**Biomaterials and Biomechanics in Bioengineering**, *Vol. 3, No. 1 (2016) 37-46*
DOI: http://dx.doi.org/10.12989/bme.2016.3.1.037 

# Enhanced stability of NADH/dehydrogenase mixture system by water-soluble phospholipid polymers

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*(Received November 19, 2015, Revised December 2, 2015, Accepted December 19, 2015)*

**Abstract.** To maintain activity in a coenzyme/enzyme mixture system, such as $\beta$-nicotinamide adenine dinucleotide (NADH)/dehydrogenase, the water-soluble 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers as an additive were synthesized and investigated for their stabilizing function. The inhibitor for the NADH/dehydrogenase reaction was spontaneously formed when the NADH was stored in the dehydrogenase solution. Therefore, we hypothesized that if the additive polymer could interact with an inhibitor without any adverse effect on the dehydrogenase, the activity in the NADH/dehydrogenase mixture could be maintained. We selected lactose dehydrogenase (LDH) as the enzyme, and the NADH was dissolved and incubated at 37°C in the LDH solution containing the polymers. The phospholipid polymers used in this study were poly(MPC) (PMPC), poly(MPC-*co*-3-trimethylammonium-2-hydroxypropyl methacrylate chloride) (PMQ) and poly[MPC-*co*-potassium 3-methacryloyloxypropyl sulfonate ($MSO_3$)] ($PMMSO_3$). The poly($MSO_3$) was used as a reference. For the PMQ and $PMSO_3$ aqueous solutions, the activity of the NADH/LDH mixture system decreased with incubation time as the same level or lower than that in the Tris buffered solution in the absence of the polymers. However, for the poly(MPC-*co*-$MSO_3$) ($PMMSO_3$) aqueous solution, the activity of the NADH/LDH mixed system was six times higher than that in the buffered solution even after a 3-days incubation. The LDH activity was 1.5-1.8 times higher in the presence of the $PMMSO_3$ compared with that in the $PMSO_3$ solution. The mixture of two polymers, poly(MPC) and poly($MSO_3$), did not produce any stabilization. Thus, both the MPC and $MSO_3$ units in the polymer chain had important and cooperative effects for stabilizing the NADH/LDH mixture.

**Keywords:** 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer; charged polymers; polymeric additive; NADH/LDH mixture system; stabilization

## 1. Introduction

Enzymatic analysis is being applied in various fields such as biological science, medical science, food science and environment science due to its excellent biological specific reaction (Bartolini *et al*. 2003, Marsh and Danielson 1991, Mogele *et al*. 1992, Monosil *et al*. 2012, Talalak *et al*. 2015). In many cases, the $\beta$-nicotinamide adenine dinucleotide (NADH) is used as a coenzyme in the dehydrogenase-catalyzed reaction. However, it is known that the NADH is an

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http://www.techno-press.org/?journal=bme&subpage=7 ISSN: 2465-9835 (Print), 2465-9959 (Online)

unstable reagent in some aqueous solutions (Yamauchi *et al*. 1981). It was readily oxidized by hydrogen ion and the inhibitors for the NADH/dehydrogenase reaction are formed during storage. Therefore, when enzyme dehydrogenase was stored with the NADH, the activity of the enzymatic system decreased due to the formation of the inhibitors. It is very important to stabilize both the enzyme and coenzyme for accurate measurement. The purpose of this study is to maintain the activity of the enzyme dehydrogenase, lactate dehydrogenase (LDH) for a long period in an aqueous medium even in the presence of the NADH. For this purpose, we attempted to trap the NADH inhibitor with water-soluble polymers based on a specific interaction between them. The requirements of the polymer are to have an electrical charge for entrapment of the spontaneously formed NADH inhibitor and to inhibit the conformational change in the LDH. In addition, the polymer should suppress the oxidation of NADH. Several charged polymers, such as polyethyleneimine (PEI) and poly (allyl vinyldimethylammonium) chloride are known to have positive effects on the enzyme stability (Andersson and Hatti-Kaul 1999). However, they could not apply this to the mixture of the NADH/dehydrogenase system. To obtain a very suitable polymer, we designed a new water-soluble polymer having hydrophilic phospholipid polar groups such as the 2-methacryloyloxyethyl phosphorylcholine (MPC) units (Ishihara *et al*. 1990, Ishihara *et al*. 1999, Ishihara and Fukazawa 2014, Iwasaki and Ishihara 2012, Ueda *et. al*. 1992). The MPC polymers are well known to maintain and stabilize the enzyme structure (Lin *et al*. 2013, Miyamoto *et al*. 2004, Sakaki *et al*. 1999, Sakaki *et al*. 2000). In this communication, the property and performance of the MPC polymers with a charged group for maintaining the enzyme activity by suppressing the formation of the inhibitors are described.

