)_6]$, NaOH, chitosan (deacetylation: 75%−80%, 50000−190000 Da) and H_2O_2 were purchased from Aladdin Reagent Inc. All other chemicals were analytical grade. Fenton solution was prepared mixing of potassium ferrocyanide and hydrogen peroxide with a molar ratio of 1:6. During the tests, Milli-Q water (18.2 M / cm) was used.

2.2 *Ginkgo leaf extract preparation*

Weigh 1.0 g ginkgo biloba powder in 25 mL 70% ethanol solution. Ultrasonic assisted extraction of flavonoids from ginkgo biloba leaves (extraction power: 80w, time: 40min, temperature: 70 ℃). Then, the solution was centrifuged (4 ℃ 4000 r/min) for 10 min, the supernatant was collected, and the volume was fixed to 25 mL volumetric bottle with 70% ethanol solution. The supernatant was filtered with 0.22 m filter membrane, and stored in a refrigerator at 4 ℃ for further storage.

2.3 *Hydrogel preparation*

The preparation of metal ion crosslinking hydrogels is based on the method reported by Li *et al*. (Fu *et al*., 2018a) In a typical procedure, a certain amount of zinc acetate solution is added to 5 ml of chitosan solution (1% acetic acid). After 1 minute of vigorous shaking, 0.1M of sodium hydroxide is slowly added until the cross-linking reaction is initiated. Hydrogels form as the pH slowly increases. The depolymerization of hydrogels is a process in which the Fenton reagent or hydrogen peroxide is slowly added. Ultrasound is used to speed up the depolymerization process.

2.4 *Flavonoids activity determination*

All electrochemical measurements were performed using CHI 832 electrochemical workstation with a conventional three-electrode system comprised of platinum wire as auxiliary electrode, a 3M Ag/AgCl electrode as reference and a glassy carbon electrode as working electrode. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV), with pulse amplitude of 50 mV, pulse width of 0.05 s and pulse period of 0.5 s are used to detect the activity of flavonoids. Comparing the changes of metal ion redox peaks before and after the addition of flavonoids can be used to measure the activity of flavonoids in ginkgo biloba extract.

3 Results and discussion

Figure 1 shows the activity of flavonoids in ginkgo biloba leaves detected by hydrogel. Zinc ions and chitosan previously formed a supramolecular network. This network is gradually destroyed in the presence of reactive oxygen species, so the metal ions in the crosslinking are released. In the crosslinking state, metal ions cannot move freely, so cyclic voltammetry can only detect relatively weak metal ion signals. But when the metal ions are released, the electrochemical signal is enhanced. If flavonoids are added in this process, reactive oxygen species will react with the flavonoids molecules first, thus slowing the destruction of supramolecular structures in hydrogels. By comparing the electrochemical behavior of adding flavonoids or not, we can measure the activity of flavonoids.

Chitosan is a polysaccharide molecule, which is composed of glucosamine (GlcN) and n-acetylglucosamine (GlcNAc) and b-1,4-glycosidic bonds. During the disaggregation process, GlcN and GlcNAc are released simultaneously (Kwok *et al*., 2018; Mawad *et al*., 2015; Yadav *et al*., 2019). We analyzed the depolymerized products of chitosan by high performance liquid chromatography. Figure 2A shows the HPLC chart of chitosan after depolymerization. It can be seen that the main decomposition products are GlcNAc and GlcN.

