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“Ready-to-Use” Hollow Nanofibers Membrane-Based Glucose Testing Strips

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ABSTRACT: A novel “ready-to-use” glucose test strip based on polyurethane hollow nanofibers membrane was fabricated through facile co-axial electrospinning. By involving glucose oxidase and horseradish peroxidase in the core-phase solution, and chromogenic agent either in core-solution (in case of 2,2’-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) was used) or in the shell-phase solution (in case of o-dianisidine was used) for co-axial electrospinning, in situ co-encapsulation of the two enzymes within the hollow nano-chamber and incorporation of chromogenic agents either inside the nano-chamber or in the shell of the hollow nanofibers was realized. Such unique “all-in-one” feature enabled the prepared hollow nanofibers membrane-based test strips to be applied either as colorimetric sensors in solution or as optical biosensor operated in “dip-and-read” mode. When was used as colorimetric biosensors in solution, the test strip with o-dianisidine as chromogenic agent shows excellent linear response range between 0.01 mM to 20 mM and a high apparent lumped activity recovery of 62.1% as compared to the reaction rate of the free bi-enzyme system. While the activity recovery of the test strip with ABTS as chromogenic agent is only 18.0%, and the test strip is found unstable due to spontaneous-oxidation of the ABTS. The o-dianisidine test strip was also applied as an optical biosensor, visible rufous color was quickly developed on the surface of the membrane upon dropping 10 μL of glucose sample, and an excellent correlation between differential diffusive reflectance of the test strip at 440 nm and glucose concentration was obtained in the range of 0.5-50 mM. The test strips also exhibited excellent long-term storage stability with half-life at 25 °C as
long as four months.

**KEYWORDS:** Glucose test strip; Hollow nanofiber; Co-axial electrospinning;

Optical biosensor
1. INTRODUCTION

Multifarious biological substances, such as enzyme, antibody, antigen, microorganism, cell, are used as biorecognition elements of biosensors for a wide range of applications from detecting hazardous chemicals in the atmosphere to measuring glucose in human body.\textsuperscript{1-5} Enzymes are the most selective and specific ones that enable more precise detection.\textsuperscript{6-8} One of the biggest obstacles in developing enzyme-based biosensor is loss of activity in environments slightly outside of the biocompatible regimes. With the development of nano technologies, applications of a variety of nanomaterials including nanoparticles\textsuperscript{9-12} and nanofibers\textsuperscript{13-16} in fabricating enzyme-based biosensors have been proven to afford the biosensors high sensitivity and much enhanced stability. In particular, nanofibrous membrane as enzyme carrier has shown more advantages over other nanostructured materials due to their high porosity, high surface-area-to-volume ratio, interconnectivity, and easy to prepare via facile electrospinning technique; therefore electrospun nanofibrous membranes have been widely used for fabricating biosensors for sensitive and quick detection.\textsuperscript{17-20}

Another challenge for biosensor fabrication is that many of detection systems need involvements of multienzymes and coenzymes, or chromogenic agents,\textsuperscript{21, 22} whose incorporation into the bio-recognition elements by traditional enzyme immobilizing process is very difficult because coenzymes and chromogenic agents could not be retained in most types of the carriers. This problem could not be well solved by using nanofibers yet. Currently, most of nanofiber enzyme-based biosensors were fabricated through adsorbing enzymes onto the surface of the pre-prepared nanofibers,\textsuperscript{13,15,16} which cannot adsorb coenzyme or chromogenic agents efficiently.\textsuperscript{23} Therefore,
developing “ready to use” type biosensor by efficiently immobilizing all of these required elements in proper carrier materials is promising but a challenging task.

In one of our previous works, a novel hollow nanofiber-based artificial cells were fabricated through a facile co-axial electrospinning by involving two enzymes and the shared coenzyme in the core-phase electrospinning solution, so that in situ co-immobilization of the multienzyme system was realized. This brand-new designing of multienzyme artificial cells have been proven to afford the multi-step biotransformation a high efficiency and much enhanced enzyme stability. What is more important, the unique advantage of in situ co-immobilization strategy provides us opportunities to fabricate “ready to use” type biosensors by cooperating all the required elements in the hollow nanofibers, so that the limitation of traditional nanofiber-based biosensor can be greatly overcome.