## 2. Experimental methods

### *2.1 Materials*

MPC was purchased from NOF Co., Ltd. (Tokyo, Japan), which was synthesized by a previously reported method. Potassium 3-methacryloyloxypropyl sulfonate ($MSO_3$) and PEI (50 wt% aqueous solution, weight-averaged molecular weight (Mw)=5.0-6.0×$10^4$) were purchased from Tokyo Kasei (Tokyo, Japan). Polyallylamine (PAA) (20 wt% aqueous solution, Mw=1.5×$10^4$) was purchased from Nitobo (Tokyo, Japan). LDH (porcine muscle, EC1.1.1.27, suspension in 2.1 M $(NH_4)_2SO_4$), pyruvic acid (sodium salt), and the NADH reduced form were purchased from the Sigma-Aldrich Chemical Co. (USA)

### *2.2 Synthesis of water-soluble polymers*

Poly(MPC) (PMPC) and poly(MPC-*co*-3-trimethylammonium-2-hydroxypropyl methacrylate chloride) (PMQ) were synthesized by a conventional radical polymerization of the corresponding monomers (Ishihara *et al*. 1990, Ueda *et al*. 1992) and they were provided by NOF. Poly($MSO_3$) ($PMSO_3$) and poly(MPC-*co*-$MSO_3$) ($PMMSO_3$) were synthesized by a conventional radical polymerization technique in degassed water using 4, 4'-azobis(4-cyanovaleric acid) as the initiator. The polymerization was carried out at 60 °C for a specific time. The reaction mixture was poured into a large amount of an acetone and methanol mixture (100/30 by volume) to purify the formed polymer by precipitation. The chemical structure of the polymers was confirmed by $^1$H-NMR ($\alpha$-300, JEOL, Tokyo, Japan) in $D_2O$ and FT-IR (FT-IR-615, Jasco, Tokyo, Japan). The Mw and

