Determination of polyanion utilizing a promoted glucose oxidase enzymatic reaction by ε-poly-l-lysine

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Abstract
In this paper, a sensitive determination method for polyanion using a glucose oxidase (GOx) enzymatic reaction with ferricyanide ion is described. We previously reported that the GOx enzymatic reaction was significantly promoted by a cationic polymer of ε-poly-l-lysine (εPL), and the enzymatic reaction could be utilized for the determination of εPL. Generally, polycation stoichiometrically forms polyion complex with polyanion. Thus, it is expected that the promotion effect of εPL on the enzymatic reaction is interfered by polyanion, and the enzymatic reaction is also applicable to the determination of polyanion. Predictably, the promotion effect of εPL was stoichiometrically interfered by polyanions, such as polyvinyl sulfate and polyacrylate, and the interference effect allowed for the determination of the polyanions. The detection limit of polyanion was estimated to be ~ 0.3 μeq L⁻¹. As a preliminary application, the proposed method was applied to the determination of anionic polymer of heparin in a human plasma.

Keywords Polyanion · Glucose oxidase · ε-Poly-l-lysine · Heparin

Introduction
There are many important natural and synthetic polyelectrolytes, and the sensitive determination is sometimes imperative in their applications. For example, heparin is a highly negatively charged glycosaminoglycan and is widely used as a drug to prevent blood coagulation. The higher dose of heparin, however, often induces some adverse effects, and the measurement of heparin level is of crucial importance in the medical applications. The typical and well-established methods for monitoring the heparin level include the activated clotting time assay, the activated partial thromboplastin time assay and the anti-Xa assay. These assays, however, are indirect and amenable to clinical settings. Therefore, chemical assays are expected to offer some advantages over the traditional methods, and significant effort has been made to develop novel methods for measuring heparin concentration [1–4].

In our previous studies, we reported that a kind of polyand multivalent cations significantly promote a glucose oxidase (GOx) enzymatic reaction with ferricyanide ion ([Fe(CN)₆]³⁻) [5, 6], and the GOx enzymatic reaction allows for detection and determination of the cations [7–9]. This analytical method utilizes catalytic reaction (that is, the GOx enzymatic reaction), and therefore, exhibits a remarkable high sensitivity for spectrophotometric and colorimetric methods. In fact, the detection limit for the cations is 30–100 times lower than other spectrophotometric and colorimetric ones [7, 8, 10–13]. Interestingly, due to the high sensitivity, this method allows for the detection and/or determination of biosynthesized poly- and multivalent cations secreted in the culture broth for the producing microorganisms without any pretreatments [7, 8], implying that the method is applicable to a variety of biological samples.

ε-Poly-l-lysine (εPL) is a basic homopolymer of l-lysine residues with linkages between α-carboxyl groups and ε-amino groups, and exists as polycationic species in acidic and neutral condition [12, 14]. ε-Poly-l-lysine significantly promotes the GOx enzymatic reaction [5, 15], and can be determined by measuring the enzymatic reaction rate [7]. Basically, polycation stoichiometrically and quantitatively forms polyion complex with polyanion. Thus, polycationic εPL is expected to stoichiometrically form polyion complex with polyanions, and the promotion effect of εPL would be...
interfered by polyanion, and be utilized for the determination of polyanion with a high sensitivity.

In this paper, we show that the determination method for εPL using the GOx enzymatic reaction is applicable to the sensitive determination of polyanion. In addition, as a preliminary application, the proposed method was applied to the determination of anionic polymer of heparin in human plasma.

**Experimental**

**Chemicals**

Glucose oxidase from *Aspergillus niger* was purchased from Toyobo. The concentration of GOx was determined spectrophotometrically using the absorption coefficient of 18,240 M⁻¹ cm⁻¹ at 460 nm [16]. ε-Poly-L-lysine was kindly donated by JNC Corporation. Potassium polyvinyl sulfate (PVSK) titration solution and methyl glycol chitosan (MGCH) titration solution, poly-γ-glutamic acid (PGA, M.W. 1,500,000–2,500,000), polyacrylic acid (PAA, M.W. 25,000) were purchased from Wako Pure Chemical Industries. Poly(styrene sulfonic acid) sodium salt (PSSNa, M.W. 70,000) was purchased from Alfa Aesar. Catalase and alginic acid (ALG) were purchased from Nacalai Tesque. Heparin (HP, 231 unit mg⁻¹) was purchased from Sigma-Aldrich. Benzyldimethyltetradecylammonium chloride dihydrate (zephiramine) and 2-morpholinoethanesulfonic acid (MES) were purchased from Dojindo Laboratories. Human Plasma (pool, citric acid) was purchased from Cosmo Bio. They were used without further purification. Other chemicals were of reagent grade and were used as received.

