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The electrochemical behaviour of ferrocene in a photocurable poly(methyl methacrylate-co-2-hydroxylethyl methacrylate) film for a glucose biosensor

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Abstract

A single-step fabrication of a glucose biosensor with simultaneous immobilization of both ferrocene mediator and glucose oxidase in a photocurable methacrylic film consisting of poly(methyl methacrylate-co-2-hydroxylethyl methacrylate) was reported. The entrapped ferrocene showed reversible redox behaviour in the photocured film and no significant leaching of both entrapped ferrocene and enzyme glucose oxidase was observed because of the low water absorption properties of the co-polymer films. From electrochemical studies, ferrocene entrapped in the co-polymer film demonstrated slow diffusion properties. A linear glucose response range of 2–11 mM was obtained at low applied potential of +0.25 V. The glucose biosensor fabricated by this photocuring method yielded sensor reproducibility and repeatability with relative standard deviation of <10% and long-term stability of up to 14 days. The main advantage of the use of photocurable procedure is that biosensor membrane fabrication can be performed in a single step without any lengthy chemical immobilization of enzyme.

Keywords: Photocurable; Methacrylate polymers; Ferrocene; Immobilization; Glucose biosensor

1. Introduction

Glucose determination is one of the major measurements performed in clinical analysis because of diabetes mellitus condition. The determination of glucose most frequently employed a biosensor based on glucose oxidase (GOD). In earlier application, the catalytic conversion of glucose to gluconolactone by the enzyme GOD was coupled to dioxygen with the production of hydrogen peroxide, which was then detected electrochemically. However, the use of dioxygen caused fluctuations in the biosensor response, especially when the oxygen tension varied. This problem was later overcome by introducing mediators to replace oxygen as the mediator of electron transfer. Among the mediators investigated, ferricinium ions from the oxidation of ferrocene or its derivatives were the most useful mediator [1]. These mediators were immobilized in carbon paste electrode and successful glucose and galactose biosensors were constructed [2,3].

However, the use of ferrocene immobilized in a carbon paste matrix has some disadvantages when the biosensor is to be used as an implantable device for glucose monitoring. This is because the mediators can diffuse away from the electrode surface to the surrounding and caused contamination in the tissue. A number of ways have been attempted to resolve leaching of ferrocene mediators and the most common solution is by covalent attachment of ferrocene onto some polymer networks. Some examples of polymers (so called redox polymers) that have been used are polysiloxane [4,5], cross-linked polyallyamine [6], polypyrrole [7], polyaniline [8], polyacrylamide [9,10] and copolymers of methacrylate [11,12].

The prevention of leaching by chemical attachment of ferrocene to a polymer matrix typically involved compli-

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cated synthesis procedures of many steps. For some of the polymers used, they were not compatible with the immobilization of GOD, thus extra step for GOD immobilization by other means, e.g. glutaraldehyde coupling or employing a composite mixture of GOD and ferrocenyl polymers with carbon paste [5,10,12], was also performed. The complex procedures used in the fabrication of the biosensor lead to unfriendly manufacturing technology. Therefore, a simple fabrication procedure that not only prevents leaching of ferrocene but also that of GOD is desirable in terms of manufacturing.

In this paper, we describe a one-step procedure for the fabrication of a glucose biosensor membrane that utilized a photocuring technique. The advantage of the procedure is to immobilize simultaneously both the ferrocene mediator and GOD in a polymer formed from photopolymerisation. The leaching of both of these entrapped membrane components was prevented by tuning the hydrophilicity of the photocurable poly(2-hydroxylethyl methacrylate) hydrogel with methyl methacrylate. Electrochemical behaviour of the ferrocene containing methacrylate films and the analytical performance of the resulting glucose biosensor are discussed.

2. Experimental

2.1. Reagents

Ferrocene, enzyme glucose oxidase (GOD), glucose monohydrate and phosphate buffer were obtained from Fluka; monomer 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) were obtained from Sigma and Aldrich respectively. The photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DMPP) was obtained from Aldrich. Phosphate buffer was prepared in distilled water, and glucose solutions were prepared by dissolving appropriate amounts in 0.1 M phosphate buffer (pH 7.0). Glucose was allowed to mutarotate for 24 h before use. Phosphate buffer (0.1 M, pH 7.0) used as electrolyte in electrochemical measurements was prepared in 0.1 M NaCl.