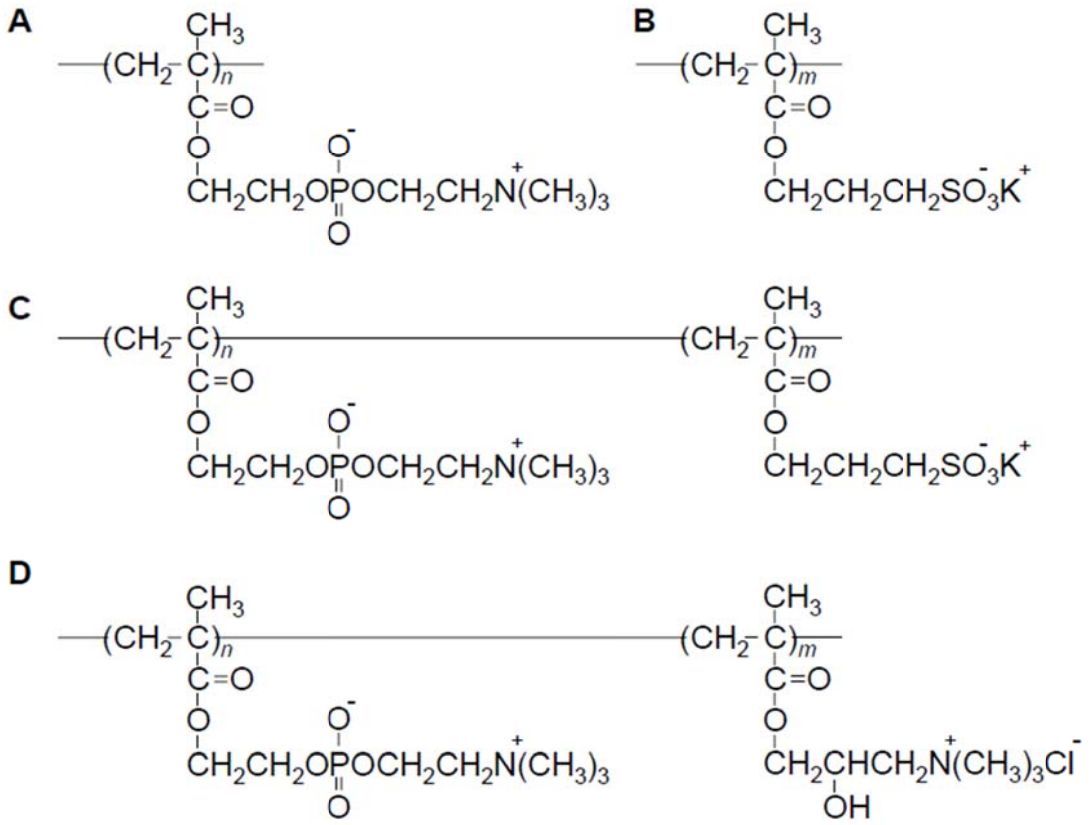


Fig. 1 Chemical structure of water-soluble polymers used in this study. (A) PMPC, (B) $PMSO_3$, (C) $PMMSO_3$, and (D) PMQ

number-averaged molecular weight (Mn) of the polymers were determined by gel permeation chromatography (GPC: Jasco, Tokyo, Japan) with poly(ethylene oxide) standards. The 300 mM phosphate buffer (pH 7.5) was used as the eluent for the GPC measurement at a flow rate of 0.5 mL/min. In Fig. 1, the chemical structures of the water-soluble polymers used in this study are shown.

### *2.3 Measurement of the NADH stability*

The NADH stability was investigated using the change in the UV adsorption spectra. The NADH was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing a given concentration of the polymers (0.10-2.0 wt% PMPC, PMQ, $PMSO_3$, $PMMSO_3$, and 0-0.10 wt% PAA, PEI). The final concentration of NADH was 0.25 mM. These solution were stored at 37 °C for 7 or 8 days and the adsorption spectra were obtained in the UV region from 250 nm to 450 nm.

### *2.4 Capillary electrophoresis*

The formation of NADH inhibitor was examined by measurement of the capillary

electrophoresis. (CAPI-3300, Otsuka Electronics, Osaka, Japan). The capillaries a 75 $\mu$m-internal diameter and 50 cm-length were used and maintained at 25°C. The voltage was applied at 20 kV, and a 50 mM Tris-HCl buffer (pH 7.5) was used as the medium for electrophoresis. The NADH solution (16.5 mM) was electrophored for 20 min and detected using a UV detector at 260, 290, and 340 nm.

### *2.5 Measurement of LDH activity*

Stock polymer solutions of PMPC, PMQ, $PMSO_3$, and $PMMSO_3$ were prepared using a 50 mM Tris-HCl buffered solution (pH7.5) with and without NADH. Also, a mixture of PMPC and $PMSO_3$ (PMPC/$PMSO_3$) was prepared with the same buffered solution. The composition of PMPC/$PMSO_3$ was 50/50 by mole ratio. The LDH solution was mixed with the polymer solution to obtain a solution with 1.1 mg / mL enzyme, 1.0 wt% of the polymer and 6.6 mM NADH. To the reaction mixture composed of 2.8 mL of 100 mM Tris-HCl buffer (pH 7.5) was added 0.10 mL of the enzyme solution. The initial rate of consumption of the NADH at 25°C was spectrophotometrically monitored at 340 nm. One unit of LDH activity was defined as the amount of enzyme causing the oxidation of 1 mmol of NADH per minute under the specified condition.

## 3. Results and discussion

### *3.1 Synthesis of water-soluble polymers*

Table 1 shows the synthetic results of the anionic polymers, $PMSO_3$ and $PMMSO_3$. The polymerization was homogeneous in the aqueous solution. The chemical structure of $PMMSO_3$ and $PMSO_3$ was confirmed by spectroscopic methods. The mole fraction unit of the MPC in the $PMMSO_3$ was 0.45. Every prepared polymer was water soluble at room temperature.