**Colloidal titration**

In the evaluation of the proposed method, normality (eq L⁻¹) was used as concentration of polyion. To obtain equivalent weights for εPL and polyanions at pH 6.0, colloidal titrations were conducted. PVSK and MGCH were used as the anionic and cationic titrants for the determination of εPL and polyanions. The normality of PVSK solution was determined by colloidal titration using zephiramine [17]. Zephiramine which was dried at 70 °C under diminished pressure was used as a primary standard. The normality of MGCH solution was determined by colloidal titration of the standardized PVSK solution. The end point of colloidal titration for PVSK by zephiramine was determined by indicator method using toluidine blue [18], and the other colloidal titrations by turbidimetric method [19]. The turbidity of the solution was measured by transmittance at 420 nm with a spectrophotometer (JASCO V-630). The typical procedure of the colloidal titration describes below by taking the titration of MGCH by PVSK as an example.

An about 250 μeq L⁻¹ MGCH solution was prepared by diluting the commercially available MGCH solution (1/400 N) with 2 mM MES solution (pH 6.0). 2 mL of the MGCH solution was poured into an optical cell, and the transmittance at 420 nm was measured. A 50 or 20 μL of 500 μeq L⁻¹ PVSK solution was consecutively added into the MGCH solution set in the spectrophotometer with stirred. After the each addition of PVSK, the transmittance at 420 nm was recorded, and the titration curve was made.

**Evaluation of the enzymatic reaction rate**

The enzymatic reaction rate, $v_E$, in the presence of εPL and polyanion was obtained by following a time-dependent decrease in the absorbance at 420 nm, $A_{420}$, after the addition of GOx to the assay solution [5, 7]. The parameter $A_{420}$ corresponds to the absorption maximum of ferricyanide ion, and the $A_{420} \text{ vs. }$ time curve was recorded at 30 °C using the spectrophotometer (JASCO V-630) equipped with a Peltier thermostat. The component of assay solution was basically 200 mM glucose, 1.0 mM $K_3[Fe(CN)₆]$, 400 unit mL⁻¹ catalase and 10 mM MES (pH 6.0). The catalase was added to decompose $H_2O_2$ which is produced by the GOx reaction with $O_2$ [7]. εPL and polyanion were added into the assay solution at required concentrations before the addition of GOx. These concentrations were described in the corresponding part.

**Results and discussion**

**Colloidal titration**

Promotion effect of εPL is confirmed in the range of pH 5–8, and reaches a maximum at pH 6.0 [5]. Since the εPL effect is employed for the determination of polyanion in the proposed method, the method should be conducted at pH 6.0 to maximize the sensitivity. However, εPL and some polyanions (such as PAA) are not fully ionized at pH 6.0 [12, 14, 18, 19]. It is known that the polion complex formation between polycation and polyanion stoichiometrically takes place. Thus, in the evaluation of the proposed method, it is reasonable to use normality for expressing the concentration of polyanion. To obtain the normality, equivalent weights for polyanions were estimated by colloidal titration. The estimated values of the equivalent weight are summarized in Table 1.

**Determination of εPL and polyanion**

In the proposed determination method for polyanion, as the prerequisite, it is required that εPL can be determined...
by the GOx enzymatic reaction. Plot a in Fig. 1 shows
the dependence of $v_E$ on the concentration of εPL, $c_{PL}$. As reported previously [7], in the lower $c_{PL}$ range, εPL did not promote the GOx enzymatic reaction. However, in the range of $c_{PL}$ from 0.70 to 3.5 μeq L$^{-1}$, the $v_E$ linearly increased with $c_{PL}$, indicating that the $c_{PL}$ can be determined by measuring $v_E$. The regression line described in this figure was given as

$$v_E/\mu\text{M s}^{-1} = (1.40 \pm 0.03)(c_{PL}/\mu\text{eq L}^{-1}) + (-0.75 \pm 0.06)$$

with the mean square of errors (MSE) = 0.0035. Since εPL did not promote the GOx enzymatic reaction in the lower $c_{PL}$ range, the detection limit was defined as $3(MSE)^{1/2}/1.40$, and calculated to be 0.67 μeq L$^{-1}$. The detection limit was lower than previous one [7], likely due to the lower setting of the buffer concentration of MES.

