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Tailored monoclonal antibody as recognition probe of immunosensor for ultrasensitive detection of silk fibroin and use in the study of archaeological samples

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ABSTRACT

The ultrasensitive detection of fibroin in unearthed silk relics has great significance for investigating the origin and transmission of silk. In this study, an anti-fibroin monoclonal antibody was successfully prepared through animal immunization. Next, a label-free electrochemical immunosensor was fabricated using layer-by-layer self-assembly technology, and an indirect enzyme-linked immunosorbent assay (ELISA) was proposed. The two methods exhibited excellent sensitivity and specificity in the detection of silk fibroin, while the immunosensor showed a wider quantitative detection range (0.1–100 ng mL^{-1}) and a lower detection limit (0.051 ng mL^{-1}) than ELISA (10–100 ng mL^{-1} and 8.71 ng mL^{-1}). Furthermore, the performance of the immunosensor was superior in archaeological sample detection. Taking advantage of the well-prepared monoclonal antibody, the two proposed immunological assays demonstrate tremendous potential for the ultrasensitive detection of silk fibroin, which can make great contributions to exploring the origin and transmission routes of ancient silks.

1. Introduction

Silk is a symbol of Chinese culture due to its time-honored use throughout history. China is generally considered the birthplace of silk, and recently, an increasing number of silk relics have been unearthed (You et al., 2017a). As precious material remains, silk relics appear throughout Chinese history for the past thousands of years and bear witness to social vicissitudes. Since the Western Han Dynasty, ancient trading routes have been opened up, which are collectively called the Silk Road (Glausiusz, 2009). Silks were exported to many countries in Europe, Africa and Asia through overland and cross-ocean trade. The Silk Road built relationships among numerous ancient regimes, and it immensely boosted the development of economy and culture (Li et al., 2017; You et al., 2017b). Therefore, studying silk relics has great significance for understanding the rise and fall of ancient civilizations in Eurasia (Lu et al., 2016; Toniolo et al., 2012).

Silk is composed of fibroin and sericin. In general, a degumming technology is applied to remove the sericin before weaving; thus, the main ingredient of silk relics is fibroin (Sehnal and Zurovec, 2004). However, like the majority of biomacromolecules, silk fibroin is easily damaged by temperature, humidity, light, acids, bases and microorganisms (Zhang et al., 2011). Thus, unearthed silk relics usually have undergone severe degradation and deterioration, and some of them even remain only as silk traces in clay (Degano et al., 2011; Garside and Wyeth, 2007). Because of the poor condition of preservation, it is extremely difficult for scholars to distinguish silk relics from other funerary objects or to detect the silk micro traces in soil. Hence, there is an imperative demand for the reliable and accurate detection of ancient silks.

During recent years, a variety of immunological detection methods have been proposed by our group to identify ancient silks, including enzyme-linked immunosorbent assay (ELISA) (Liu et al., 2015b), immunofluorescence microscopy (IFM) (Liu et al., 2015b) and immunochromatographic strips (ICS) (Liu et al., 2015a; You et al., 2017b). Among them, ELISA is the most widely used technique because of its high sensitivity and specificity, simple operation, and low cost (Arslanoglu et al., 2011; Clark and Adams, 1977; Heussner et al., 2014; Listed, 1976; Weimin et al., 2006). However, a great proportion of

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ancient silks have been degraded to peptide fragments or even amino acids, causing extremely low content of silk fibroin remained (sometimes lower than the detection limits of above-mentioned immunoassays). On the other hand, considering the complicated preservation environment, there would be various impurities in cultural heritage, resulting in a high background. Therefore, it may show false negative/positive results during the detection of ancient silks using conventional immunological techniques and a novel ultrasensitive method possessing lower background is urgently needed.

It is noteworthy that recently electrochemical immunosensor, a late-model immunodetection tool, has been rapidly developed (Gautham Kumar and Mitra, 2010; Han et al., 2018; Lai et al., 2017; Liu et al., 2013; Zhao et al., 2014). Electrochemical immunosensors can be divided into two branches, i.e., labeled immunosensor and label-free immunosensor, according to the presence or absence of labeling (enzyme or other electroactive materials) (El-Moghazy et al., 2018; Radi et al., 2009; Yan et al., 2018). Because of its simple fabrication process as well as its environmental stability, brief immobilization procedure and low cost, label-free immunosensors have been more widely employed than sandwich-type immunosensors (Gopalan et al., 2013; Joung et al., 2013; Li et al., 2016). Although nowadays ultrasensitive detection of unearthed relics is highly desirable, electrochemical immunosensor has rarely been established or reported in the archaeological field.