Huge market size and simplicity of sensing reaction chemistry make diabetes a model disease for developing new biosensing concepts. Accurate and rapid determination of glucose is a crucial issue in clinical diagnostics, food industry and biotechnology. Miscellaneous techniques such as electrochemical or non-enzymatic methods, and enzymatic methods, including enzyme-amperometric and enzyme-colorimetric methods, were applied in glucose determination. Enzymatic methods, especially colorimetric sensors, have been proven to be a powerful approach and attracted much attention, on account of its simplicity and practicality, that the testing result even can be easily read by the naked eyes without aid of any expensive and sophisticated instrument. For colorimetric sensors, there are many blood glucose
test kits and test strips available on the market. Even though good selectivity and high sensitivity were obtained with these enzymatic sensors, while inevitable drawbacks such as the chemical and thermal instabilities originating from the intrinsic nature of enzymes as well as the tedious fabrication procedures still limited their analytical applications. Up to now, design and preparation of glucose sensors meeting all these demands still remain a significant challenge.

In this paper, glucose oxidase (GOD), horseradish peroxidase (HRP) and chromogenic agents were in situ co-immobilized in hollow chamber or shell of PU hollow nanofibers by coaxial electrospinning technique to fabricate “ready-to-use” biosensor for quick and sensitive glucose detection. This bioactive nanofibers membrane can be used either as colorimetric biosensor to detect glucose in solution, or using as optical biosensor in “dip-and-read” mode.

2. EXPERIMENTAL SECTION

2.1. Reagents and Chemical. Polyurethane A85E (PU) pellets with bulk density about 700 kg/m$^3$ were supplied by Xiamen Jinyouju Chemical Agent Co. (Xiamen, China). Glucose oxidase (GOD, EC 1.8.1.4) and horseradish peroxidase (HRP, EC 1.11.1.7) were supplied by Beijing Apis Biotechnology Co. (Beijing, China). 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), o-dianisidine, glycerol, and N,N-Dimethylacetylamide (DMAc) were purchased from Sigma.

2.2. Instruments and Characterizations. Morphologies of hollow nanofibers membranes were characterized by scanning electronic microscope (SEM, JSM-6700F, JEOL, Japan) and transmission electron microscope (TEM, JEM-2100UHR, JEOL,
Japan). To view cross-section of the hollow nanofibers, the collected nanofibrous membranes were frozen in liquid nitrogen, and then cut perpendicularly to the axis of the fibers to expose their hollow structure.

### 2.3. Preparation of hollow nanofiber based testing strip for glucose measurement

The bi-enzyme system for glucose colorimetric detection includes GOD, HRP and chromogenic agents. In the present work, two commonly used chromogenic agents, ABTS and o-dianisidine, were selected. A co-axial electrospinning technology, which has been well described in one of our previous work, was used to prepared hollow nanofibers membrane based testing strips for glucose measurement. Schematic illustration of the set-up of co-axial electrospinning and the reaction mechanism of bi-enzyme system for glucose detection is shown in Scheme 1. Briefly, shell-phase solution was prepared by dissolving PU in DMAc to get final polymer concentration of 23 wt%, core-phase solution was prepared by well mixing 800 μL glycerol with 200 μL ultra-pure water solution containing 1.0 mg GOD, and 1.0 mg HRP. To realize simultaneous in-situ immobilization of the chromogenic agent in the electrospun hollow nanofibers, either 30 mg/mL water-soluble ABTS was involved in core-phase solution of co-axial electrospinning or 8 mg/mL water-insoluble o-dianisidine was involved in shell-phase solution.