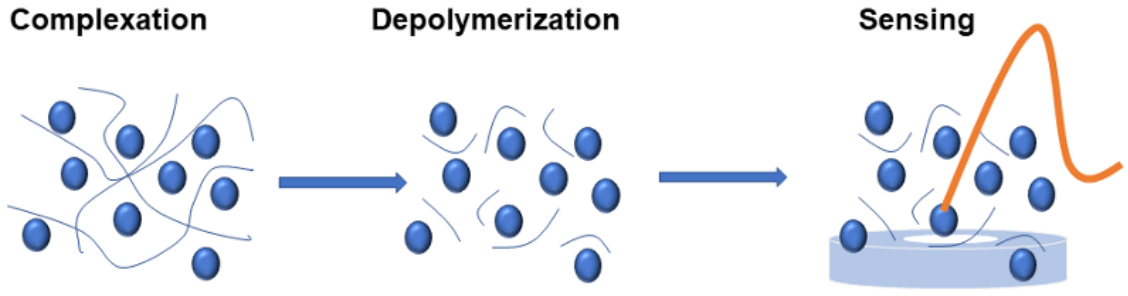

Fig. 1. Schematic diagram of the flavonoids activity measurement based on metal ions cross-linked hydrogel.

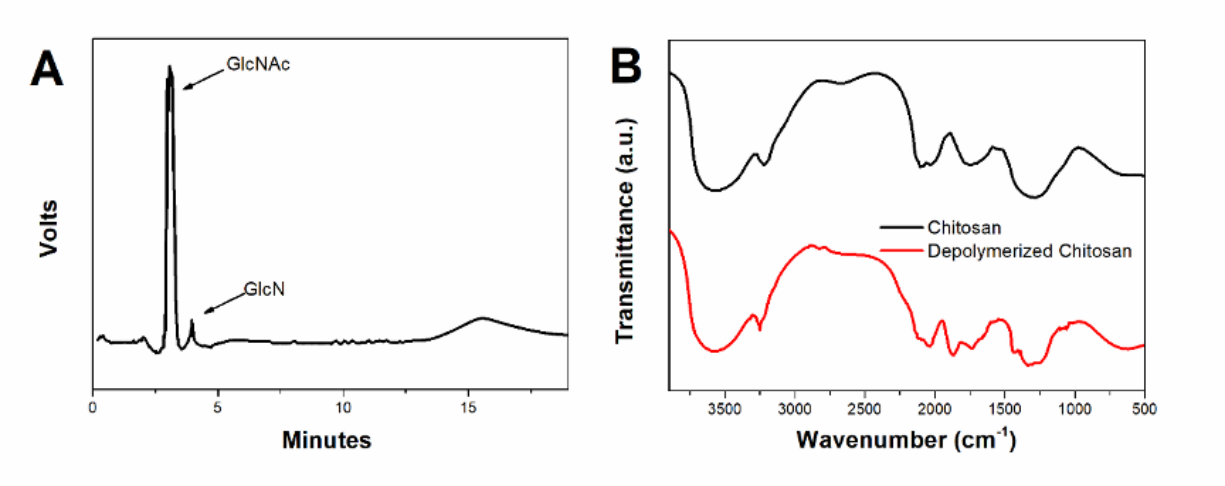

Fig. 2. (A) HPLC profiles of chitosan hydrogel and depolymerized chitosan. (B) FTIR spectra of chitosan and depolymerized chitosan.

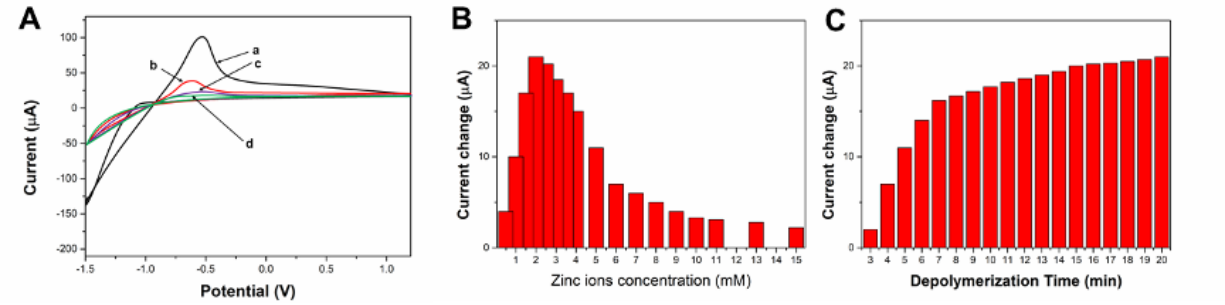

Fig. 3. (A) CVs of zinc ions (a), zinc-chitosan hydrogel (b), zinc-chitosan hydrogel with 0.01 mM H_2O_2 (c) and 0.03 mM H_2O_2 (d). The influence of the (B) zinc ion concentration and (C) depolymerization time in sensing performance.