If the polyion complex formation between εPL and polyanion takes place stoichiometrically, polyanion is also expected to be determined by measuring $v_E$. The linear dependence of $v_E$ was confirmed up to 3.5 μeq L$^{-1}$ of εPL. Here, for the determination of polyanion, 3.0 μeq L$^{-1}$ of εPL was added in the assay solution. Plot b in Fig. 1 shows the dependence of $v_E$ on concentration of polyanion (here, PVSK), $c_{PA}$, in the presence of 3.0 μeq L$^{-1}$ εPL. The $v_E$ linearly decreased with $c_{PA}$, indicating that the promotion effect of εPL was interfered by polyanion, and that the $c_{PA}$ can be also determined by measuring $v_E$. The equation of the regression line was given as

$$v_E/\mu\text{M s}^{-1} = (-1.12 \pm 0.03)(c_{PA}/\mu\text{eq L}^{-1}) + (3.56 \pm 0.06)$$

with MSE = 0.0072. If the detection limit was defined as $3(MSE)^{1/2}/1.12$, the value was determined to be 0.23 μeq L$^{-1}$. The extrapolated value of $c_{PA}$ to the $v_E = 0$ was 3.2 μeq L$^{-1}$ and closely agreed with the value of $c_{PL}$ for the assay solution (3.0 μeq L$^{-1}$). This agreement indicates that PVSK stoichiometrically form polyion complex with εPL.

The dependences of $v_E$ on $c_{PA}$ when using other polyanions are shown in Fig. 2. As in the case of PVSK, the $v_E$ linearly decreased with $c_{PA}$. The slopes, intercepts, and MSEs for the regression lines are summarized in Table 2. The regression lines for all tested polyanions were in agreement with each other. These results indicate again that the polyion complex formation between εPL and polyanion takes place stoichiometrically, and that the concentration of polyanion can be estimated by the proposed method.

### Determination of heparin in the human plasma

In previous section, we showed that heparin can be also determined by our proposed method. The detection limit

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**Table 1** Calculated and experimental values of equivalent weight at pH 6.0 for εPL, PVSK, HP, PAA, ALG, PGA and PSSNa

|          | Calculated value$^a$ | Experimental value |
|----------|----------------------|--------------------|
| εPL      | 129                  | 142                |
| PVSK     | 162                  | 169                |
| HP       | 200$^b$              | 204                |
| PAA      | 71                   | 106                |
| ALG      | 175                  | 213                |
| PGA      | 128                  | 137                |
| PSSNa    | 182                  | 289                |

$^a$The ionic groups in polions are assumed to be fully ionized

$^b$Calculated using an average molecular weight and charge reported by Ref. [24]
Table 2 Calculated values of the slope, intercept and MSE of the regression lines for the plots of $v_E$ vs. $c_{PA}$ in Fig. 2

|        | Slope        | Intercept     | MSE   |
|--------|--------------|---------------|-------|
| HP ($n=4$) | $-1.15 \pm 0.06$ | $3.64 \pm 0.09$ | 0.016 |
| PAA    | $-1.20 \pm 0.04$ | $3.60 \pm 0.07$ | 0.011 |
| ALG    | $-1.09 \pm 0.07$ | $3.5 \pm 0.1$   | 0.025 |
| PGA    | $-1.20 \pm 0.06$ | $3.64 \pm 0.09$ | 0.016 |
| PSSNa  | $-1.22 \pm 0.07$ | $3.6 \pm 0.1$   | 0.028 |

Fig. 3 The dependence of $v_E$ on the concentration of $c_{PL}$ (a) and HP, $c_{HP}$ (b) in the presence of 4% plasma. In the measurement of plot $b$, 7.5 μeq L$^{-1}$ εPL was added into the assay solution. The $c_{GOx}$ was 360 nM (16 unit mL$^{-1}$), and the vertical bars denote the standard deviation ($n=4$).

was calculated to be 0.33 μeq L$^{-1}$ from the data of Table 2. In heparin analysis, the concentration of heparin, $c_{HP}$, is frequently expressed by activity of heparin (unit mL$^{-1}$), and then the detection limit was calculated to be 0.016 unit mL$^{-1}$. The detection limit is sufficiently lower than medically relevant concentrations (cardiovascular surgery: 2–8 unit mL$^{-1}$, postoperative and long-term care: 1.7–10 unit mL$^{-1}$ [3]), and hence, as a preliminary application, we applied the proposed method to determination of heparin in blood plasma.

