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Preparation of Zn^{2+}-Chelated Carboxymethyl Poly(1-Vinylimidazole) for Intracellular Zn^{2+} Delivery

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Abstract

Zinc ions (Zn^{2+}), known to be a novel intracellular second messenger related to various biological functions, have been delivered inside cells. For the intracellular Zn^{2+} delivery, Zn^{2+} has been chelated to carboxymethyl poly(1-vinylimidazole) (CM-PVIm) by mixing zinc chloride ($ZnCl_2$) or zinc acetate ($Zn(OAc)_2$) with CM-PVIm. The resulting Zn^{2+}-chelated CM-PVIm, that is, Zn^{2+}/CM-PVIm complex by mixing $ZnCl_2$ exhibited smaller particle size below 10 nm and possessed larger amount of Zn^{2+} ions, as compared to the Zn^{2+}/CM-PVIm by mixing $Zn(OAc)_2$. The both Zn^{2+}/CM-PVIm complexes exhibited no significant cytotoxicity, leading to intracellular Zn^{2+} delivery. The Zn^{2+}/CM-PVIm by mixing $ZnCl_2$ delivered larger amount of intracellular Zn^{2+} ions than that by mixing $Zn(OAc)_2$. These results suggest that the optimal Zn^{2+}/CM-PVIm complex is a useful tool for intracellular Zn^{2+} delivery to control various biological functions.

Keywords

Zinc Ion (Zn^{2+}), Carboxymethyl Poly(1-Vinylimidazole), Intracellular Delivery

1. Introduction

Zinc ion (Zn^{2+}) is known to be a novel intracellular second messenger related to various biological functions [1]. As well, Zn^{2+} is involved in the structure of all enzyme classes, a micro nutrient, and necessary for growth and development [2]. Zinc transporter proteins, divided into two major families, ZIPs to increase and ZnTs to decrease Zn^{2+} concentration in the cytosol, are responsible for keeping zinc at certain concentrations [3]. Intracellular free Zn^{2+} is considered to be in

the nM to pM range because many zinc metalloproteins have metal binding affinities in that range [4] [5] [6]. The imidazole group of histidine in a zinc metalloprotein is recognized as an excellent ligand for the tetrahedral coordination of the Zn^{2+} transition metal ion [7] [8]. The carboxyl group of aspartic or glutamic acid also coordinates Zn^{2+} in the protein [9]. These studies have inspired us to control the intracellular free Zn^{2+} concentration, without zinc transporter, by using delivery carrier containing imidazole and carboxyl groups for exploring unknown biological functions.

We have previously reported the design of carboxymethyl poly(1-vinylimidazole) (CM-PVIm) for biocompatibility [10]. The CM-PVIm is a net nonionic polyampholyte under physiological pH conditions, because the CM-PVIm possesses both imidazole and carboxyl groups, exhibiting the suppression of the nonspecific interaction with serum proteins. The imidazole groups are considered to possess a buffering capacity in the endosome, resulting in the endosomal escape by endosome membrane destabilization after protonation of imidazole groups for efficient delivery to cytoplasm [11] [12]. Furthermore, we have also reported Zn^{2+}-chelated PVIm derivatives, not polyampholyte but polycation, for efficient gene delivery [13] [14] [15] [16].

In this study, we have focused on the preparation of Zn^{2+}-chelated CM-PVIm for intracellular Zn^{2+} delivery. The resulting intracellular Zn^{2+} delivery is expected to control various biological functions.

2. Materials and Methods

2.1. Materials

Zinc chloride ($ZnCl_2$) and zinc acetate ($Zn(OAc)_2$) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals were of a special grade and were used without further purification.

2.2. Preparation of Zn^{2+}-Chelated CM-PVIm

As CM-PVIm, 18 mol% carboxymethyl PVIm were synthesized according to our previous paper [10]. 1-Vinylimidazole (VIm) (500 mg) and 2,2'-Azobis (isobutyronitrile) (AIBN) as an initiator were dissolved in 8 mL *N,N*-dimethylformamide (DMF). Radical polymerization reaction was carried out for 120 h at 60˚C. After the reaction, the content was poured into a large excess of diethylether, and the precipitate was dried. The resulting polymer (PVIm) and iodoacetic acid (180 mg) were dissolved in 8.5 mL of DMF. The reaction mixture was incubated at 40˚C for 24 h, followed by dialysis against distilled water using a Spectra/Por 7 membrane (molecular weight cutoff = 10^3) to remove unreacted iodoacetic acid. The resulting polymer (CM-PVIm) was obtained by freeze-drying.

