Encapsulation of Hemoglobin Using Condensation Reaction of Butylcyanoacetate with Formaldehyde

Hidenobu Shimizu, Masayo Tomizawa, Risei Wada, Masaru Okabe*

Summary: Hemoglobin (Hb)-encapsulating particles have been prepared in a single step by condensation reaction of butylcyanoacetate (BCNA) with formaldehyde (FA) in Hb aqueous solution. Dimethylamine was used as a base catalyst and then the reaction was performed in 0.1 M HEPES buffer (pH 7.4) at room temperature. Particle composition analysis by extraction indicated that the particles consisted of PBCNA, HEPES and Hb. The content of Hb loaded into the particles monotonously increased with increasing initial Hb concentrations. Almost all Hb molecules in aqueous solution could be recovered as particles. In addition, the encapsulated Hb maintained the oxygen-carrying capacity similar to native Hb. These results indicate that condensation reaction of BCNA with FA can provide the assembly of Hb molecules in aqueous solution.

Keywords: cyanoacetate; encapsulation; formaldehyde; hemoglobin; nanocapsules; oxygen carrier

Introduction

Encapsulation of biological molecules, especially of proteins and enzyme, is of great interest for protein delivery and bioreactor systems. Various approaches have been reported so far to encapsulate these functional molecules.[1] Encapsulation techniques are usually divided into two basic groups, namely chemical and physical processes. Physical processes include layer-by-layer (LBL) assembly,[2] complex coacervation,[3] injection inside preformed capsules,[4] macromolecular self-assembly,[5] and emulsification.[6] On the other hand, chemical processes such as *in situ* polymerization processes present the advantages of being easy to control and versatile, but their processes have not appreciably been used for protein encapsulation due to a decline of its activity during encapsulation processes.[7]

Poly (alkylcyanoacrylates) (PACNA) is biocompatible and biodegradable. Their particles are therefore promising candidates for a carrier of antisense oligonucleotides,[8] drugs,[9] or proteins.[10] PACNA particles can be obtained by anionic polymerization of ACNA[11] or condensation polymerizations of alkyl cyanoacetates with formaldehyde (FA).[12] The present paper reports on the condensation reaction of butylcyanoacetate (BCNA) with FA in protein aqueous solution. Using alkyl cyanoacetates instead of alkyl cyanoacrylate would make it possible to better control the polymerization processes. Hemoglobin (Hb), which is a typical oligomeric protein, was used as a model protein. Hb possesses the ability to transport and transfer oxygen. Hb capsules, which encapsulate the concentrated Hb inside the particles, have a potential as a red blood cell substitute, provided that the sizes could be very small.[13] Condensation reaction was initiated by a basic catalyst of dimethylamine (DMA). The reaction was also performed in 0.1 M HEPES buffer (pH 7.4) at room temperature. We then examined the influence of initial Hb concentrations on

Department of Applied Bioscience, Faculty of Applied Bioscience, Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi, Kanagawa 243-0292, Japan
Tel&Fax: +81-46-291-3148;
E-mail: shimizu@bio.kanagawa-it.ac.jp

Hb loading, entrapment efficiency, particle size, and oxygen affinity of Hb-encapsulating particles.

Experimental Part

Materials

Butylcyanoacetate (BCNA) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Formaldehyde solution and dimethylamine (DMA) solution were purchased from Kanto Chmical Co. (Tokyo, Japan). Soluble Hb powder was purchased from Oxoid Ltd. (Cambridge, UK). All chemicals were of analytical grade and used without additional purification.

Preparation of Protein Nanocapsules

Commercially available hemoglobin (methemoglobin, metHb) was dissolved in 0.1 M HEPES buffer (pH7.4). BCNA (0.5 g) and FA (0.1g) were then added into the Hb-containing buffer (99 g). Condensation reaction was initiated by the addition of DMA (0.03 g). The reaction was carried out at room temperature for 7 min. All particles were purified by repetitive centrifugation at 33,850 g for 30 min and redispersion in distilled water several times.

Determination of Encapsulated Hb

Hb-encapsulating particles were isolated by repetitive centrifugation, followed by freeze-drying. The dried samples were dispersed in acetone. In this process, BCNA can be extracted as a soluble fraction. Acetone-insoluble fraction was collected with centrifugation, followed by vacuum drying. The collected samples were dispersed in distilled water, followed by the extraction of the HEPES salt. Finally, Hb remained in distilled water as the insoluble fraction.