### *3.2 Stabilization of NADH*

The stability of NADH depended on the pH, temperature and buffer type of the buffered solution as a medium (Yamauchi *et al*. 1981, Hentall *et al*. 2001). Fig. 2 is the schematic representation of the enzymatic reaction of LDH with NADH and its side reactions. One of the side reactions forms the NADH inhibitor. To suppress this reaction, we added various polymers to the NADH solution and investigated the effect of the polymers on the stability of the NADH. In Fig. 3, the UV spectra of NADH in the solution are shown. The absorption spectra of NADH had two peaks at 260 nm and 340 nm attributed to the adenine group and the pyridine ring, respectively (Rover Jr. *et al*. 1998). After incubation, we observed both an increase in the absorbance at 260 nm and a decrease in the absorbance at 340 nm. Also, an absorbance around 300 nm appeared as a shoulder. This phenomenon indicated that the NADH was oxidized and formed inhibitors.

Fig. 4 shows the representative capillary electrophoresis chart of NADH, when the NADH solution just after preparation was injected. On the first day, the NADH was detected at 14 min at 260 nm and 340 nm. However, after 1 day of incubation, the NADH was also detected at 290 nm and it is detected about 5 min earlier than the other wavelength. These results indicate that the NADH inhibitor with a positive charge was formed for storage at 37°C.

Table 1 Synthetic results of water-soluble anionic polymers

| Abb. | MPC unit mole fraction | | Molecular weight[b)] | | Mw/Mn | Time (h) | Yield (%) |
|---|---|---|---|---|---|---|---|
| | In feed | In copolymer[a)] | Mn ($\times 10^{-5}$) | Mw ($\times 10^{-5}$) | | | |
| $PMSO_3$ | 0 | 0 | 8.0 | 28 | 3.5 | 2 | 44 |
| $PMMSO_3$ | 0.50 | 0.45 | 8.4 | 34 | 3.9 | 0.5 | 49 |

[Mpnomer]=0.3 M, [4,4-azobis(4-cyanov aleric acid)]=3 mM; polymerization was carried out at 60°C.
a) Determined by $^1$H NMR measurement.
b) PEO standard in PBS

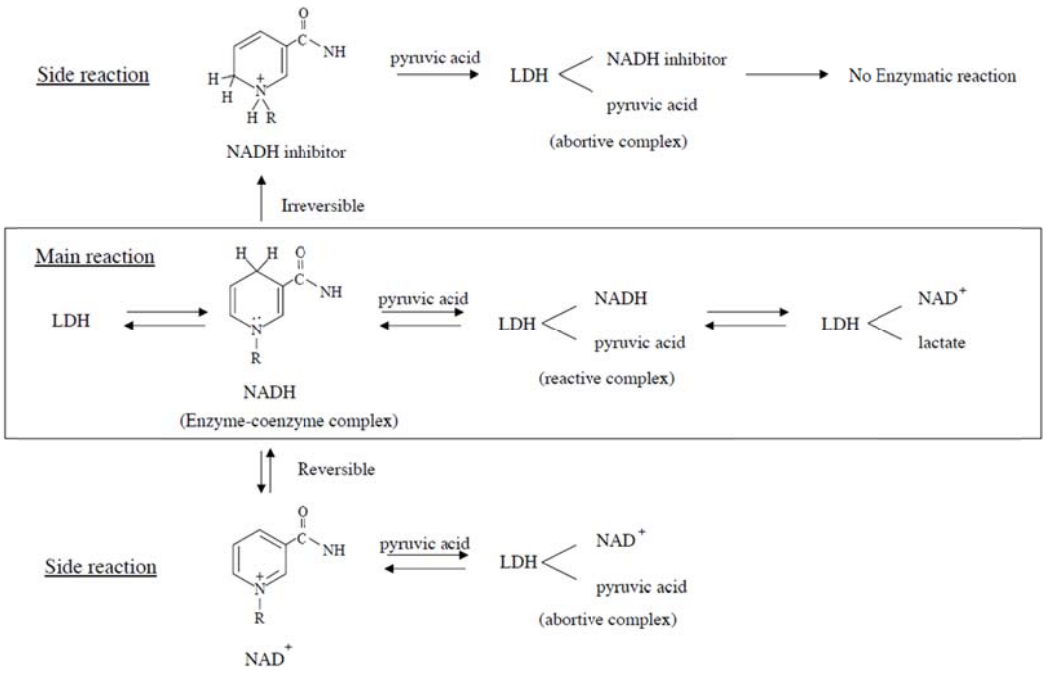


Fig. 2 Schematic representation of enzymatic reaction of LDH with NADH and its side reactions