In this work, a label-free electrochemical immunosensor and an indirect ELISA were developed to detect silk fibroin and archaeological samples. First, a tailored monoclonal antibody was designed and prepared via immunizing mice with silk fibroin. Compared with polyclonal antibodies, a monoclonal antibody recognizes only one epitope, exhibiting higher specificity. Once the hybridoma cell was selected, the monoclonal antibody could be prepared thereafter through cell propagation and purification. Then, the specific anti-silk fibroin antibody was used as a recognition probe in ELISA and electrochemical immunosensor. Considering the scarcity of silk relics, modern silks were chosen as substitutes to obtain the linear range and limit of detection (LOD) of the two methods. After that, three valuable ancient samples from different historical periods were detected and analyzed. Both the methods showed tremendous potential for the ultrasensitive identification of silk fibroin, which can make great contributions to exploring the origin and spread of ancient silks in further research.

2. Materials and methods

2.1. Materials and instruments

The goat anti-mouse immunoglobulin G-HRP antibody, 8-azaguanine, Iscove's Modified Dulbecco's Medium (IMDM) and polyethylene glycol (PEG) 1500 were purchased from Hangzhou Hua'an Biotechnology Co., Ltd. N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), 3-mercaptopropanoic acid (3-MPA), 2-morpholineethanesulfonic acid (MES), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 20 nm alumina slurry were obtained from Sigma Aldrich. Modern silk and ancient silk samples were supplied by China National Silk Museum. All other chemicals were analytically pure. A TPM Ultrapure system was applied to purify water used in all experimental processes.

Phosphate-buffered solution (PBS, pH 7.4) was prepared with KCl, Na_2HPO_4, NaCl, and KH_2PO_4. A 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl was prepared by PBS (pH 7.4) and selected as the electrolyte for electrochemical measurements because of the excellent reversibility of $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$.

All the electrochemical measurements were performed using a CHI 660E electrochemical workstation through a conventional three-electrode system, including a gold electrode, a platinum wire electrode and a saturated calomel electrode (SCE), which were used as the working electrode, auxiliary electrode and reference electrode, respectively (Chenhua Instrument Shanghai Co., Ltd, China).

2.2. Preparation of pure silk fibroin

Ten grams of raw silk and 2.5 g of Na_2CO_3 were added to 500 mL of deionized water, and the mixture was boiled and stirred for half an hour. The remaining silk fibroin was washed three times before being degummed once again. The silk fibroin obtained after treatment was washed and dried in a constant temperature oven. One gram of degummed silk fibroin was sheared and mixed with 21.3 g calcium chloride, 27.6 mL deionized water and 22.4 mL ethanol. Then, the mixture was boiled for 60 min followed by filtering. The obtained salt solution was moved to a cellulose membrane with a molar weight cut-off of 8000 D. After 3 days of dialysis, the silk fibroin solution was lyophilized and ground into powders.

2.3. Preparation of the monoclonal antibody

All the immunoassays are based on an antigen–antibody immunoreaction, which indicates that the preparation of specific antibodies possesses important significance. In recent years, a series of polyclonal antibodies have been prepared successfully by our group (Liu et al., 2015b; You et al., 2017b).

In the present work, an anti-silk fibroin monoclonal antibody was tailored through the following steps. Firstly, the antigen emulsion was obtained through mixing 1 mg of silk fibroin powder, 1 mL of saline and the same amount of Freund's complete adjuvant. Then, one hundred microliters of emulsion was injected into each rat using the abdominal multipoint injection method. The primary immunization continued for 2 weeks followed by the booster immunization. In the booster immune process, Freund's incomplete adjuvant was used, while the concentration of antigen emulsion was adjusted to 0.5 mg mL^{-1}. Each booster immune phase lasted for 2 weeks. Next, the antibody titer in rat serum was tested by indirect ELISA after three rounds of enhanced treatment. If the antibody titer was sufficient, the spleen of the rat was removed to a super clean bench. If the titer was too low, another enhanced immunization was performed until an adequate result was obtained.