Characterization

The average hydrodynamic diameters of the protein nanocapsules were measured by using a homemade light scattering apparatus equipped with an ALV 5000/60X0 Tau digital correlator and a 20 mW He-Ne laser ($\lambda = 632.8$ nm). The dispersed particles in water were allowed to equilibrate thermally before measurements were taken at 30 °C. Scattering data were collected at 90° and the time correlation functions were obtained. The average hydrodynamic diameters were calculated from the translational diffusion coefficients by using the Stokes-Einstein equation.

The spectra for Hb-loading nanocapsules were recorded (wavelength range: 380-650 nm) with a JASCO V-630 spectrophotometer.

Results and Discussion

Hb Loading and Entrapment Efficiency

Condensation reaction of BCNA with FA in HEPES buffer without Hb could provide water-dispersible particles. And then the reaction was carried out in buffer containing Hb molecules at different concentrations. As a consequence, Hb aqueous solution turned translucent as soon as the base catalyst, DMA, was added to the solution, and no precipitates were observed, suggesting that stable PBCNA dispersions were formed even in Hb aqueous solution. The reaction was completed for about 5 min. In addition, BCNA conversion reached only about 60%, indicating that FA may attack the amino groups in Hb molecules during the encapsulation process.

We then analyzed the particle composition by gravimetric method with extraction. As a result, we can find that the particles obtained by condensation reaction consisted of PBCNA, HEPES, and Hb. Figure 1 shows the weight percent of each component as a function of initial Hb concentrations. The concentration was based on the BCNA monomer weight. The content of Hb loaded into the particles monotonously increased with increasing Hb concentrations up to ca. 30%. The HEPES salt content could also increase with an increase in Hb loading. These results indicate that Hb loading can be controlled by varying initial Hb concentrations in the

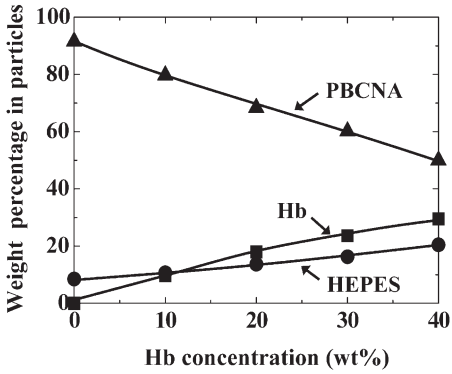

Figure 1.
Weight percent of each component in particles as a function of Hb concentration.

range of 0 to 30 wt%. On the other hand, varying the initial Hb concentrations caused little significant change in Hb encapsulation efficiency and the efficiency was almost 100%, suggesting that almost all Hb molecules in aqueous solution can be encapsulated into polymeric particles in a single step by condensation reaction of BCNA with FA.

Particle Size Analysis

Figure 2 shows the effect of initial Hb concentrations on the particle size determined by dynamic light scattering. Condensation reaction in HEPES buffer without Hb molecules could bring about water-dispersible particles with a size of 700 nm. The size decreased dramatically to less than 200 nm at performing the reaction in Hb aqueous solution, and the increased concentrations led to a gradually decreased size. These results indicate that Hb may act as surface active agents for particle formation process under condensation reactions. Hb would be therefore both inside the polymeric matrix and at the surface of the particles. The particles obtained also had a narrow size distribution.

Oxygen-carrying Capacity

The oxygen-carrying capacity is very important to consider using the Hb-encapsulating particles as an oxygen carrier. To clarify whether the encapsulated Hb could maintain the oxygen-carrying capacity of the native Hb, the capacity of the Hb nanocapsules was measured with a UV-Vis spectrometer. Figure 3 exhibits the adsorption spectra of native and encapsulated Hb. Encapsulated Hb corresponds to metHb, and Fe^{3+} was first reduced to Fe^{2+} with sodium dithionate, followed by the formation of oxy Hb. Due to the light scattering effect of the nanocapsules, the turbidity of the dispersions was a little higher than that of native Hb solution, but the spectra and the peak can be clearly observed. Encapsulated Hb in the dispersions bubbling with an oxygen gas showed a positive peak at around 410, 540, and 580 nm, which is in accord with that of

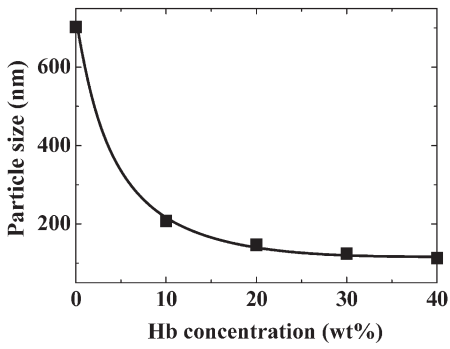

Figure 2.
Size of Hb-encapsulating particles as a function of initial Hb concentrations.