The spinneret contains two coaxial stainless steel needles, whose inner and outer diameters of the core nozzles are 0.42 and 0.72 mm, and that of shell nozzle are 0.92 and 1.28 mm, respectively. The core solution and shell solution were fed at a constant rate of 0.07 mL/h and 0.5 mL/h, respectively, through two syringe pumps. A positive
voltage of 15–17 kV was applied and an aluminium sheet was used as the collector at a distance of 25 cm away from the nozzle tip. Electrospinning was carried out at 25±3°C, humidity 10-15%. After a stable electrospinning operation was obtained, nanofibrous membrane was collected every 30 min. The collected nanofibers membrane was placed overnight in a laminar hood at room temperature to evaporate residuals solvent, and then were sealed in a plastic bag and stored in dry place.

2.4. Colorimetric Detection of Glucose Using the Testing Strip in Solution. About 3 mg of as-spun hollow nanofibers membrane with co-immobilized GOD, HRP and chromogenic agent (ABTS or o-dianisidine) was immersed into 1 mL glucose solution of different concentration prepared in PBS buffer (pH 7.0, 50 mM). All assays were conducted at 37 °C, and the generated colour in solution after 5 min reaction as a function of glucose concentration was measured using a Unico 2800 spectrophotometer at wavelength of 420 nm (A420nm for ABTS as chromogenic agent) or 440 nm (A440nm for o-dianisidine as chromogenic agent). Control test was carried out by using blank hollow nanofibers containing no enzymes and chromogenic agent. When the measurement was carried out by using free bi-enzyme system, the concentrations of GOD, HRP and chromogenic agent were all adjusted to the same values as that of above hollow nanofiber-based detecting system.

2.5. Optical Detection of Glucose by Measuring the Color Intensity Changes on the Testing Strip. The as-spun hollow nanofibers membrane containing GOD, HRP, and o-dianisidine was cut into little round-shaped test strips with diameter of 10 mm.
Then 10 µL of glucose solutions of various concentrations were dropped onto the prepared test strips. A circular rufous spot was formed on the test strip in about 30 seconds. The color intensity of the rufous spot was determined by measuring its diffusive reflectance at 440 nm with X-rite 500 spectrodensitometer. The lower the diffusive reflectance at 440 nm, the stronger the intensity of the rufous spot on the test strips. For each sample, three replicates were conducted and the average result was reported. The change in reflectance was calculated using Eq. (1):

$$\Delta R = R_1 - R_0$$  \hspace{1cm} (1)

where $R_0$ and $R_1$ are the reflectance of the paper test strip before and after reaction for 30 seconds with glucose sample solution.

To illustrate the feasibility of the hollow nanofiber membrane-based glucose test strip, it was employed to measure glucose concentration in human blood serum. Five fresh human blood serum samples, provided by a local hospital, were determined by dropping 10 µL of the sample onto the prepared test strips; thereafter the color intensity of the developed rufous spot were measured and the glucose concentration was calculated from the standard curve. For comparison, glucose concentrations were also measured using commercial glucose test kit (Beijing Leadman Biochemistry Co. LTD, Beijing), which is used for clinical blood glucose determination in hospital.

2.6. Stability of the glucose testing strip. Hollow nanofibers membrane-based glucose testing strips were sealed in plastic Ziploc bags and stored at 4°C or 25°C. The stability of the testing strips was examined by monitoring changes in reaction rate as a function of storage time with the reaction rate of freshly prepared testing strip

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**Analyst**
defined as 100%.

3. RESULTS AND DISCUSSION

3.1. Fabrication of Hollow Nanofibers Membrane-Based Testing Strip. Due to its simplicity and practicality, enzyme-colorimetric methods for glucose analysis\textsuperscript{21,33,36} based on the GOD-HRP bi-enzyme reaction are most frequently employed in clinic. The measurement could be performed either in solution using standard test kit that GOD, HRP and chromogenic agent solutions are usually separately packed thus a pre-mixing operation are required during measurement, or in “dip-and-read” mode using test strip where the GOD, HRP and chromogenic agent are loaded on the surface of testing zone by simple adsorption or spraying. In the present work, an novel hollow-nanofibers membrane-based glucose testing strip was prepared using a facile co-axial electrospinning technique, with GOD, HRP and chromogenic agent were all in situ co-immobilized in the hollow nanofiber (Scheme 1).

