# (Inorganic Nanofiber/Enzyme) Hybrid Hydrogel: Preparation, Characterization, and Enzymatic Activity of Imogolite/Pepsin Conjugate

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The hybrid hydrogel composed of tubular aluminosilicate nanofiber, "imogolite," and pepsin was prepared in a simple manner. We confirmed the formation of a network structure of imogolite in hydrogel by FE-SEM observations. Pepsin immobilized onto imogolite showed enzymatic activity after repeated reactions.

Imogolite is a kind of clay mineral widely distributed in volcanic ash soils and weathered pumice beds that was first discovered in Japan in 1962.[1] It has a hollow, fibrous structure with an outer diameter of 2.5 nm and a length from several hundred nanometers to a micrometer and has the general formula of $Al_2O_3 \cdot SiO_2 \cdot 2H_2O$.[2] The Al–OH groups at the outer surface of imogolite can interact specifically with phosphate ions[3] and alkyl phosphonyl/phosphoryl groups.[4] By utilizing the immobilization of the enzyme which has a phosphoric group onto the nanofiber, it is possible to achieve high levels of enzyme immobilization by immobilizing enzymes with a phosphoric group onto the nanofiber. Furthermore, enzyme activity can be expected to be maintained by forming a hybrid hydrogel immobilized with enzyme because imogolite can form a 3-dimensional network structure in aqueous solution.[5] The authors report herein preparation of a hybrid hydrogel via the immobilization of pepsin as a model enzyme with a phosphoric group and evaluation of its aggregation structure and enzyme activity.

The (imogolite/pepsin) hybrid hydrogel was prepared by adding 10 mL of pH = 3.1 pepsin solution (1 mg/mL) to the 10 mL of pH = 3.1 imogolite solution (0.5 mg/mL). The mixture was incubated at 37 °C by shaking at 120 rpm for 4 h. After that, it was centrifuged at 3000 rpm for 15 min and rinsed 3 times with pH = 3.1 acetic acid solution to obtain the hybrid hydrogel. Figure 1 shows a photograph and schematic representation of the structure of the hybrid hydrogel. Pepsin has a phosphoric group

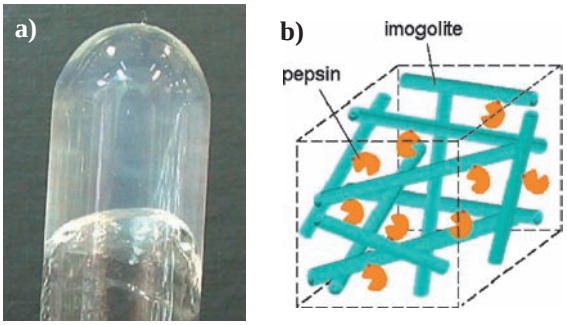


**Figure 1.** a) Photograph and b) schematic representation of (imogolite/pepsin) hybrid hydrogel.

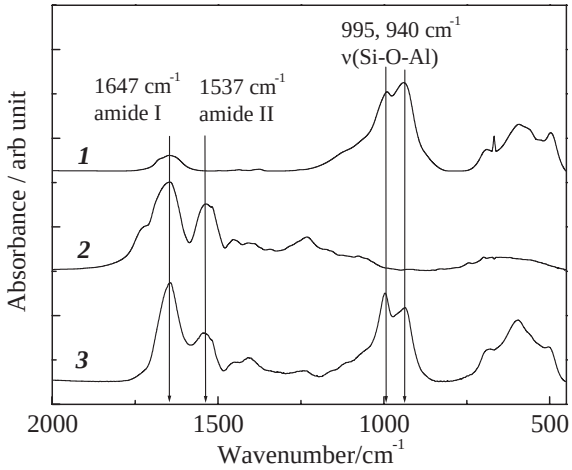


**Figure 2.** IR spectra of (1) imogolite, (2) pepsin, and (3) hybrid hydrogel immobilized with pepsin.

at the serine of the 68th residue.[6] The Al–OH groups of imogolite are positively charged and dispersed under acidic conditions (pH below 5) in aqueous solution by the electrostatic repulsion among them and pepsin is negatively charged under pH = 3.1 because its isoelectric point is 1.0.[7] This hybrid hydrogel was formed due to the electrostatic interaction between imogolite and pepsin as well as the interaction between Al–OH groups of the imogolite surface and the phosphoric group of pepsin. To the best of our knowledge, this is the first report of a hybrid hydrogel prepared via the immobilization of enzyme onto inorganic nanofibers.