2.2. Apparatus and measurements

The electrochemical measurements were performed with an Autolab PGSTAT 12 Pontentiostat/Galvanostat. A one-compartment cell with a working volume of 5 mL was used. The working electrode is a carbon paste screen-printed electrode coated with ferrocene-containing copolymer film, whereas saturated calomel electrode (SCE) and a glassy carbon electrode were used as reference electrode and auxiliary electrode respectively. The electrodes used for the preparation of working electrode are screen-printed carbon paste electrodes with active surface 4 mm in diameter designed by Scrint Cyclic voltammetry was studied between 0.0 and 0.70 V versus SCE. The amperometric measurements of the glucose biosensor were performed at 0.25 V versus SCE. All experiments were performed in 0.1 M phosphate/0.1 M NaCl buffer (pH 7.0).

2.3. Preparation of ferrocene containing copolymer films

The procedure used for the preparation of photocurable methacrylic films is similar to those reported before [13,14]. A mixture was prepared by mixing an appropriate amount of monomer 2-hydroxyethyl methacrylate (HEMA), methyl methacrylate (MMA) and photoinitiator DMPP in a vial. An appropriate amount of ferrocene was then added into the mixture. The same amount of Fc was used in preparing all films. The mixture was then exposed to UV radiation to yield the co-polymer film poly(methyl methacrylate-co-2-hydroxylethyl methacrylate) or MH. Polymer films containing physically entrapped ferrocene (Fc/MH) were prepared for the examination of their hydrophilicity character and ferrocene leaching behavior.

2.4. Evaluation of water absorption, ferrocene and enzyme GOD leaching

A mixture of monomer HEMA, MMA and initiator DMPP without ferrocene was prepared in a vial. The mixture was then deposited onto a supporting material with a known weight, and then exposed to UV light. The photocopolymer formed was weighed together with the supporting material and thus the weight of the blank photocopolymer (MH) was obtained. The copolymer together with the supporting material was then exposed to 0.1 M phosphate buffer. After 5 min, the weight was recorded and the water content of the copolymer after 5 min was then calculated as reported by Bayramolu et al. [15]. The water absorption of the copolymer was determined with duration of exposure to water.

As for ferrocene leaching test, Fc/MH polymer film was exposed to 0.1 M phosphate buffer and after 5 min a fixed volume of the phosphate buffer was taken out. This step was repeated several times in a 4-h period. The samples collected were then analysed using a Perkin-Elmer atomic absorption spectrometer (AAS) to determine the presence of iron quantitatively, which would be related to the amount of ferrocene that leached out from the polymer into the solution.

The Fc/MH copolymer films incorporated with enzyme GOD were prepared and also exposed to 0.1 M phosphate buffer. After 5 min, a fixed volume of the phosphate buffer was taken out. This step was repeated several times in a 4-h period. The buffer was then assayed with diluted Bradford reagent and the absorbance was monitored at 595 nm using a Varian Cary 100 UV–Vis spectrophotometer.

2.5. Preparation of electrodes and glucose biosensors

Mixtures of HEMA, MMA, DMPP and Fc were deposited on screen-printed carbon paste electrodes. This

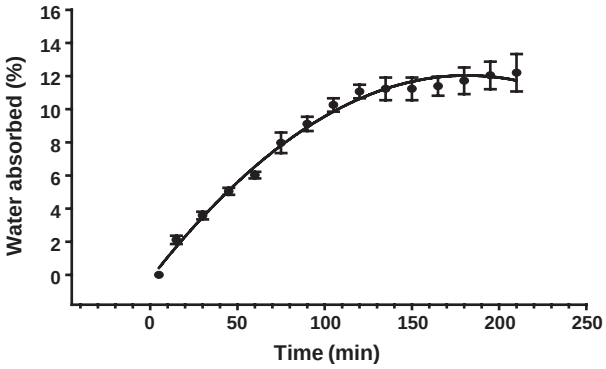

Fig. 1. The water absorption profile of the MH copolymer.