Figure 1 shows the SEM and TEM images of the as-electrospun hollow nanofibers with the in situ immobilized GOD/HRP bi-enzyme system inside the hollow chamber of the fibers. Averagely, the fibers have an outer diameter ranging from 800 to 1000 nm, inner diameter about 500 nm, and the thickness of the shell was about 400 nm.

\textbf{<Figure 1>}

It has been suggested that identification of an appropriate chromogenic agent is one of the key factors in the development of test strips for glucose analysis.\textsuperscript{33} In the present work, water soluble ABTS and non-polar o-dianisidine were chosen as chromogenic agents, whose in situ co-immobilization were therefore realized by
involving the ABTS in core-phase solution for co-axial electrospinning or involving the o-dianisidine in shell-phase solution. Correspondingly, two types of hollow nanofibers membrane-based test strips were fabricated, the first one with GOD, HRP and ABTS were all inside the hollow chamber of fibers (referred as ABTS-test strip); while for the second one, two enzymes were inside the hollow chamber, but o-dianisidine was incorporated within the shell of the fibers (referred as o-dianisidine-test strip). This unique ‘all-in-one’ feature will enable the prepared test strips to be applied either in solution as colorimetric sensors or as optical biosensors that can be operated in the “dip-and-read” mode.

3.2. Colorimetric detection of glucose using the testing strip in solution. The hollow nanofibers membrane-based testing strips were firstly evaluated for colorimetric detection of glucose in solution. Figure 2 shows that when 3 mg as-prepared hollow nanofibers membrane-based o-dianisidine-test strip was immersed into glucose solution, the coupled reaction by GOD and HRP was initiated and a brown color was developed in the glucose solution due to conversion of the colorless reduced o-dianisidine to its corresponding oxidized products, whose maximal absorbance wavelength are at 440 nm. During 5 min measurement, the absorbance increased linearly with time and upon removal of the testing strip by simply picking up with a forceps, the reaction stopped, indicating that there was no leakage of immobilized enzymes. By measuring the absorbance of solution at 5 min over a range of glucose concentration, a standard curve for glucose assay was developed with linear range for glucose assays from 0.01 mM to 20 mM, and correlation coefficient $r$
of 0.990. The limit of detection was estimated to be 0.05 μM at a signal-to-noise ratio (S/N) of 3.

*Figure 2*

By comparing the reaction rates of o-dianisidine oxidization catalyzed by immobilized and free formations of the bi-enzyme system at glucose concentration of 20 mM, the apparent lumped activity recovery of the immobilized bi-enzyme system was calculated approximately to be 62.1%. The high activity of o-dianisidine-test strip system is attributed to the following two reasons: firstly, the GOD and HRP molecules retained inside the hollow chamber of nanofibers were remained in free formation, and the cascade reaction could even be enhanced due the spatial confining effects from nano-scaled hollow chamber; secondly, the chromogenic agent o-dianisidine was embedded in the wall of the hollow fiber, therefore its oxidized product could be quickly released to solution. The normal concentration of glucose in human serum ranges from 3.9 to 6.4 mM; therefore the testing strips can potentially be used as a colorimetric biosensor to detect the glucose concentration in sera.

When ABTS-test strip was used for glucose measurement, green color was developed in the glucose solution due to conversion of the colorless reduced ABTS to its corresponding oxidized products, whose maximal absorbance wavelength is at 420 nm (Figure S1). The standard curve for glucose assay was developed with linear range for glucose assays from 0.01 mM to 25 mM, correlation coefficient $r$ of 0.998, the limit of detection of 0.1 μM estimated at a signal-to-noise ratio (S/N) of 3. Compared with the high apparent lumped activity recovery of the o-dianisidine-test strip (62.1%),
the value for ABTS-test strip was calculated to be only 18.0%. This much lower apparent lumped activity recovery is mainly attributed to encapsulation of ABTS inside the chamber of the hollow fiber, the oxidized product of encapsulated ABTS has to diffuse across the fiber wall to color the solution. In contrast, in the o-dianisidine-test strip, the chromomeric agent was embedded in the wall of the hollow fiber thus enabling its oxidized product a quick release to solution. Furthermore, it was found that the ABTS-test strip is unstable. Figure S2 shows that ABTS-test strip turns to green gradually in air due to significant spontaneous-oxidation of the ABTS, while the color of nanofibrous membrane with o-dianisidine remains unchanged. Therefore, the following study was focused on the evaluation of o-dianisidine-test strip.