Each resulting CM-PVIm was mixed with $ZnCl_2$ or $Zn(OAc)_2$ (Zn^{2+}/imidazole = 0.5) and incubated at room temperature for 24 h, followed by dialysis against distilled water using a Spectra/Por 7 membrane (10^3 molecular weight cut-off) to remove free Zn^{2+}. The control sample of $ZnCl_2$ or $Zn(OAc)_2$ in the absence of

CM-PVIm was also dialyzed under the same conditions.

2.3. Atomic Absorption Spectrometry

Under acidic conditions, which were achieved by the addition of 1 M HCl to the resulting dialysis fluid, the Zn^{2+} content in the CM-PVIm was determined by atomic absorption spectrometry at 213.9 nm using an atomic absorption spectrophotometer AA-6200 (Shimadzu Co., Kyoto, Japan).

2.4. Size and ζ-Potential Measurement

The size and ζ-potential of the resulting Zn^{2+}/CM-PVIm complexes were measured in the resulting dialysis fluid. The size of each sample was measured by a dynamic light scattering (DLS) method using an electrophoresis light scattering spectrophotometer (ELS-Z2, Otsuka Electronics Co., Ltd., Tokyo, Japan) and the zeta potential was measured by ELS with electrodes.

2.5. Cell Viability Assay

HepG2 cells (from Riken Bioresource Center Cell Bank), human hepatoblastoma cell line, were cultured in tissue culture flasks containing Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated of fetal bovine serum (FBS). The cells were seeded at 1×10^4 cells/well (100 μL/well) in a 96-well plate and incubated at 37˚C in a 5% CO_2 incubator, overnight. After the addition of 20 μL of the sample containing the Zn^{2+}/CM-PVIm complexes or the control sample containing $ZnCl_2$ or $Zn(OAc)_2$ alone, as well as CM-PVIm alone, the cells were incubated at 37˚C for 24 h. After washing the medium with PBS (+), by further incubated for 4 h, the cell viability was measured using the Alamar Blue assay [17] in triplicate.

2.6. Determination of Intracellular Zn^{2+} Ions

To HepG2 cells seeded at 1×10^4 cells/well in a 96-well plate, 20 μL of the sample containing the Zn^{2+}/CM-PVIm complexes or the control sample containing $ZnCl_2$ or $Zn(OAc)_2$ alone was added according to the cell viability assay. After incubation for 24 h, the cells were washed with PBS (+) twice, followed by the addition of 20 μL of cell culture lysis reagent (25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) (Promega, Madison, WI). After 5 min incubation, 100 μL H_2O was added. Subsequently, 400 μL of 0.4 M HCl was added to 120 μL of the resulting cell lysate diluted to 1.5 mL with H_2O. The concentration of Zn^{2+} in the resulting sample was determined by atomic absorption spectrometry at 213.9 nm using an atomic absorption spectrophotometer AA-6200 (Shimadzu Co., Kyoto, Japan).

3. Results and Discussion

Figure 1 shows the design of Zn^{2+}-chelated CM-PVIm with two Zn^{2+}-chelating

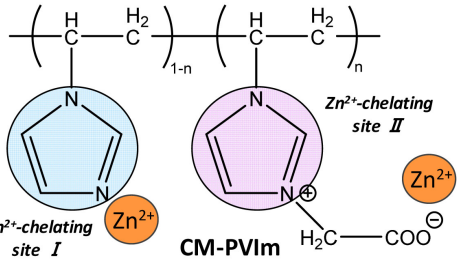

Figure 1. Design of Zn^{2+}-chelated CM-PVIm, that is, Zn^{2+}/CM-PVIm complex.

sites, that is, imidazole and carboxyl groups. To examine the effect of counter anions on the chelation to CM-PVIm, we mixed CM-PVIm with $ZnCl_2$ or $Zn(OAc)_2$, as shown in **Figure 2**, followed by the removal of free Zn^{2+}. The interaction between Zn^{2+} ions and CM-PVIm is considered to form a cross-linking structure with measurable particle size and ζ-potential. After mixing CM-PVIm with $ZnCl_2$, as shown in **Figure 3**, the particle size was 6.6 nm and the ζ-potential was +16.3 mV. On the other hand, larger particle size (79.6 nm) and lower ζ-potential (+8.74 mV) were exhibited by mixing with $Zn(OAc)_2$. These results suggest that the Zn^{2+} ions chelated to the imidazole and/or carboxyl groups of CM-PVIm and that counter anions affected the resulting cross-linking structure by the difference in their coordination interaction with CM-PVIm polyampholytes.