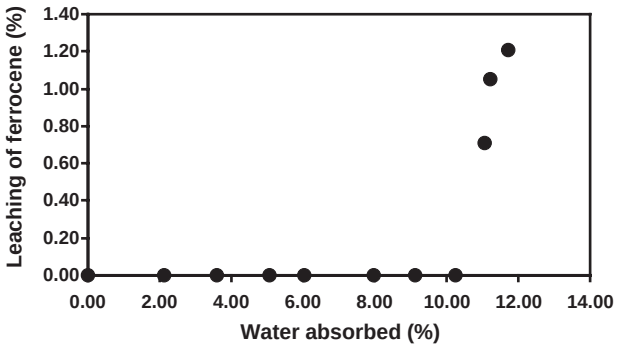

Fig. 3. A correlation between the amount of Fc leached out and the water absorbed by the MH copolymer.

was then exposed to UV radiation and a thin layer of film with thickness ca. 170 μm was formed. These electrodes were then used in the investigation of the redox behaviour of the immobilized ferrocene. Glucose biosensors based on Fc/MH films were constructed by including the enzyme GOD into the mixture before photocuring. The amount of Fc or GOD used in all films (Fc/MH–GOD) was the same.

3. Results and discussion

3.1. Water absorption of MH copolymer and leaching of Fc and GOD

Fig. 1 depicts the water absorption profile by the MH copolymer in a 3 1/2-h period. There is a steady rate of water absorption in the first 2 h and the amount of water absorbed reaches a maximum of approximately 12% by weight after 3 h. HEMA co-polymers containing immobilised vinyl ferrocene (0.14 mol fraction, co-polymer Mw=55,500) prepared by solution polymerisation can absorb up to 20% of water [12]. This shows that the level of water absorption for the MH copolymer is lower and it is more hydrophobic although the copolymer is also HEMA based. The hydrophobic nature of the HEMA based copolymer is mainly due to the dense polymer network result from photopolymerisation employed in this work and the introduction of MMA monomer.

The water absorption behaviour of the MH co-polymer may have influence over the leaching of the mediator Fc entrapped in the polymer by photocuring process. Below 100 min, no iron was detected using an atomic absorption spectrophotometer. However, leaching of Fc began after approximately 100 min of exposure to buffer solution (Fig. 2). A plot of correlation between the amount of Fc leached out and water absorbed (Fig. 3) demonstrated that when the water absorption reached approximately 10% (w/w), Fc leaching began. Therefore, below 10% of water absorption, it seems that Fc could not leach out of the MH film and only 2.2% of Fc was found to leach out after 4 h of exposure to the buffer solution. Leaching studies conducted on entrapped GOD in the MH film also showed that no GOD was detectable after the polymers were exposed to water for 4 h. These studies have demonstrated that by using a hydrophobic co-polymer such as MH to physically immobilise Fc and GOD, the lost of the mediator Fc through leaching can be delayed and minimised whilst that of enzyme GOD leaching can be eliminated altogether.

3.2. Electrochemical behavior of the ferrocene-containing copolymers

The cyclic voltammograms of Fc/MH film were obtained between 0.00 and +0.70 V in a phosphate buffer after the

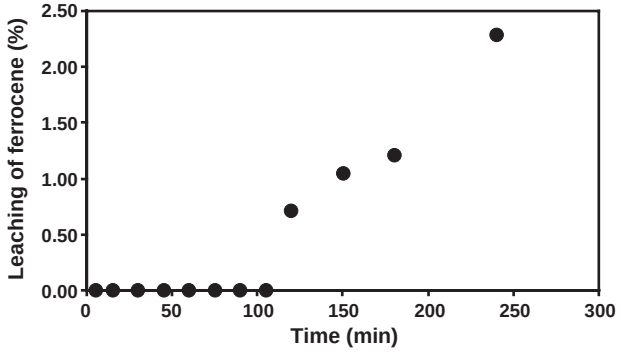

Fig. 2. The leaching profile of the entrapped Fc in a MH film with time. Leaching of Fc only occurred after 100 min of exposure to buffer.