3.3. Evaluation of hollow nanofibers membrane-based glucose test strip as optical biosensor. Using the hollow nanofibers membrane-based test strip in solution as colorimetric biosensor has shown excellent sensitivity and broad detection range. The “all in one” feature of the test strip also makes it possible to be operated in “dip-and-read” mode as an optical biosensor. Figure 3 shows that in response to 10 µL glucose samples of different concentration, visual rufous color spot was quickly developed on the surface of the o-dianisidine-test strip and the intensity of color reached to maximum in about 30 s, thereafter kept stable for about 10 min. More importantly, the color intensity increased with the increasing of glucose concentration.

By measuring the diffusive reflectance of the rufous spot and plotting the differential reflectance (ΔR) against the logarithm of the glucose concentration,
calibration curve for glucose measurements could be developed. Result shown in Figure 3(B) indicated that within the tested glucose concentration range (0.1-50 mM), an excellent correlation between the $\Delta R$ and the glucose concentration was obtained, with a regression correlation coefficient of 0.999.

*Figure 3*

In illustrate the feasibility of the hollow nanofiber membrane based o-dianisidine-test strip in potential clinical diagnose, it was employed to measure glucose in human blood serum. Five fresh human blood samples were analyzed by both the commercial glucose test kit based on spectrophotometric method and the o-dianisidine-test strip. The results were summarized in Table 1. As can be seen, the glucose concentrations determined by hollow nanofibers membrane-based test strips were satisfactory and in good agreement with values measured using the spectrophotometric method. Thus, these results indicated the suitability of the hollow nanofibers membrane-based glucose test strips to practical applications.

*Table 1*

The o-dianisidine-test strip also showed excellent storage stability. As shown in Figure 4, when the test strips were stored at 4°C, no loss of activity was found for a 4-month period. Even at 25°C, about 50% of the original activity was retained after storage for four months. Because GOD and HRP were confined inside the nano-chamber of the hollow fibers, it was considered that the spatial confining effect provided the enzymes unique stabilizing mechanism. Such excellent storage stability makes the prepared test strips attractive for potential clinic applications.
4. CONCLUSIONS

In this paper, we proposed a simple strategy to fabricate a hollow nanofibers membrane-based test strips for glucose measurement. Using a co-axial electrospinning technique, both GOD and HRP were in situ encapsulated inside the chamber of the hollow fiber, at the same time chromogenic agent, o-dianisidine or ABTS, was also simultaneously immobilized in the hollow fiber. Such unique “all-in-one” feature of the hollow fibers membrane enabled the prepared test strips to be applied either in solution as colorimetric sensors or as optical biosensors operated in “dip-and-read” mode. Owing to the good stability and high activity, the strips with o-dianisidine as chromogenic agent showed excellent linear response range between 0.01 mM to 20 mM when was used as colorimetric biosensors for glucose in solution. As an optical biosensor, visible rufous color was quickly developed on the surface of the membrane by dropping 10 μL of glucose sample, and an excellent correlation between ΔR and the glucose concentration was obtained over a glucose concentration ranges from 0.1-50 mM. The test strips also exhibited excellent long-term storage stability. The broad detection range and excellent stability make the prepared test trips suitable for practical clinic application. Moreover, the facile preparation process of hollow nanofibers membrane and the advantages of simultaneous in situ co-immobilization approach for multiple substances are expected to promote the development of a great variety of biosensors that need mutlienzymes and coenzymes or chromogenic agents for measurement.
ACKNOWLEDGMENTS

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REFERENCES


Caption of Figures:

SYNOPSIS TOC BRIEFS: Fabrication and application of hollow nanofibers membrane based test strip for glucose detection

Scheme 1. Schematic illustrations of bi-enzyme reaction for glucose measurement and the setup for co-axial electrospinning to prepare hollow nanofibers membrane-based glucose testing strips. During co-axial electrospinning, GOD, HRP, and chromogenic agent (ABTS or o-dianisidine) were simultaneously in-situ immobilized in the hollow nanofibers membrane.