The splenocytes were extruded from the spleen to fuse with myeloma cells. One week before cell fusion, the activity of SP2/0 myeloma cells was enhanced by cultivation in 8-azaguanine. Next, the spleen cells were carefully blown out to a dish on a super clean bench. The well-cultivated myeloma cells were resuspended in medium before mixing with spleen cells in a 1:10 ratio. After that, 1 mL of PEG (1500) was gently dropped into the bottom of a centrifuge tube. Then, 100 μL of fused hybridoma cell suspension was added to each well of a 96-well plate and cultured for 7 days. An indirect ELISA was used to select positive hybridoma cells, and the chosen cells were then propagated. Finally, the cell supernatant was collected and purified using a Protein A column, and the monoclonal antibody (1.36 mg mL^{-1}) was stored at − 20 °C.

All animal experiments were performed according to the national standard GB 14925-2001 and the guidelines issued by the Ethical Committee of Zhejiang Sci-Tec University.

2.4. Indirect ELISA for detection of silk fibroin

The silk fibroin powders were first diluted with a carbonate buffer (pH 9.6) solution. Next, an indirect ELISA was proposed as in our previous work (Liu et al., 2016; Wang et al. 2018a, 2018b; Zhou et al., 2014) to obtain the linear range and limit of detection (LOD). Several various proteins (100 ng mL^{-1} in carbonate buffer), i.e., human serum protein (HSA), bovine serum albumin (BSA), ovalbumin (OVA), bovine collagen and pig collagen, were chosen as possible interference antigens in the specificity test.

2.5. Fabrication of label-free electrochemical immunosensor

The label-free electrochemical immunosensor was fabricated through a layer-by-layer self-assembly method. A gold electrode (AuE, φ = 3 mm) was polished with 20 nm Al_2O_3 suspension grout followed by ultrasonic cleaning for 5 min with ethanol and deionized water separately. The AuE was then immersed in 0.5 M H_2SO_4 and activated using cyclic voltammetry (CV) technology. After 40 cycles of scanning from −0.4 V to 1.6 V at a scan rate of 0.2 V/s, the AuE was washed thoroughly, followed by the modification process.

First, the AuE was incubated in 0.25 M 3-MPA for 12 h to form a 3-MPA monomolecular layer on the surface of AuE via strong Au–S chemical bonds. After that, the electrode was washed, followed by incubation in 0.05 M/0.03 M EDC/NHS solution containing 0.1 M MES for 5 h; hence, the carboxyl end group of 3-MPA was activated to form NHS esters. Next, 10 μL of the monoclonal antibody HAPK0111(1.36 mg mL^{-1}) was dropped onto the electrode surface, and the AuE was incubated at 37 °C for 60 min. During the above process, the amino group of the antibodies connected with the NHS ester through amido bonds. Afterwards, the electrochemical immunosensor was washed thoroughly with PBS (pH 7.4) three times, and the AuE was blocked with BSA (0.2% in PBS) for 30 min to lower the nonspecific binding level. The fabrication process of the immunosensor is illustrated in Scheme 1.

2.6. Electrochemical measurements for the detection of silk fibroin

Following the fabrication process, 20 μL of silk fibroin solution was placed on the surface of the AuE. After incubation at 37 °C for 1 h, the electrochemical immunosensor was washed thoroughly with PBS (pH 7.4) three times to remove silk fibroin that had not been captured by the monoclonal antibody.

For the determination of silk fibroin, a differential pulse voltammetry (DPV) method was proposed. The experimental parameters of DPV were set as a potential range of −0.2–0.6 V, an amplitude of 0.05 V, an increment potential of 0.004 V and a pulse width of 0.05 V. The linear range, sensitivity and specificity were determined by the DPV method.

2.7. Detection of archaeological samples using both methods

Three valuable archaeological samples from different historical periods were friendly provided by China National Silk Museum. The original appearance of these precious samples is demonstrated in Fig. 1. As shown in Fig. 1A, sample 1# was extracted from Nanhai No. 1 archaeological site, a Southern Song period (approximately 800 years ago) shipwreck found along the route of the ancient Maritime Silk Road. Sample 2# was excavated from the Chu period (approximately 2300 years ago) tomb in Anji County, Zhejiang Province (Fig. 1B). As illustrated in Fig. 1C, sample 3# was unearthed from the Wei-Jin period (approximately 2000 years ago) tomb in Yingpan, Xinjiang Uygur Autonomous Region.