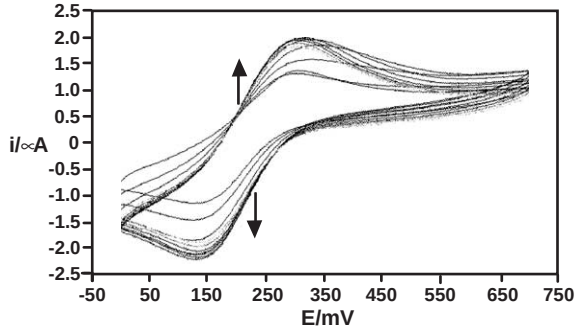

Fig. 4. Cyclic voltammograms of a Fc/MH-coated SPE in 0.1M phosphate/0.1 M NaCl buffer (pH=7.0). Scan rate: 5 mV s^{-1}.

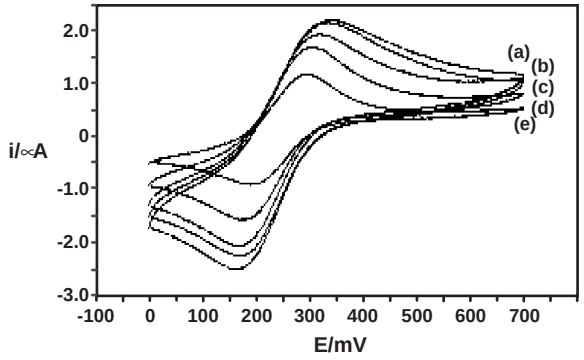

Fig. 5. Cyclic voltammograms of a Fc/MH-coated electrode in 0.1M phosphate/0.1 M NaCl buffer at scan rate of (a) 10, (b) 7.5, (c) 5, (d) 2.5 and (e) 1 mV s^{-1}.

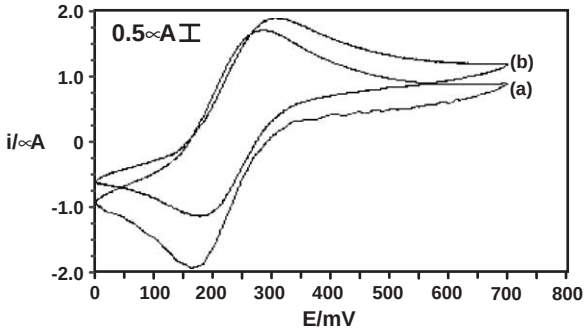

Fig. 6. Cyclic voltammograms of a Fc/MH–GOD electrode in 0.1M phosphate buffer/0.1M NaCl (pH=7.0) in the (a) absence and (b) presence of glucose. Scan rate: 5 mV s^{-1}.

film was deposited onto a screen-printed carbon paste electrode (SPE). Altogether 20 scans were performed but only 10 of the scans were shown in Fig. 4.

The anodic and cathodic currents of the Fc/MH film rise continuously with potential scans until a distinct redox couple of Fc occurred and reached steady state after 13–16 cycles. As the Fc/MH film was scanned further, increase in stability of the measured current was observed and this probably due to the reorientation of the Fc entrapped in the MH film. After equilibrium was established, the peak potentials E_{pa} and E_{pc} values remained essentially constant. The steady-state values for E_{pa} and E_{pc} of the cyclic voltammograms shown in Fig. 4 are 0.294 V and 0.156 V, respectively.

When the scan rate was altered from 1 to 10 mV s^{-1}, the cyclic voltammograms of the Fc/MH films demonstrated that both i_{pa} and i_{pc} shifted in response to the change in the scan rate (Fig. 5). The details of various current and potential parameters of the cyclic voltammograms depicted in Fig. 5 are tabulated in Table 1.

As the scan rate was increased, E_{pa} shifted to more positive values whilst E_{pc} became more negative. Hence, smaller values of ΔE_p at low scan rate were obtained when compared with fast scan (Table 1). The smallest ΔE_p (104 mV) attained was at scan rate of v=1 mV s^{-1} (i.e. $v^{1/2}$=0.03 $V^{1/2}$ $s^{-1/2}$). When the scan rate was increased by 10 times, almost a two-fold increase in ΔE_p was observed. The large ΔE_p value indicates that the charge-transfer process is quasi-reversible. The changes of the ΔE_p value with scan rates are attributed to the partial control of the charge-transfer step with respect to that of the diffusion step when the scan rates increase. In theory, when the charge-transfer is fast and completely reversible, there should be no change in ΔE_p with scan rates [16]. The observation here is consistent with ferrocene derivatives incorporated in a nafion film [17] or covalently immobilized in a cross-linked polyallylamine polymer [6]. However, the i_{pa}/i_{pc} is close to theoretical value of 1 for all the scan rates used (Table 1).