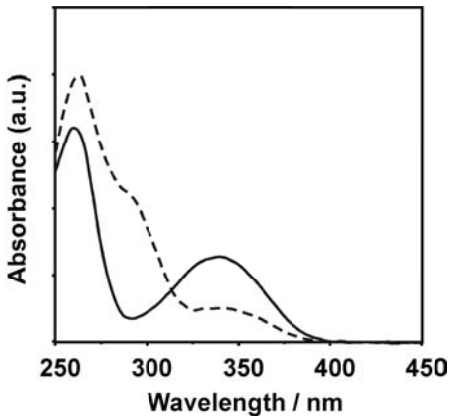


Fig. 3 UV spectra of NADH in 50 mM Tris buffered solution(pH 7.5). Before (solid line) and after a 7-day-incubation at 37°C (dotted line) are indicated

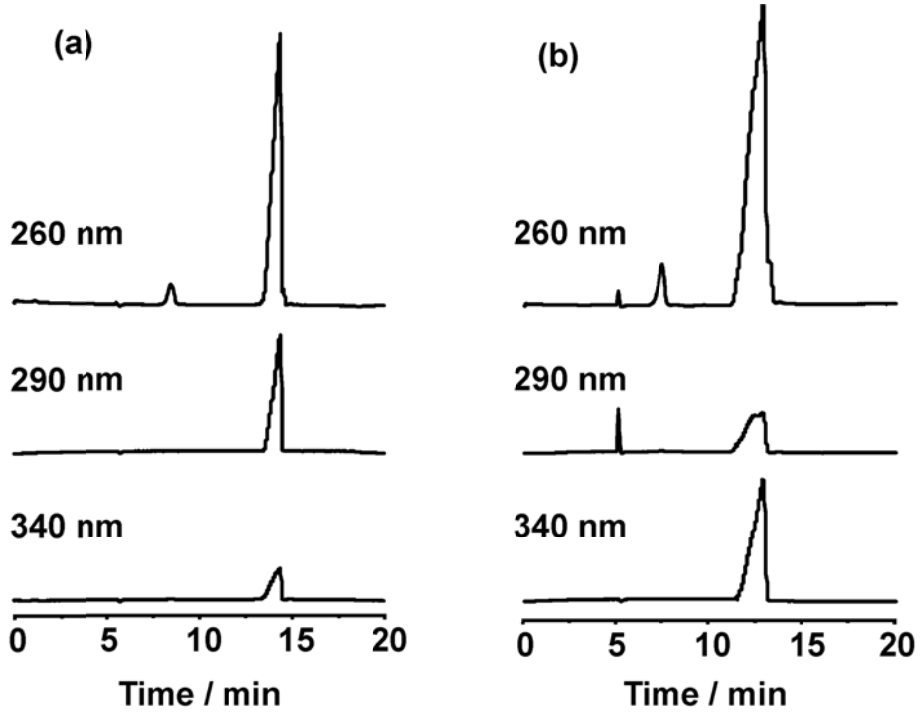


Fig. 4 Migration time of NADH during electrophoresis. Before (a) and after a 1-day incubation at 37°C (b)