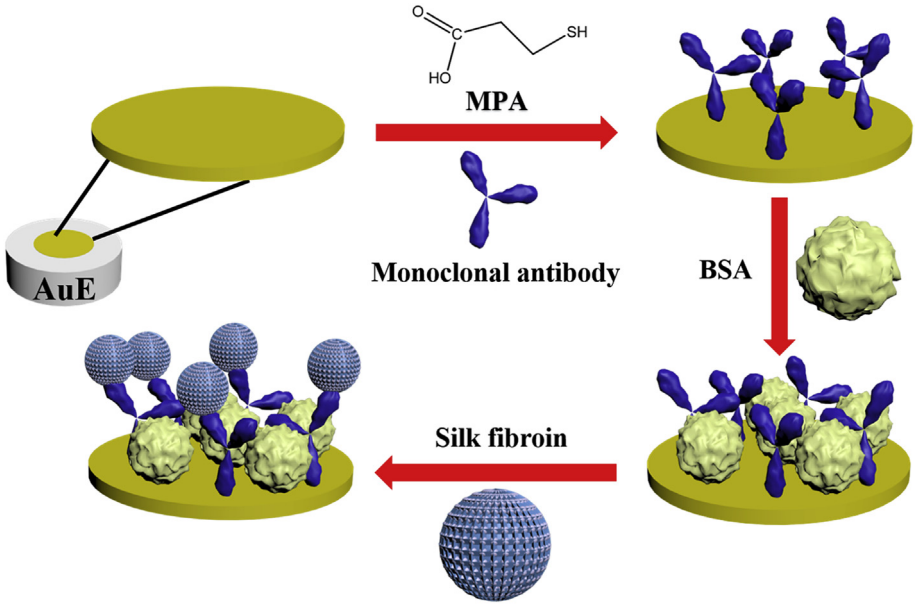
All ancient samples were treated with a protein extracting solution, which was used in the preparation of pure silk fibroin. Next, the archaeological samples were determined by the as-established indirect ELISA and label-free electrochemical immunosensor. Considering that sample 1# was preserved in the form of soil, its solution was diluted by a gradient and measured by both methods to obtain more accurate information.

3. Results and discussion

3.1. Indirect ELISA for detection of silk fibroin

An indirect ELISA was performed using the tailored monoclonal antibody. The dilution ratio of the primary antibody (1.36 mg mL^{-1}) was 1:1000, while that of the secondary antibody (2 mg mL^{-1}) was 1:5000. The optical density (OD) values of various silk fibroin solution concentrations (1 ng mL^{-1}, 10 ng mL^{-1}, 20 ng mL^{-1}, 30 ng mL^{-1}, 40 ng mL^{-1}, 50 ng mL^{-1}, 60 ng mL^{-1}, 70 ng mL^{-1}, 80 ng mL^{-1}, 90 ng mL^{-1}, 100 ng mL^{-1}, 500 ng mL^{-1} and 1000 ng mL^{-1}) were obtained at a wavelength of 450 nm, and the standard curve is shown in Fig. 2A. As the concentration of silk fibroin increased gradually from 1 to 1000 ng mL^{-1}, the variation trend of the OD values displayed an "S" type growth. Within the concentration range of 1–10 ng mL^{-1}, the OD value changed little, which indicates insufficient antigens. On the other hand, the rise in the OD value became slower again when the concentration exceeded 100 ng mL^{-1} because the antibodies had completely immunoreacted with the silk fibroin.

Remarkably, in the concentration range of 10–100 ng mL^{-1}, the OD



Scheme 1. A schematic illustration of the fabrication process for the label-free electrochemical immunosensor.