We used atomic absorption spectrometry to determine the amount of Zn^{2+} ions chelated to the CM-PVIm. The concentration of Zn^{2+} ions chelated to CM-PVIm by use of $ZnCl_2$ and $Zn(OAc)_2$ was 1.3, and 0.51 mol%, respectively, based on all the imidazole groups of the CM-PVIm (**Table 1**).

The calculated amount of the Zn^{2+} chelated to the CM-PVIm is in proportion to the ζ-potential. Conversely, that of the Zn^{2+} is in inverse proportion to the particle size, presumably due to higher condensation of CM-PVIm polyampholyte chains by larger amount of the Zn^{2+} ions. Namely, taking these results into account, free Zn^{2+} ions are considered to be easy of access to imidazole and/or carboxyl groups of CM-PVIm without steric hindrance by use of $ZnCl_2$, because $ZnCl_2$ released 3-fold higher concentration than $Zn(OAc)_2$ [18].

As shown in **Figure 4**, the cell viability in the presence of CM-PVIm alone and the resulting Zn^{2+}/CM-PVIm complex for use of intracellular Zn^{2+} delivery. We confirmed almost 100% cell viability in the presence of CM-PVIm alone, up to the concentration of 16 mg/mL, which was higher than Zn^{2+} delivery experimental conditions (**Figure 4(a)**). Furthermore, the Zn^{2+}/CM-PVIm complexes by use of both $ZnCl_2$ and $Zn(OAc)_2$ exhibited no significant cytotoxicity, as well as $ZnCl_2$ and $Zn(OAc)_2$ alone, under the experimental conditions (**Figure 4(b)**).

Based on the lack of significant cytotoxicity of the Zn^{2+}/CM-PVIm complexes, as shown in **Figure 5**, the intracellular Zn^{2+} delivery by the Zn^{2+}/CM-PVIm complexes was examined. When $ZnCl_2$ or $Zn(OAc)_2$ was added to the cells in the absence of CM-PVIm, intracellular Zn^{2+} hardly increased. On the other hand, the Zn^{2+}/CM-PVIm complex led to increase in intracellular Zn^{2+}, succeeding in

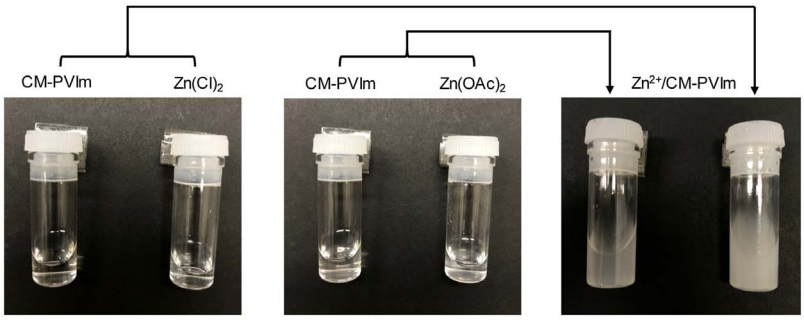

Figure 2. Typical images just after mixing CM-PVIm with $ZnCl_2$ or $Zn(OAc)_2$.

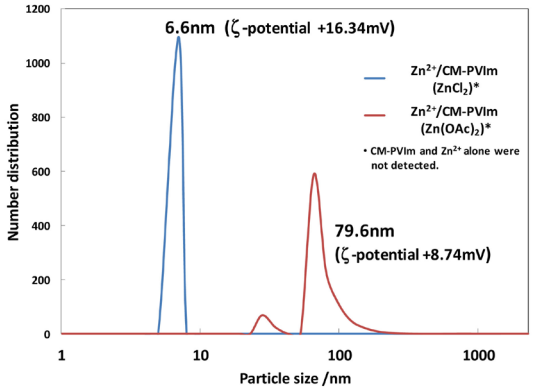

Figure 3. Particle size and ζ-potential of the Zn^{2+}/CM-PVIm complexes.