At the same time, HPLC analysis also showed that the depolymerized chitosan still contained higher molecular weight polysaccharides (Krolicka *et al.*, 2018; Singh *et al.*, 2017). Therefore, we further studied with infrared spectroscopy. Figure 2B shows the FTIR patterns of chitosan before and after depolymerization. Peaks above 3000 cm^{-1} are due to the hydrogen bonding network and water in the sample. Compared with the original chitosan, the peaks of the depolymerized samples decreased slightly, indicating that the ordered structure between the lattices was reduced. In addition, the peak at 1320.6 cm^{-1} corresponds to GlcNAc. Based on the above results, we can find that GlcNAc attached to zinc ions mainly participates in the electrochemical redox reaction. Cyclic voltammetry was used to study the electrochemical behavior of zinc ions in chitosan hydrogels. Figure. 3A shows a CV curve (c, d) of a hydrogel (b) prepared with 2 mM zinc ion (a), and 0.01 and 0.03 mM hydrogen peroxide added to the hydrogel. A glassy carbon electrode (GCE) was used as the working electrode. It can be seen from curve a that the reduction peak of zinc ion is at −1.13 V. A more negative current indicates that metal zinc is deposited on the electrode surface. During the forward scan, an anode peak appears near 1.06 V, which can be attributed to the dissolution with zinc. In contrast, this redox reaction is significantly suppressed in the hydrogel (b). The currents at both the cathode and anode are significantly reduced, which can be attributed to the fact that zinc ions are in a supramolecular cross-linked state and cannot diffuse to the working electrode surface in time for redox reactions (Chen *et al.*, 2017; Cheng *et al.*, 2017; Krolicka *et al.*, 2018; Pella *et al.*, 2018; Sayyar *et al.*, 2015; Singh *et al.*, 2017). The hydrogel began to depolymerize after the addition of hydrogen peroxide, and the redox behavior of some

zinc ions recovered. As shown in the figure, after adding 0.01 mM hydrogen peroxide, the reduction current in the CV scan increased, indicating that more zinc ions reached the electrode. For comparison, we increased the hydrogen peroxide concentration and the current was further increased.

Based on the above results, we have reason to suspect that excessive zinc ions will cause an increase in the background current in the hydrogel system. At the same time, insufficient zinc ions cannot synthesize hydrogels. Therefore, the concentration of zinc ions is very important in this system. As shown in Figure 3A, a continuous decrease in the zinc ion concentration can effectively increase the current difference. Further reducing the zinc ion concentration Although the background current can continue to be reduced, the difference in current also becomes smaller. Therefore, the optimal condition has a zinc ion concentration of 2 mM (Figure 3B). The depolymerization time is also an important parameter. Figure 3C shows the change in current with depolymerization time. We can see that the response increases rapidly at the beginning, then continues to increase over time, but slows down. For ease of operation, we decided to use 15 minutes as the optimal time.

During the depolymerization of chitosan hydrogel, the current signal of zinc ions can be used to measure the content of hydrogen peroxide. Figure 4A records the response of hydrogel with different concentrations of hydrogen peroxide. The results showed that the oxidation peak of zinc ions increased with the increase of hydrogen peroxide concentration. Figure 4B shows the relationship between the current response and hydrogen peroxide concentration.