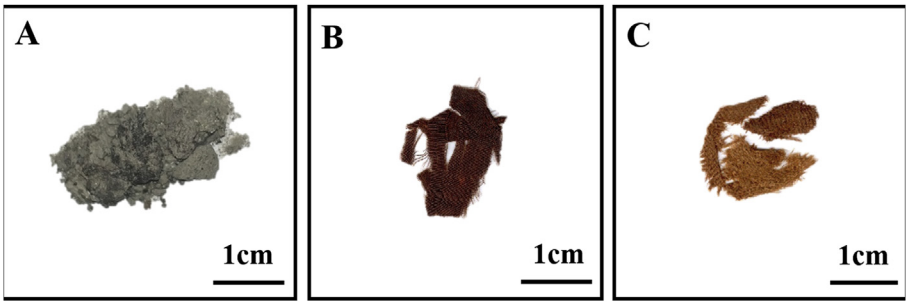


Fig. 1. Digital images of ancient samples. (A) Sample 1#, soil sample extracted from the Nanhai No. 1 archaeological site, a Southern Song period shipwreck. (B) Sample 2#, silk fragment from the Chu period tomb in Anji County, Zhejiang Province. (C) Sample 3#, silk fragment from the Wei-Jin period tomb in Yingpan, Xinjiang Uygur Autonomous Region.

value and the fibroin concentration exhibit an obvious linear relationship (Fig. 2B). The region was linearly fitted, and the regression equation was y = 0.0164 + 0.0471 C (R^2 = 0.9957) with an LOD (S/N = 3) of 8.71 ng mL^{-1}. In comparison, indirect ELISA based on a monoclonal antibody showed a lower detection limit than our previously reported method using polyclonal antibodies, which showed the higher sensitivity of monoclonal antibody as well as its potential in future studies (You et al., 2017a).

The specificity of the indirect ELISA was further investigated. Considering the diversity of funerary objects, especially the existence of proteinaceous materials, HSA, BSA, OVA, bovine collagen and pig collagen were selected as interference antigens. As shown in Fig. 2C, the OD values of all interfering samples were below the cut-off, while that of silk fibroin gave an extremely high value. This result revealed the favorable selectivity of ELISA.

All of the above results illustrate the excellent sensitivity and specificity of the established monoclonal antibody based-indirect ELISA, which ensure the extensive application prospects of this method in future immunological detection.

3.2. Fabrication of label-free electrochemical immunosensor

Prior to fabrication, it is extremely important to ensure the cleanliness of the immunosensor surface. Each AuE was polished and washed thoroughly followed by activation in 0.5 M H_2SO_4. The activation process was characterized by CV (Fig. S1), with the increase of scan cycles, the reduction peak rose up to a point, but after approximately 30 cycles, the scan became stable, indicating that the AuE had been completely cleaned and activated.

To further investigate the surface cleanliness of the AuE, CV was next used within the scan range of −0.3–0.6 V in a 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. Usually, by definition, the surface is considered inclusion-free when the voltage difference between the oxidation peak and reduction peak is less than 100 mV. As shown in Fig. S2, the voltage difference was 86.3 mV, indicating that the layer-by-layer self-assembly process can proceed.

The modification with 3-MPA and the monoclonal antibody was determined by DPV assay (Fig. S3). The peak current demonstrated a downtrend which indicated the gradual decrease in electron transfer efficiency between the electrolyte and the AuE surface. Hence, the DPV results indicated the immobilization of poor-conductivity materials on the electrode surface (3-MPA and antibody), which verified that the electrochemical immunosensor had been successfully fabricated.