The blood plasma was not completely transparent and the components of blood plasma interfered with the promotion effect of εPL. Thus, the used amount of plasma in the determination of heparin needs to be reduced as much as possible. From some preliminary experiments, we decided to use 120 μL of blood plasma (that is, 4% of total volume of the assay solution) for construction of the calibration curve for heparin in plasma. Additionally, 1 mM EDTA was added into the assay solution in this experiment for suppressing the interference effect of Fe(II) ion through the Fenton reaction [7].

At first, the dependence of $v_E$ on $c_{PL}$ in the presence of 4% (v/v) blood plasma without heparin was investigated (plot a in Fig. 3). A linear increase in $v_E$ with $c_{PL}$ was obtained in the $c_{PL}$ range of 2.8–8.4 μeq L$^{-1}$ although the linearity became worse compared to that in the absence of plasma. The equation of the regression line was given as

$$v_E/\mu\text{M s}^{-1} = (0.85 \pm 0.08)(c_{PL}/\mu\text{eq L}^{-1}) + (-1.8 \pm 0.5)$$

with the MSE = 0.14. The values of slope and intercept were smaller than those obtained in the absence of blood plasma (Eq. 1) indicating that the promotion effect of εPL was interfered by the components of blood plasma.

In clinics, one of the commonly used methods for heparin monitoring is the activated clotting time assay. The measurable range is reported to be 2.3–4.2 unit mL$^{-1}$ [1]. The required $c_{PL}$ to cover the heparin concentration range in our method is calculated to be about 4 μeq L$^{-1}$ from the data in Tables 1 and 2, and the activity value of heparin. Here, for the determination of heparin in plasma, 7.5 μeq L$^{-1}$ εPL was added in the assay solution.

The dependence of $v_E$ on $c_{HP}$ in the presence of 4% plasma with 7.5 μeq L$^{-1}$ εPL is shown by plot $b$ in Fig. 3. As in the case of the absence of the plasma, a linear decrease in $v_E$ was confirmed in the $c_{HP}$ range of 0.84–5.0 μeq L$^{-1}$. The concentration range corresponds to the $c_{HP}$ range in the blood plasma from 1.0 to 6.0 unit mL$^{-1}$. The linearity was improved compared to the dependence of $v_E$ on $c_{PL}$ in the presence of the plasma, likely due to the relatively strong interaction between εPL and heparin. The regression line was given as

$$v_E/\mu\text{M s}^{-1} = (-0.87 \pm 0.03)(c_{HP}/\mu\text{eq L}^{-1}) + 5.05(\pm 0.09)$$

with MSE = 0.016, and the detection limit was calculated to be 0.44 μeq L$^{-1}$ (0.52 unit mL$^{-1}$ in the blood plasma). The detection limit was comparable to or somewhat higher than other reported methods [1–4, 20, 21]. However, the reported methods have been basically achieved by complicated molecular design of the detection probes and required cumbersome synthesis for their preparation [4, 22, 23]. Contrarily, our method can be carried out with commercially available reagents and simple procedure. In heparin analysis for biological media, other polyanions, such as dermatan sulfate and chondroitin sulfate, may exist as a potential competitor. Thus, the selectivity for heparin would be an important issue to be overcome for the practical applications. Nonetheless, taking into account the merits and sensitivity, our method may became a suitable candidate for determination of heparin in medical and biological samples.

Conclusions

In this study, we showed that the promotion effect of εPL on the GOx enzymatic reaction was interfered by polyanions, and the GOx enzymatic reaction was also utilized to the
determination of polyanion. The detection limit was estimated to be ~0.3 μeq L\(^{-1}\). As a preliminary application, the method was applied to the determination of heparin in human plasma. The linear calibration curve covered a medically relevant concentration range of heparin, and the performance of the proposed method was comparable to those of many reported ones. The proposed method can be carried out with commercially available reagents and simple procedure. Thus, the method may become useful for determination of polyanion in biological samples.

Data availability statement  All data generated or analyzed during this study are included in this published article.

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