If the process of charge-transfer in the Fc/MH films is assumed to be reversible, the Randles–Sevik equation may provide information regarding the diffusion behaviour in the polymer films. The dependence of the anodic current on the scan rates for Fc/MH films can be examined by using a plot of the current i_{pa} against $v^{1/2}$ according to the Randles–Sevik equation. A strong linear relationship, i.e. i_{pa} (10^{-7} A cm^{-2})=160.9$v^{1/2}$ (V s^{-1})$^{1/2}$+2.56 (R^2=0.987) was observed for such a plot. This linearity indicates that electron transfer to and from the redox centers of the ferrocene compounds has occurred [9] and from the slope of the plot, the product $D^{1/2}C$ can be determined where D is the approximate estimation of the diffusion coefficient for the diffusion-like transport of charge through the polymer film and C is the concentration of the redox centers in the film. The $D^{1/2}C$ values estimated from the slopes are 2.37×10^{-10} (mol cm^{-2} $s^{-1/2}$). At a Fc concentration of approximately 5.652×10^{-5} mol cm^{-3}, a typical concentration used in this work, the D value is estimated to be 1.76×10^{-11} cm^2 s^{-1}. This value is much lower than that reported for Fc in an acrylamide–acrylic acid hydrogel film where the D value is in the order of 10^{-6} cm^2 s^{-1}, which is similar to the diffusion of free ferrocene in aqueous solution [10]. The low diffusion characteristic of Fc in the MH copolymer may be attributed to the low water absorption and the dense polymer network.

Table 1
Electrochemical data obtained from cyclic voltammograms of Fc/MH electrode at different scan rates

$v^{1/2}$	i_{pa} ($\times10^{-7}$ A)	i_{pc} ($\times10^{-7}$ A)	i_{pa}/i_{pc}	E_{pa}	E_{pc}	$E_{1/2}$	ΔE_p
0.10	9.608	−9.561	1.0	0.342	0.167	0.255	0.175
0.09	7.969	−8.502	0.9	0.336	0.170	0.253	0.166
0.07	6.762	−6.837	1.0	0.322	0.168	0.245	0.154
0.05	5.547	−5.989	0.9	0.303	0.171	0.237	0.132
0.03	3.778	−3.580	1.1	0.294	0.190	0.242	0.104

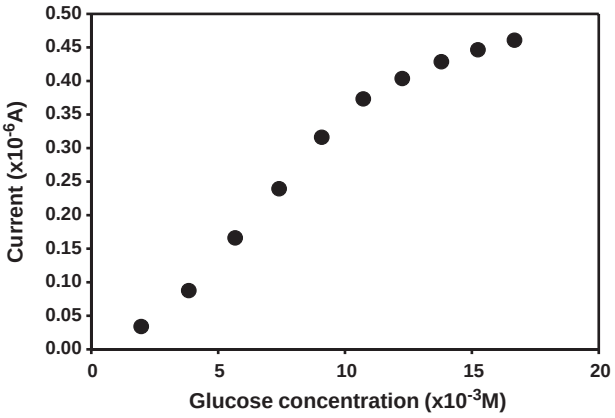

Fig. 7. The changes of current with glucose concentrations of a Fc/MH–GOD enzyme electrode in 0.1 M phosphate buffer/0.1 M NaCl (pH=7.0) at applied potential of 0.25 V versus SCE.

3.3. Electrochemical behavior of the MH copolymer films with both ferrocene and GOD entrapped simultaneously

The electrochemical behavior observed for the Fc/MH–GOD enzyme electrode is the same as described above. As compared to the non-enzyme electrode, the equilibrium at the Fc/MH–GOD electrode can be established more rapidly (10 scans compared to 13–16 scans). After the 10th scan, the anodic peak current remained essentially constant but small reduction in cathodic peak current was observed. Besides, a smaller ΔE (0.129 V) was observed for the enzyme electrode.