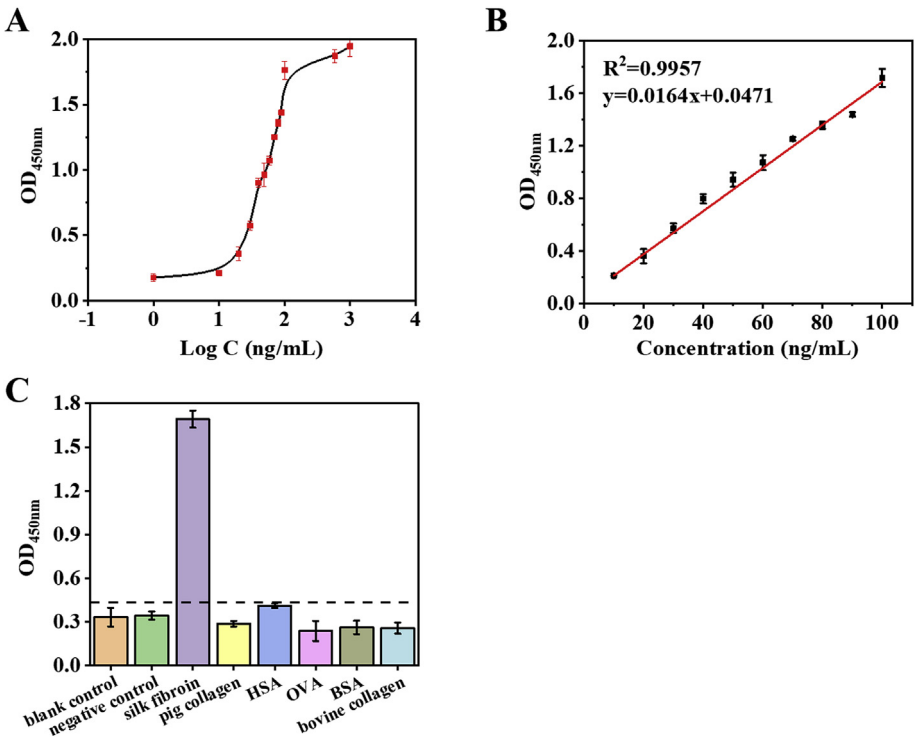


Fig. 2. (A) Standard curve of ELISA for different concentrations of silk fibroin. (B) Calibration curve of ELISA for different concentrations of silk fibroin. (C) ELISA results for possible interference antigens (100 ng mL^{-1}).

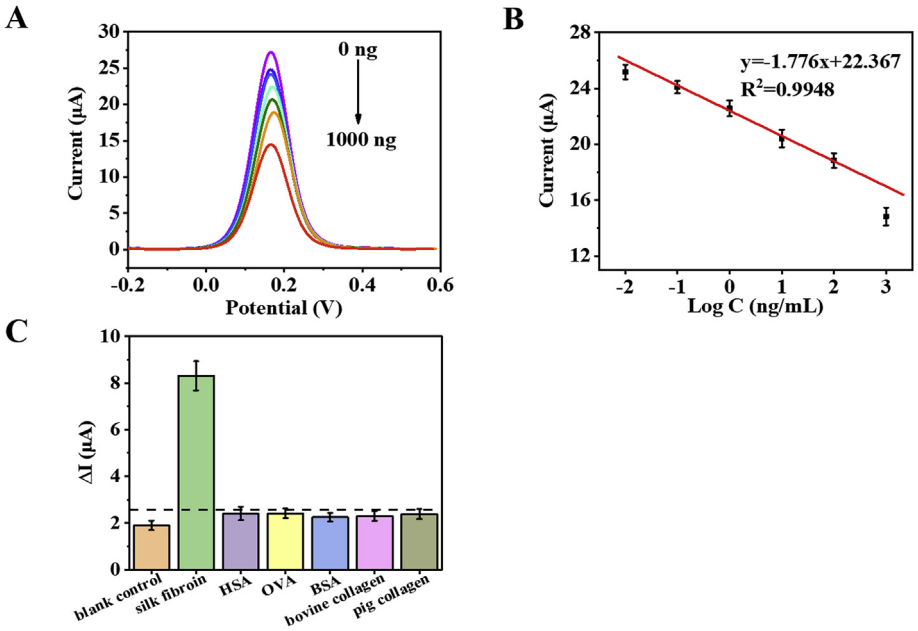


Fig. 3. (A) DPV curves of the immunosensor for different concentrations of silk fibroin. (B) Calibration curve of the immunosensor for different concentrations of silk fibroin. (C) The electrochemical immunosensor results for possible interference antigens (100 ng mL^{-1}).

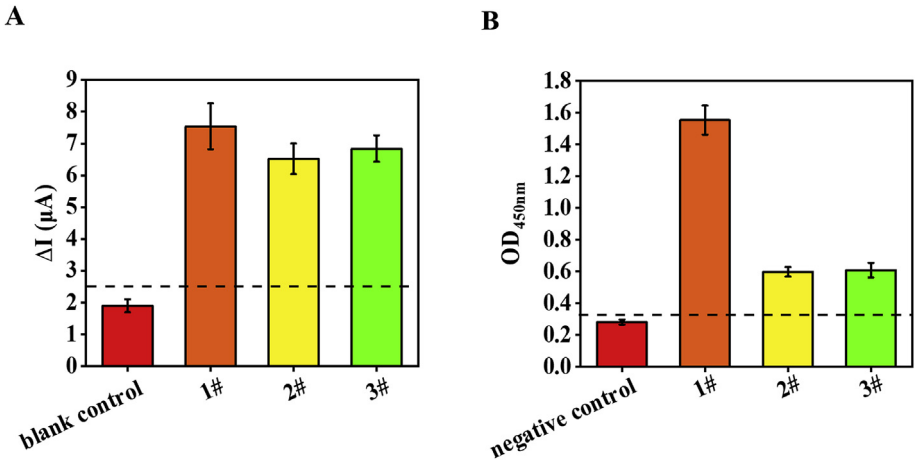


Fig. 4. (A) The electrochemical immunosensor results for ancient samples. (B) The ELISA results for ancient silk samples.