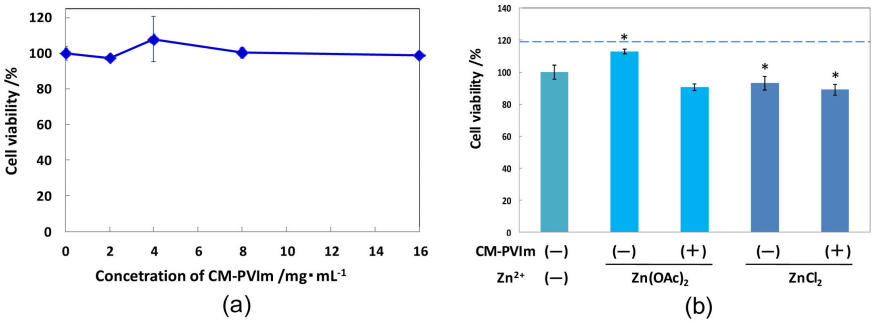

Figure 4. Effect of CM-PVIm (a) and Zn^{2+}/CM-PVIm (b) on the viability of HepG2 cells. Symbols and error bars represent the mean and standard deviation of the measurements made in paired wells ((a) n = 3 or (b) n = 4). * means that the highest value was dismissed (n = 3).

Table 1. Amount of Zn^{2+} chelated to CM-PVIm.

Conditions	Abs. (213.9 nm)	
	$Zn(OAc)_2$	$ZnCl_2$
CM-PVIm (−)	0.0027	0.0030
CM-PVIm (+)	0.0610	0.1667
Zn^{2+} concentration/mg·L^{-1}*	2.0×10^{-4}	5.6×10^{-4}
Chelated Zn^{2+}/mol% per VIm*	0.51	1.28

*CM-PVIm (−) as blank.

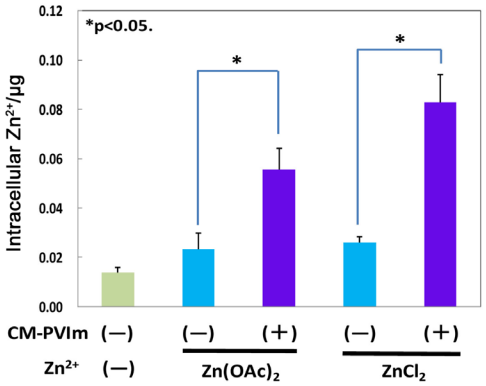

Figure 5. Cellular uptake of Zn^{2+} by the Zn^{2+}/CM-PVIm complexes. Symbols and error bars represent the mean and standard deviation of the measurements made in paired wells (n = 3). Statistical significance ($p < 0.05$) is indicated when compared to the absence of CM-PVIm.

intracellular Zn^{2+} delivery. The resulting amount of intracellular Zn^{2+} treated with the Zn^{2+}/CM-PVIm complex by use of $ZnCl_2$ was higher than that by use of $Zn(OAc)_2$. These results suggest that the Zn^{2+}/CM-PVIm complex with larger amount of Zn^{2+} as well as smaller particle size is more suitable for intracellular Zn^{2+} delivery.

From an applied point of view, because zinc transporter-8 (ZnT8), to deliver Zn^{2+} from the cytosol to insulin granules, is known to regulate hepatic insulin clearance [19], the optimization of our intracellulr Zn^{2+} delivery system has the possibility of use for type 2 diabetes therapy.

4. Conclusion

We have chelated Zn^{2+} ions to CM-PVIm by mixing $ZnCl_2$ or $Zn(OAc)_2$. The resulting Zn^{2+}/CM-PVIm complex by mixing $ZnCl_2$ exhibited smaller particle size and possessed larger amount of Zn^{2+} ions, as compared to the Zn^{2+}/CM-PVIm by mixing $Zn(OAc)_2$, delivering larger amount of intracellular Zn^{2+} ions without cytotoxicity. Collectively, from these results, the optimal Zn^{2+}/CM-PVIm complex is considered to be a useful tool for intracellular Zn^{2+} delivery to control various biological functions.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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