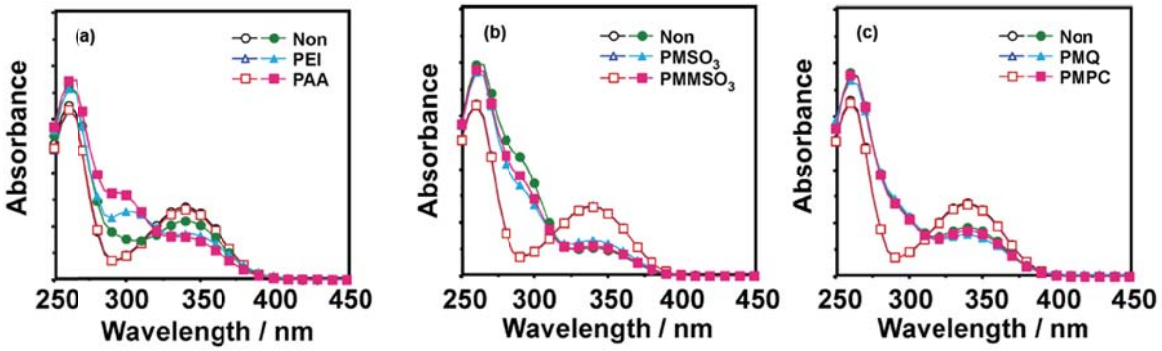


Fig. 5 Effect of the addition of various polymers on the NADH stability. The polymers used were (a) 0.1 wt% PEI and PAA; (b) 1 wt% $PMSO_3$ and $PMMSO_3$; (c) 1 wt% PMQ and PMPC. The storage conditions were (a) pH 7.9 at 25°C for 7 days; (b) pH 7.5 at 25°C for 8 days; (c) pH 7.6 at 37°C for 7 days. The open and solid symbols represent the absorption spectra of NADH in various polymer solutions before and after incubation

### *3.3 Effect of polymer addition on NADH/LDH mixture system*

The NADH stability in various polymer solutions was investigated using the UV-visible adsorption spectra. We then found that one of the NADH inhibitors had a positive charge. In this study, the water-soluble polymers, such as $PMSO_3$ and $PMMSO_3$, were synthesized, and their stabilizing function for LDH in the presence of NADH was investigated. Fig. 5 shows the time dependence of the stability of NADH in various polymer solutions. All polymers did not have an

influence on the stability of NADH before incubation, however, we observed the change in the stability of NADH by polymers after incubation. In the PEI and the PAA aqueous solutions (0.1 wt%), the NADH was significantly oxidized and formed a significant amount of an inhibitor compared to that in the control solution. This is due to the fact that the oxidation of the NADH was accelerated by a lone pair of electrons of the PEI and the PAA bond of a proton from the pyridine ring of the NADH (Simon *et al.* 2002). In the $PMSO_3$ and the $PMMSO_3$ aqueous solutions (0.1 wt%), the absorption spectra of the NADH were the same as that of the control solution. However, the highly concentration solutions of the $PMSO_3$ and the $PMMSO_3$ (1.0 wt%) effectively suppressed the increased absorbance around 300 nm. In the PMPC and the PMQ aqueous solutions, the absorption spectra of the NADH were the same as that of the control and there was no concentration dependence of the added polymer.

Fig. 6 shows the relative activity of the LDH in the various polymer solutions. The relative activity of LDH decreased with storage time, but it depended on the chemical property of the polymer added to the solution. In the PMQ solution, the LDH activity was higher than that in the Tris-buffered solution (pH 7.5). It has already been reported that PEI has a positive effect on the LDH activity and stability [6]. PMQ is one of the polycations that showed the same effect as PEI. In the $PMSO_3$ solution, the LDH activity was lower than that in the Tris-buffered solution, suggesting that the $PMSO_3$ strongly interacted with the LDH therefore, the conformation of LDH may be changed, and the activity of the LDH decreased. However, in the $PMMSO_3$ aqueous solutions, the activity of LDH was at the same level as that in the Tris-buffered solution. This result is considered to be due to the fact that the $PMMSO_3$ had no significant effect on the LDH conformation. Fig. 7 shows the relative activity of the LDH in the presence of NADH (NADH/LDH mixture system). The LDH stored with NADH significantly lost its original activity after a 1-day incubation compared with that in the absence of NADH. This is due to the NADH inhibitor spontaneously formed during storage as shown in Fig. 2. In the $PMMSO_3$ solution, the activity of the NADH/LDH mixture system doubled after the 1-day incubation and six times higher after a 3-days incubation compared to the Tris-buffered solution. However, in the PMQ and $PMSO_3$ aqueous solutions, it was at the same level or lower than that in the control. Though the PMQ could maintain the LDH activity without NADH, it could not entrap the NAHD inhibitor,