The immobilization of pepsin was confirmed by IR measurement using the KBr method. Figure 2 shows IR spectra of imogolite, pepsin, and hybrid hydrogel immobilized with pepsin. The absorption peaks at 995 and 940 $cm^{-1}$ can be attributed to the stretching vibration of Si–O–Al in imogolite,[8] and those at 1647 and 1537 $cm^{-1}$ to amide I and II bands in pepsin, respectively.[9] The immobilization of pepsin by the interaction between pepsin and imogolite was indicated, since the absorption peaks of pepsin and imogolite were observed simultaneously and the intensity ratio of absorption peaks from imogolite at 995 and 940 $cm^{-1}$ was changed before and after immobilization. Furthermore, the same absorption peaks from amide I and II bands before and after immobilization suggested that pepsin was not denatured. The amount of immobilized pepsin was estimated by thermogravimetric analysis (TGA). The maximum value was approximately 1.8 mg of pepsin per 1 mg of imogolite.[10] Assuming a single-fiber dispersion of imogolite, this maximum value indicates that ca. 71% of the surface of imogolite is covered with pepsin molecules.

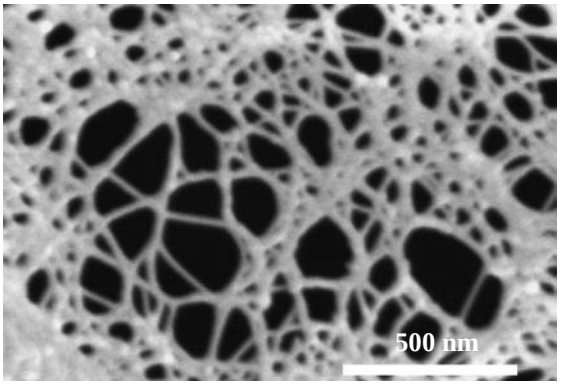


**Figure 3.** FE-SEM image of the hybrid hydrogel immobilized with pepsin.

Confocal laser scanning microscopy (CLSM) and field-emission scanning electron microscopy (FE-SEM) were used to evaluate the dispersion state of pepsin in the hybrid hydrogel and the network structure of hybrid hydrogel.[11] Pepsin was found to be finely dispersed in the hybrid hydrogel, and the fluorescent images of pepsin and imogolite showed a similar morphology (figure not shown) due to the immobilization of pepsin onto the imogolite surface. Figure 3 shows the FE-SEM image of the hybrid hydrogel with a 99.7% water content. The 3-dimensional network structure of the hybrid hydrogel composed of imogolite could be directly observed. The average pore size of the hybrid hydrogel in this image was 108 nm.

The enzyme activity of immobilized pepsin in the hybrid hydrogel was evaluated from hydrolysis of hemoglobin at pH = 3.1.[12] $\Delta A_{280}$/min of free pepsin was 0.183, and that of immobilized pepsin in the hybrid hydrogel was 0.048. Therefore, immobilized pepsin in the hybrid hydrogel retained ca. 26% of its enzyme activity compared with free pepsin in aqueous solution. The enzyme activity of immobilized pepsin was apparently decreased. Similar behavior was observed on the enzyme immobilized at the inner surface of halloysite.[13] This decrease could be ascribed to the slow diffusion of substrate into the network structure of the hybrid hydrogel shown in Figure 3 as well as to inhibition of the diffusion of substrate to the active site of immobilized pepsin due to the steric hindrance of the imogolite network. In the case of pepsin immobilized in the hybrid hydrogel, it can be easily recovered from the reaction system and can repeatedly react with the substrate. The change in enzyme activity was investigated in the repeated reaction. Figure 4 shows the change in enzyme activity of immobilized pepsin in the hybrid hydrogel. In fact, the enzyme activity of immobilized pepsin was retained after four reactions, with a slight decrease in activity as the number of reactions increased.

In conclusion, the authors successfully prepared a hybrid hydrogel immobilized with enzyme having a phosphoric group. Although the enzyme activity of immobilized pepsin in the hybrid hydrogel was slightly decreased, the immobilized pepsin could react repeatedly after the enzyme reaction. This method of enzyme immobilization should be applicable to enzymes that have phosphoric groups or to which phosphoric groups have been introduced.