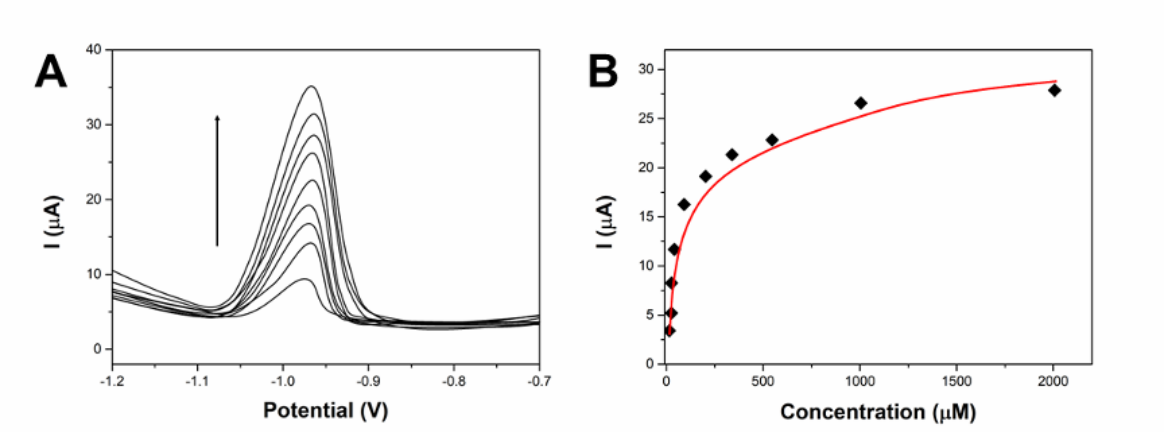

Fig. 4. (A) DPV profiles of the hydrogel along with the depolymerization with different amount of H_2O_2 injection. (B) The relationship between the DPV peak current and the H_2O_2 concentration.

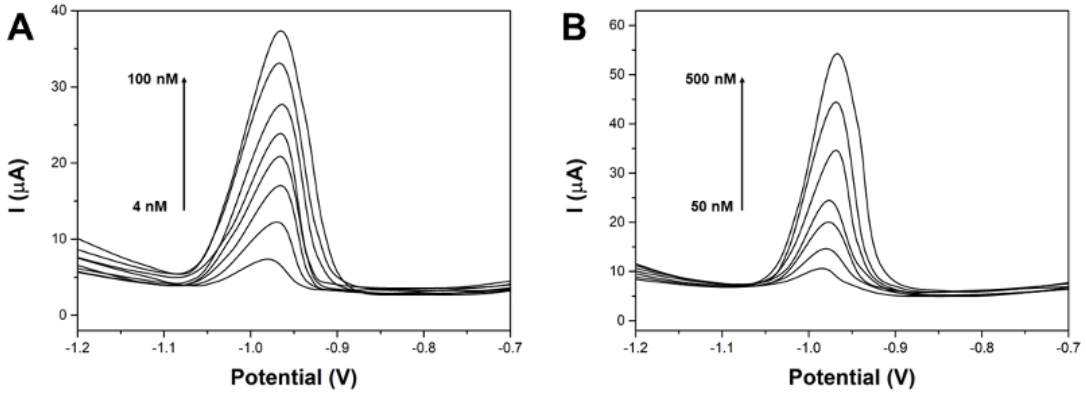

Fig. 5. (A) DPV profiles of the hydrogel along with the depolymerization with different amount of Fenton injection. (B) The relationship between the DPV peak current and the Fenton concentration.