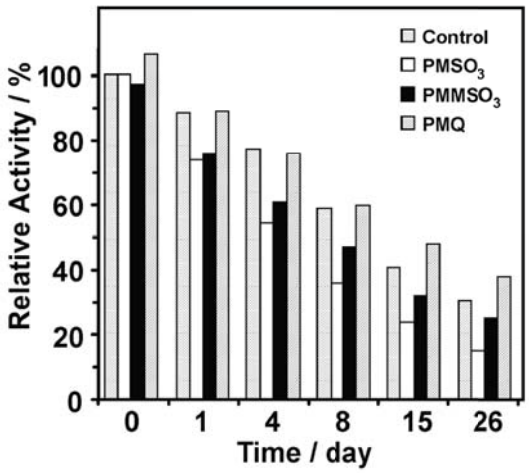


Fig. 6 Relative activity of LDH in various polymer solutions at 37°C

and the activity of the NADH/LDH mixed system was at the same level as that in the Tris-buffered solution. A further investigation of the NADH/LDH mixed system using the PMPC/$PMSO_3$ was then carried out. In the PMPC/$PMSO_3$ aqueous solution, the activity of the NADH/LDH mixed system was at the same level as the Tris-buffered solution, though the monomer unit concentrations of both the MPC and $MSO_3$ units are the same as that in the $PMMSO_3$. It is clear that the $MSO_3$ units in the polymer could interact and attract the NADH inhibitor. As shown in Fig. 8, we considered role of water-soluble polymers for stabilizing the NADH/LDH mixed system. It is considered that the high negative charge density in the $PMSO_3$ chains may affect the structure of LDH. Thus, the activity of the NADH/LDH mixed system decreased even in the presence of $PMSO_3$. The PMPC/$PMSO_3$ had no significant effect on the activity of the NADH/LDH mixed system. The MPC units in the polymer reduced the density of the negative charges of the $MSO_3$ units and may reduce the adverse effects of the $MSO_3$ units on the LDH conformation. This result suggested that it is necessary to have an MPC unit within one molecule. Based on these previously mentioned results, the water-soluble polymer having both phosphorylcholine group and sulfonate group, $PMMSO_3$, is useful for maintaining the activity of the NADH/LDH mixed system.

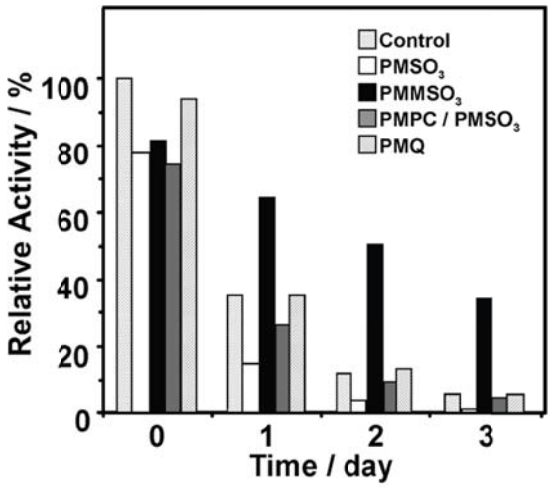


Fig. 7 Relative activity of LDH in various polymer solutions containing NADH at 37°C

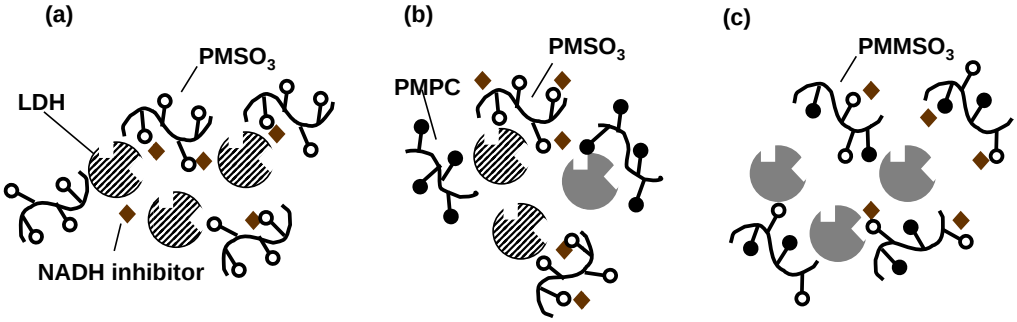


Fig. 8 Representative scheme of role of polymers in NADH/LDH mixed system. (a) $PMSO_3$, (b) PMPC / $PMSO_3$, (c) $PMMSO_3$

## 5. Conclusions

The water-soluble phospholipid polymer that could trap the NADH inhibitor without affecting LDH was synthesized by a conventional radical polymerization. It is considered that the sulfuric group in the polymer can trap the NADH inhibitor, and the MPC unit stabilized the LDH. We concluded that the addition of $PMMSO_3$ in the NADH/dehydrogenase mixed system is effective for maintaining the activity, and it leads to make obtaining a higher performance and more convenient of enzymatic analysis.

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