The present work was supported in part by a Grant-in-Aid for the 21st Century COE Program, "Functional Innovation of Molecular Informatics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Scientific Research (A)(2) (No. 15205028), and Japan-Korea Basic Scientific Cooperation Program from Japan Society for the Promotion of Science. The authors also acknowledge the financial support of P&P Green Chemistry of Kyushu University. CLSM and FE-SEM observations of the hybrid hydrogel were performed at the Collabo-station II, Kyushu University.

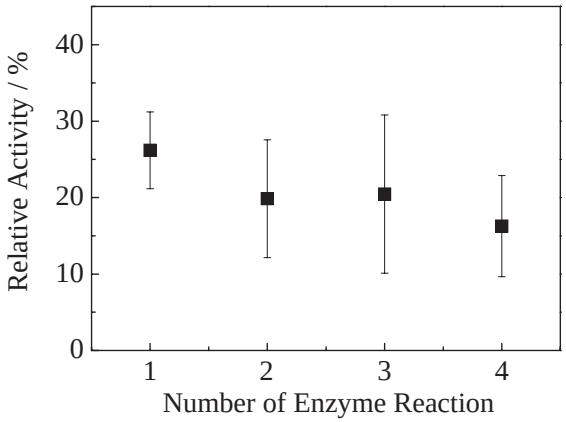


**Figure 4.** The enzyme activity change of immobilized pepsin during the repeated reaction at 37 °C.

**References and Notes**

1 N. Yoshinaga, S. Aomine, *Soil Sci. Plant Nutr.* **1962**, *8*, 22.
2 P. D. G. Cradwick, V. C. Farmer, J. D. Russell, C. R. Masson, K. Wada, N. Yoshinaga, *Nat. Phys. Sci.* **1972**, *240*, 187.
3 R. L. Parfitt, A. D. Thomas, R. J. Atkinson, R. St. C. Smart, *Clays Clay Miner.* **1974**, *22*, 455.
4 a) K. Yamamoto, H. Otsuka, S.-I. Wada, A. Takahara, *Chem. Lett.* **2001**, 1162. b) K. Yamamoto, H. Otsuka, S.-I. Wada, A. Takahara, *J. Adhes.* **2002**, *78*, 591. c) K. Yamamoto, H. Otsuka, S.-I. Wada, D. Sohn, A. Takahara, *Polymer*, **2005**, *46*, 12386.
5 A. P. Phillips, A. M. Wierenga, *Langmuir* **1998**, *14*, 49.
6 a) A. R. Sielecki, A. A. Fedrov, A. Boodhoo, N. S. Andreeva, M. N. G. James, *J. Mol. Biol.* **1990**, *214*, 143. b) J. Tang, B. S. Hartley, *Biochem. J.* **1970**, *118*, 611.
7 A. Tiselius, G. E. Henschen, H. Svensson, *Biochem. J.* **1938**, *32*, 1814.
8 M. Johnson, T. J. Pinnavaia, *Langmuir* **1990**, *6*, 307.
9 L. A. Forato, R. Bernardes-Filho, L. A. Colnago, *Anal. Biochem.* **1998**, *259*, 136.
10 The sample for TGA was prepared by heating the freeze-dried hybrid hydrogel in toluene at 84 °C, which is azeotropic temperature between $H_2O$ and toluene, and residual toluene was removed by vacuum drying.
11 The sample for CLSM observation was prepared with hybrid hydrogel composed of pre-labeled imogolite and pepsin. Imogolite and pepsin were labeled with fluorescein 5-isothiocyanate and adenosine 5′-triphosphate, Alexa Fluor® 647 2′-(or-3′)-*O*-(*N*-(2-aminoethyl)-urethane), hexa(triethylammonium) salt, respectively. For FE-SEM observation, hybrid hydrogel was dehydrated by freeze-drying and the specimen was prepared by pre-coating dehydrated hybrid hydrogel with $OsO_4$.
12 Each sample of hybrid hydrogel and free pepsin solution was mixed with 2.5 mL of 2.5 wt % hemoglobin solution and incubated at 37 °C for 10 min. Next, 5 mL of 5 wt % trichloroacetic acid was added to the mixture and the reaction mixture was incubated at 37 °C for 1 h. After centrifugation and filtration, the filtrate products were measured by UV–vis spectroscopy to estimate the reaction rate, $\Delta A_{280}$/min. $\Delta A_{280}$/min shows the change in absorbance at 280 nm.
13 D. G. Shchukin, G. B. Sukhorukov, R. R. Price, Y. M. Lvov, *Small* **2005**, *1*, 510.

Published on the web (Advance View) January 14, 2006; DOI 10.1246/cl.2006.194