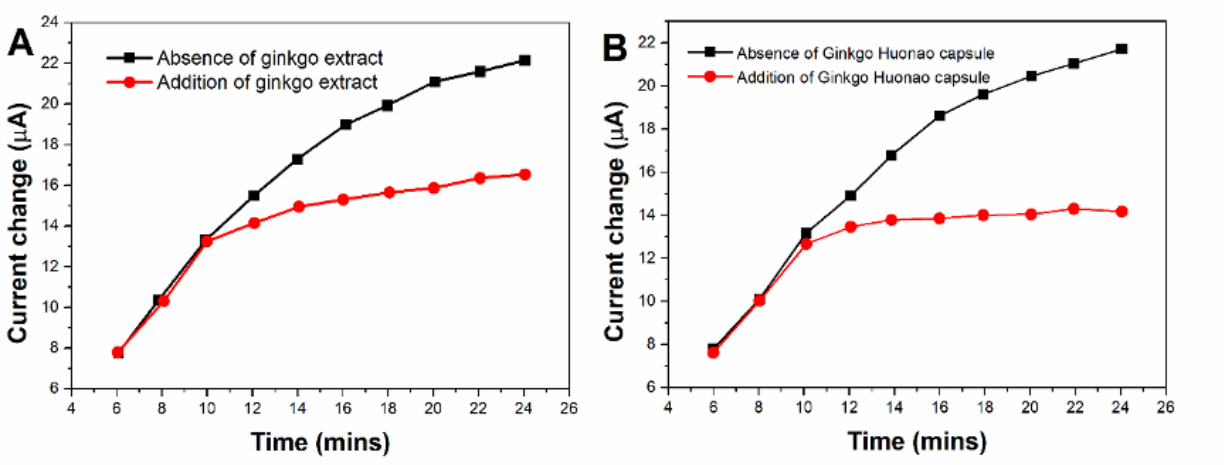

Fig. 6. (A) The current change of the hydrogel with or without addition of ginkgo extract. (B) The current change of the hydrogel with or without addition of Ginkgo Huonao capsule.

It can be seen that the depolymerization process of hydrogel dropped rapidly in the first 30 minutes and almost completed after 2 hours. The results show that the low molecular weight part of shell polymerization is easy to depolymerize. At the same time, those that crystallize well can keep the structure (Alves *et al*., 2018; Fu *et al*., 2018b; Szymańska and Winnicka, 2015). Figure 5 shows the DPV response of adding different concentrations of Fenton solution to hydrogels. After 15 min of depolymerization, the peak value of zinc ions increased. According to the results, the current of zinc ions in the range of 4-100 nM is proportional to the logarithm of hydroxyl radical concentration.

The presence of flavonoids could react with the hydroxyl radical, which hindered the depolymerization performance. Figure 6A shows the DPV response changes of the zinc ions when the addition of the ginkgo leaf extract. It can be seen that, the addition of the ginkgo extract significantly lower the depolymerization process, which reflect the flavonoids activity. Figure 6B shows the relationship of the amount of ginkgo extract and the current change. A positive relationship between the amount of ginkgo extract and the current change was observed. We further used Ginkgo Huonao capsule has an real example. A similar result has been noticed, suggesting the proposed hydrogel electrochemical method can be potentially used for flavonoids activity determination of ginkgo sample.

We also tested the injection of ascorbic acid with 10-folds of some interference species such as glucose and sucrose. These compounds were found to have statistically insignificant effect on the intensity of the peak currents. The reproducibility of the proposed sensing platform was tested using 5 freshly synthesized hydrogels towards ginkgo leaf extract. A relative standard deviation (RSD) of 4.2% was obtained. The life-time stability of the chitosan hydrogel was tested once per week towards ginkgo leaf extract for one month using the same bath of chitosan hydrogel. The current difference decreased by approximately 7.22% after one month. The chitosan hydrogel showed a good reproducibility with an acceptable stability.

Conclusions

In conclusion, we proposed a hydrogel based method for electrochemical evaluation of flavonoids activity. Zinc ions and chitosan have been used for hydrogel synthesis. Then, the H_2O_2 or Fenton solution have been used for initiating the depolymerization process. This process can be delayed in the presence of flavonoids, which also reflect the their activity. Therefore, we used this method for the determination of flavonoids activity in ginkgo sample. In addition, the chitosan hydrogel showed a good reproducibility with an acceptable stability.

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