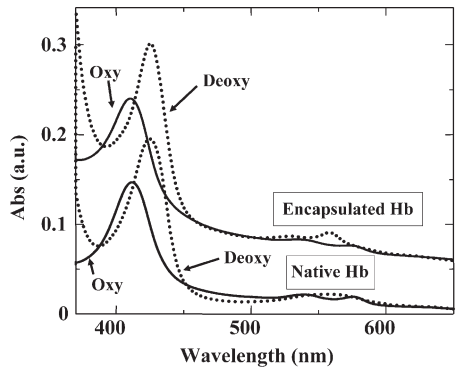

Figure 3.
UV/Vis absorption spectra of encapsulated and native Hb.

native oxy Hb. After passing a nitrogen gas to the dispersions, the peak that corresponded to the encapsulated Hb in the deoxidized state shifted to 430 and 555 nm, which is also agreement with that of native deoxy Hb. By changing the atmosphere, the spectra of both encapsulated Hb reversibly converted in spite of the Hb content of the particles. These results indicate that the encapsulated Hb would maintain the oxygen-carrying capacity similar to native Hb.

Conclusion

Hb with oxygen-carrying capacity was successfully encapsulated in a single step by condensation reaction of BCNA with FA in HEPES buffer containing Hb. The resulting protein nanocapsules have a unimodal size distribution and a size of less than 200 nm, depending on the Hb initial concentrations. Furthermore, encapsulated Hb retained its activity. We believe that this *in situ* polymerization process can be applicable for other proteins.

[1] [1a] S. W. Ghosh, Ed., "*Functional Coating*", Wiley-VCH, Weinheim **2006**; [1b] F. Caruso, Ed., "*Colloids and Colloid Assemblies*", Wiley-VCH, Weinheim **2004**.
[2] F. Caruso, H. Mohwald, *J. Am. Chem. Soc.* **1999**, *121*, 6039.
[3] A. Harada, K. Kataoka, *Macromolecules* **1998**, *31*, 288.
[4] X. Li, X. LI, J. Zhang, S. Zhao, J. Shen, *J. Biomed. Mater. Res.* **2008**, *85A*, 768.
[5] [5a] T. Nishikawa, K. Akiyoshi, J. Sunamoto, *J. Am. Chem. Soc.* **1996**, *118*, 6110; [5b] T. Kida, K. Inoue, T. Akagi, M. Akashi, *Chem. Lett.* **2007**, *36*, 940.
[6] [6a] G. Zhu, S. R. Mallery, S. P. Schwendeman, *Nat. Biotechnol.* **2000**, *18*, 52; [6b] S. Takeoka, T. Ohgushi, K. Terase, T. Ohmori, E. Tsuchida, *Langmuir* **1996**, *12*, 1755; [6c] J. Zhao, C.-S. Liu, Y. Yuan, X.-Y. Tao, X.-Q. Shan, Y. Sheng, F. Wu, *Biomaterials* **2007**, *28*, 1414.
[7] N. Behan, C. Birkinshaw, *Macromol. Rapid Commun.* **2001**, *22*, 41.
[8] G. Lambert, *J. Disper. Sci. Technol.* **2003**, *24*, 439.
[9] S. C. Yang, H. X. Ge, Y. Hu, X. Q. Jiang, C. Z. Yang, *J. Appl. Polym. Sci.* **2000**, *78*, 517.
[10] [10a] C. Chauvierre, M. C. Marden, C. Vauthier, D. Labarre, P. Couvreur, L. Leclerc, *Biomaterials*, **2004**, *25*, 3081; [10b] S. Watnasirichaikul, N. M. Davies, T. Rades, I. G. Tucker, *Pharm. Res.* **2000**, *17*, 684.
[11] C. Limouzin, A. Caviggia, F. Ganachaud, P. Hemery, *Macromolecules* **2003**, *36*, 667.
[12] M. T. Peracchia, D. Desmaele, P. Couvreur, J. d'Angelo, *Macromolecules* **1997**, *30*, 846.
[13] T. M. S. Chang, *Trends Biotechnol.* **2006**, *24*, 372.