Fig. 6 depicts the current response of an Fc/MH–GOD enzyme electrode in the absence and presence of glucose. The increase in the anodic current observed in the presence of glucose indicates that the catalytic reaction does enhance the oxidation current of the Fc/MH–GOD enzyme electrode similar to that reported by Saito and Watanabe [12]. The presence of glucose and GOD(FAD) leads to the formation of GOD(FADH2), which subsequently reduces ferrocenium ions to ferrocene. Thus, regenerating the ferrocene that can be oxidised at the applied potential and this causes an increase in the anodic current when glucose is present. The decrease of the reduction current of the ferrocenium ions was caused by the more rapid electron-transfer reaction from FADH2 (coenzyme) to the ferrocenium ions and the electron-hopping reaction between ferrocenium ions and ferrocene. Thus this allows the enzyme electrodes to be used in direct determination of glucose.

Table 2
The repeatability of five Fc/MH–GOD electrodes exposed to 4.9 mM of glucose solution

Electrode no.	Mean response ($\times 10^{-8}$ A) (n=5)	Standard deviation	Relative error (RSD) (%)
1	11.76	1.60	13.65
2	12.06	0.62	5.17
3	11.95	1.20	10.00
4	12.28	0.37	3.01
5	11.52	0.39	3.35

Potential used=0.25 V versus SCE.

Table 3
The reproducibility of five Fc/MH–GOD electrodes exposed to 4.9 mM of glucose solution for five times

Batch of analysis (n=5)	Mean response ($\times 10^{-8}$ A)	Standard deviation	Relative error (RSD) (%)
1	12.68	0.75	5.86
2	11.97	0.46	3.87
3	11.33	0.55	4.89
4	12.05	0.55	4.60
5	11.35	1.32	11.58

Potential used=0.25 V versus SCE.

For the construction of a glucose biosensor, the applied potential was chosen at 0.25 V, a value where the ferrocene–ferrocenium redox couple yielded a sharp current change. The current response of an Fc/MH–GOD electrode to changes in glucose concentrations at applied potential 0.25 V versus SCE is shown in Fig. 7. A good linear relationship between the glucose concentrations and the measured current was obtained in the range of 2–11 mM glucose for the Fc/MH–GOD electrode.

3.4. Analytical performance of the glucose biosensor based on MH copolymer films

The repeatability and reproducibility of the Fc/MH–GOD electrodes are shown in Tables 2 and 3 respectively. The repeatability of the electrodes was established by testing a single electrode for five times in 4.9 mM glucose solution and this was performed for five different electrodes. The relative standard deviation (RSD) obtained is 3.0–13.6%. For the electrode reproducibility, a batch of five different electrodes was tested each time until five test batches were carried out. This yielded a RSD values of 3.8–11.6%. The low values of RSD shows that glucose biosensors based on photocurable MH film can be used to determine glucose with satisfactory precision.

Apart from good reproducibility and repeatability, a study of Fc/MH–GOD electrodes under dry storage at 4 °C for a period of 2 weeks demonstrated that the Fc/MH–GOD enzyme electrodes remained stable and exhibited a 94.2±3.6% of the original response of 4.9 mM glucose even after 2 weeks' period. This compares favorably with a similar glucose biosensor using GOD–Fc–Nafion film but the Nafion film electrode observed a 20% drop in response in the first 8 h of operation [17]. Such an initial drop of response was not observed in the present Fc/MH–GOD electrodes.

4. Conclusions

A simple and single-step method for the fabrication of a ferrocene-mediated glucose biosensor was demonstrated. With the immobilization of ferrocene and GOD simulta-

neously in a HEMA–MMA based copolymer by a photo-curing procedure, amperometric biosensor for glucose measurements can be performed at low potential, i.e. +0.25 V (vs. SCE). The hydrophobic nature of the copolymer film has prevented the rapid leaching of both ferrocene and GOD and this has contributed to the good analytical performance of the biosensor. The glucose biosensor exhibited satisfactory long-term stability and only <10% drop in current response was observed after 14-day storage. Both the RSD values for the reproducibility and repeatability were below 10%. This polymer matrix may be useful for the incorporation of other oxidase enzymes and ferrocene derivatives for the construction of various types of biosensors for application in bioanalysis.

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