3.3. Electrochemical measurements for the detection of silk fibroin

To investigate the sensitivity of the immunosensor, silk fibroin solution was diluted by a gradient (0.01 ng mL^{-1}, 0.1 ng mL^{-1}, 1 ng mL^{-1}, 10 ng mL^{-1}, 100 ng mL^{-1}, and 1000 ng mL^{-1}, respectively) and used in DPV tests (Fig. 3A). As the concentration of the sample solution increased, the peak current showed a declining trend. This finding reveals that more silk fibroin had bound to the antibodies, and the contact between the redox probe and AuE was limited due to the reduction in electron transfer efficiency.

The peak current and logarithm concentration of silk fibroin were found to have a good linear relationship. As shown in Fig. 3B, the regression equation was y = - 1.776 Log C + 22.367 (R^2 = 0.9948), and the LOD (S/N = 3) was 0.051 ng mL^{-1}. Compared with indirect ELISA, the electrochemical immunosensor exhibited a wider quantitative detection range (0.1 ng mL^{-1} - 100 ng mL^{-1}) and a lower LOD. The excellent performance of this assay may be attributed to the following reasons. In the label-free electrochemical immunosensor, the antibodies were modified on the electrode surface through covalent binding to form a bio-sensitive layer, and the antigen were captured by the layer via specific immune recognition. Moreover, the DPV method directly monitors changes in electrical signals when the properties of immunosensor surface have been altered after the formation of immune complex, which enhances the sensitivity of the immunosensor.

Specificity is an essential evaluation standard for immunoassay capability. The same interference antigens as in ELISA (HSA, BSA, OVA, bovine collagen and pig collagen) were chosen to examine the selectivity of the immunosensor, and carbonate buffer solution (pH 9.6) was used as a blank control. The ΔI was defined as the difference in peak current before and after sample incubation, and the dotted line was the positive cut-off. As illustrated in Fig. 3C, only silk fibroin gave a positive result, which reveals the high selectivity of the proposed electrochemical immunosensor.

3.4. Detection of archaeological samples using electrochemical immunosensor and ELISA

Three valuable archaeological samples from different historical periods were treated with the protein extracting solution (the concentrations for samples 1#, 2# and 3# were 10 mg mL^{-1}, 100 ng mL^{-1}

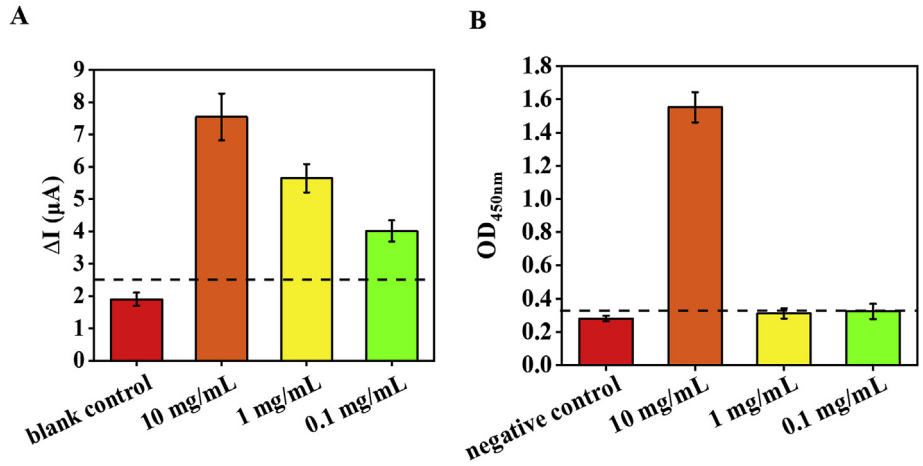


Fig. 5. The quantitative analysis of ancient sample 1# using (A) electrochemical immunosensor and (B) indirect ELISA.

and 100 ng mL^{-1}, respectively) and then detected by the label-free electrochemical immunosensor and indirect ELISA. As shown in Fig. 4A and Fig. 4B, all the samples gave positive results in both immunological methods, revealing that the two assays can effectively identify silk fibroin in these cultural relics.