Table 1. Determination of glucose in blood serum samples using commercial test kit and the hollow nanofibers membrane-based test strip

Fig. 1. Characterization of hollow nanofibers prepared by co-axial electrospinning. (A, B) Cross-sectional SEM images, (C) TEM images.

Fig. 2. Calibration curves for glucose assay obtained by using (△) free and (▲) hollow nanofibers membrane based GOD/HRP bi-enzyme systems with o-dianisidine as chromogenic agents. Inset figure shows the data point for 0.01, 0.1, and 0.5 mM glucose solution measured with o-dianisidine-test strip. Concentrations of enzymes and chromogenic agents for the free system was adjusted to the same as that of the immobilized system. Picture in right hand shows the photographs of the detection of different concentration of glucose obtained by digital camera after removal of the
o-dianisidine-test strips.

**Fig. 3.** Optimal detection of glucose by measuring the color intensity changes on the testing strip with o-dianisidine as chromogenic agent. (A) Differential reflectance spectra of the membrane test strips upon reaction with glucose solutions of different concentrations for 30 s. (B) Correlation between the $\Delta R$ of test strips and the log of glucose concentration. The inset picture demonstrates the visual color change in response to the change in glucose concentration of the o-dianisidine-test strip. The diameter of the membrane is 1 cm.

**Fig. 4.** Stability of the hollow nanofibers membrane test strips with o-dianisidine as chromogenic agent.
Scheme 1

(1) \[ \text{O}_2 + \beta\text{-D-Glucose} + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \beta\text{-D-Glucose-δ-lactone} + \text{H}_2\text{O} \]

(2) \[ \text{H}_2\text{O}_2 + \text{ABTS}(\text{reduced}) \xrightarrow{\text{HRP}} \text{H}_2\text{O} + \text{ABTS}(\text{oxidized}) \]

OR \[ \text{H}_2\text{O}_2 + \text{o-Dianisidine}(\text{reduced}) \xrightarrow{\text{HRP}} \text{H}_2\text{O} + \text{o-Dianisidine}(\text{oxidized}) \]
Table 1. Determination of glucose in blood serum samples using commercial test kit and the hollow nanofibers membrane-based test strip with o-dianisidine as chromogenic agent

<table>
<thead>
<tr>
<th>Blood serum samples</th>
<th>Glucose concentration $^a$ (mM)</th>
<th>Glucose concentration $^b$ (mM)</th>
<th>Bias $^c$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$3.80 \pm 0.13$</td>
<td>$3.85 \pm 0.22$</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>$4.37 \pm 0.09$</td>
<td>$4.41 \pm 0.24$</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
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<td>$4.48 \pm 0.19$</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>$4.77 \pm 0.21$</td>
<td>$4.81 \pm 0.25$</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>$5.89 \pm 0.26$</td>
<td>$5.93 \pm 0.31$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ Determined by commercial glucose test kit.

$^b$ Determined by hollow nanofibers membrane-based test strip with o-dianisidine as chromogenic agent.

$^c$ Calculated by dividing the difference between values of glucose concentration determined from these two methods with the values determined by commercial glucose test kit.
Figure 2

![Graph showing concentration of glucose (mM) vs. absorbance (A440).

The graph includes two linear equations:

1. \( y = 0.04x + 0.025 \) with \( r^2 = 0.9807 \)
2. \( y = 0.066x + 5.25 \times 10^{-9} \) with \( r^2 = 0.9998 \)

The graph also shows a table with concentrations: 0 mM, 5 mM, 10 mM, 15 mM, 20 mM.

The legend indicates: o-Dianisidine as chromogenic agent.]}
Figure 3

![Graph showing reflectance vs. wavelength for different glucose concentrations.](image)

\[ \Delta R = -17.57 \log(C_{\text{glucose}}/\text{mM}) - 12.83 \]

\[ r^2 = 0.9992 \]
Figure 4

![Graph showing residual activity over time at 25°C and 4°C](image-url)