Because sample 1# was preserved in the form of soil, its solution was further diluted by a gradient (10 mg mL^{-1}, 1 mg mL^{-1} and 0.1 mg mL^{-1}, respectively) and detected by both methods to obtain more accurate information. As shown in Fig. 5, the immunosensor specifically recognized silk fibroin in all concentrations of the sample solution (Fig. 5A), while the ELISA exhibited positive result only when the soil sample concentration was 10 mg mL^{-1} (Fig. 5B). In comparison, the DPV results decreased gradually during the diluting process of sample 1#, while the OD value sharply fell below the positive cut-off when the concentration of soil sample changed from 10 mg mL^{-1} to 1 mg mL^{-1}. The results proof our above conclusion that the immunosensor performs superior in detection range. On the other hand, calculated from the regression equation for the immunosensor, the silk fibroin content of sample 1# at each concentration was 0.344% (10 mg mL^{-1}), 0.298% (1 mg mL^{-1}) and 0.356% (0.1 mg mL^{-1}), respectively. The coefficient of variation (CV) was 9.20%, confirming that the electrochemical immunosensor has excellent performance in the quantitative detection for silk fibroin in ancient samples, while the ELISA is more applicable to qualitative detection.

3.5. Relationship between content of silk fibroin and different age of the ancient silks

The relationship between content of silk fibroin and different age of the ancient silk plays an important role in understanding the origin and transmission of silk. Thus, several representative archaeological samples from different historical periods which exhibit similar preserve situations were detected by the electrochemical immunosensor (the concentration for each sample was 100 ng mL^{-1}). As shown in Fig. S4, the silk fibroin content of sample 2#, 3#, 4#, 5# and 6# (The burial duration of samples decreases with the increasing of sample number) were 9.41%, 13.85%, 43.89%, 35.90% and 22.37%, respectively. The results indicated the burial duration is not the only reason for the decreasing of silk fibroin content in ancient silks. Analogous to most of natural protein materials, silk fibroin has a complex multilevel structure and is easily damaged by altering external circumstances. Therefore, other factors, i.e. the complex burial environment and the preservation conditions after excavation, can also influence the content of silk fibroin in archaeological samples. In general, the silk fibroin content of ancient silk is determined by the synergistic effect of multiple factors.

4. Conclusions

In the present work, a label-free electrochemical immunosensor and an indirect ELISA were established to determine silk fibroin and ancient silks. These two monoclonal antibody-based methods demonstrated excellent sensitivity and specificity. In comparison, the quantitative detection range and detection limit of ELISA were 10–100 ng mL^{-1} and 8.71 ng mL^{-1}, while the electrochemical immunosensor showed a wider quantitative detection range (0.1–100 ng mL^{-1}) and a much lower detection limit (0.051 ng mL^{-1}). In addition, the performance of the immunosensor was superior in archaeological sample detection, confirming its outstanding ability in both qualitative and quantitative tests, while the ELISA was more applicable to qualitative identification. Although so far, the electrochemical immunosensor was only used to determine ancient silks, considering its versatility, the immunosensor exhibits tremendous application prospect in the whole archaeological analysis field in future research. In summary, our proposed methods reveal enormous potential for the ultrasensitive detection of archaeological silk samples, which could make great contributions to investigating the origin and transmission routes of ancient silks, even to exploring the rise and fall of ancient civilizations along the Silk Road.

CRediT authorship contribution statement

Jin Li: Writing - original draft, Conceptualization, Methodology. **Yi Ouyang:** Investigation. **Linshuai Liu:** Investigation. **Chengyu Zhu:** Investigation. **Junjing Meng:** Conceptualization. **Hailing Zheng:** Resources, Data curation. **Yang Zhou:** Writing - review & editing, Resources. **Junmin Wan:** Writing - review & editing. **Zhiwen Hu:** Supervision. **Bing Wang:** Writing - review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111709.

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