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Effective encapsulation of laccase in an aluminium silicate nanotube hydrogel

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Aluminium silicate nanotubes (ASNT), with a length to width ratio of four, were synthesized from aluminium chloride and sodium silicate, and the ASNT hydrogel was easily prepared by adjusting to pH 7. The hydrogel nanotube concentration was 1.5 wt%. Laccase, a type of oxidase, was encapsulated during ASNT hydrogel formation. This encapsulation method will have fewer negative effects on the relatively unstable enzyme because of the milder conditions used, which are different from sol–gel silica formation by acidic catalysis using strong acids such as hydrochloric acid. The obtained hydrogels were fully characterized by various methods such as field-emission scanning electron microscopy and Fourier transform infrared spectroscopy. The ASNT-hydrogel encapsulated enzyme worked well; notably, laccase-ASNT hydrogels prepared from the shortest nanotubes exhibited higher activity than the free laccase in solution because of an improvement in substrate affinity of the encapsulated enzyme. Tryptophan fluorescence spectroscopy indicated that the highly ordered structure of laccase was not altered once bound to nanotubes within the hydrogel. Notably, after nine repeated reactions, laccase encapsulated in ASNT hydrogel retained its activity. The cycle performance of the encapsulated enzyme indicates no enzyme was released from the ASNT hydrogel. In addition, the laccase-ASNT hydrogel was easily used to prepare transparent thin films on glass cover slips, while still maintaining enzyme activity. Another oxidase, myoglobin, was also encapsulated in the same type of ASNT hydrogel. Although free myoglobin in solution demonstrated oxidation activity, the bound protein’s activity was remarkably decreased due to changes in its tertiary structure.

Introduction

Encapsulation technologies have attracted considerable attention because of an increased interest in fields such as biotechnology, medicine, pharmaceuticals, catalysis, ecology and nutrition.1–3 Encapsulation can concentrate and shield biomolecules to protect them in a defined volume and to create a single compartment that represents a microenvironment separated from the outer environment. Biomolecules have industrial applications based on various chemical and physical methods such as solvent evaporation, coacervation, interfacial polymerization and matrix (organic or inorganic) entrapment.4,6 As an effective encapsulation technique, there are several strategies for encapsulating biomolecules in sol–gel silica materials.7–10 However, most of the strategies are based on the usual process, in which alkoxysilanes are mixed with an alcohol and polymerized in the presence of acidic or basic conditions. Although this method has been used successfully for many years, it involves heating the particles to temperatures well above ambient temperature and the use of highly basic pH; both conditions are destructive for proteins and other biomolecules. As other techniques of enzyme immobilisation within sol–gel matrices, aqueous routes have been introduced to overcome the difficulties regarding alcohol release and polymerisation conditions.11 However, taking aside the alcohol release issue, other problems regarding porosity and surface charge could be responsible for the partial loss of function of the immobilised enzymes within pure silica hydrogels.12

The typical aluminium silicate nanotube material, imogolite, is a naturally occurring single-walled nanotubular aluminium silicate with an empirical formula of (OH)1-Al2O3SiOH.13–15 Imogolite has an inner diameter of around 1.0 nm, an external diameter of around 2.5 nm and a length ranging from hundreds of nanometres to the micrometre scale. The inner surface of the nanotubes comprises Si–OH groups, while the outer surface is covered in Al–OH groups. These hydroxyl groups make the nanotubes hydrophilic, and the positive charges on the outer surface under acidic condition allow the
bioremediation. In addition, laccase has a high catalytic activity, and with 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as its electron donor, laccase can be efficiently coupled to carbon electrodes. One approach towards the design of an implantable, membrane-less and bio-compatible biofuel cell comprises catalysing the oxidation of glucose at the anode using either glucose oxidase or glucose dehydrogenase enzymes, coupled to the reduction of dioxygen at the cathode by a dioxygen-reducing enzyme such as laccase. However, various laccase immobilisation methods on different solid supports have not been able to successfully immobilise the enzyme with retention of its full catalytic activity.

Myoglobin (Mb) is an oxygen-transport protein in mammalian muscle. Mb has a molecular weight (M_w) of about 17000 and contains a single electro-active iron heme as a prosthetic group. The Fe(III)/Fe(II) redox couple of the heme protein Mb yielded standard electron-transfer rate constants about 1000-fold larger on pyrolytic graphite electrodes than free Mb in solution. In addition, Mb catalyzed the epoxidation of styrene with the oxidants (H_2O_2 or tert-butyl hydroperoxide) in organic solvents. It is essential to develop adsorption and immobilization procedures and carrier materials for further bioanalytical utilization.

In this study, we investigated the encapsulation of oxidative enzyme, laccase, in ASNT hydrogel. We focused on two different aspects, namely, the length of nanotubes used, which will maintain enzyme activity and the conformation change of the enzyme in the hydrogel. With the protein immobilized in the ASNT hydrogel, the confinement effect tends to stabilize the protein molecules. First, we synthesized an ASNT hydrogel with various nanotube lengths (1, 2, 4 and 7 days ageing) to understand the effect on enzyme encapsulation and catalytic activity. Second, the conformational change of laccase was discussed. For this, the enzyme was encapsulated in ASNT hydrogel, and changes to its tertiary structure were estimated by fluorometric techniques. In addition, transparent films from the laccase-ASNT hydrogel were easily produced by a drop-coating method, and these enzyme films worked well.

The purpose of this work was to investigate the use of the oxidative enzymes, laccase and myoglobin, encapsulated in ASNT hydrogel as a new technology for stable biocatalysis. Our investigation revealed superior catalytic activity in the optimized enzyme-ASNT hydrogel composite and excellent reusability associated with structural stability.

**Experimental**

**Materials**

Aluminium chloride (AlCl_3·6H_2O) was purchased from Kanto Chemical Co., Tokyo, Japan. Sodium silicate (Na_2SiO_3·nH_2O; SiO_2= 20.3 wt%, Na_2O=40.1 wt%) was purchased from Kishida Chemical Co., Osaka, Japan. Laccase (from *Trametes versicolor*, Cat. NO. 51639; M_w = 70 kDa; pI = 3.5; molecular size = 8 nm × 9 nm × 9 nm) and Mb (from equine skeletal muscle, Cat. NO. M0630; M_w = 17.6 kDa; pI = 7.3) were obtained from Sigma–Aldrich, St. Louis, MO, USA. ABTS was acquired from Tokyo Chemical Industry Co., Tokyo, Japan. Fluorescein labelling Kit-NH_2 was obtained from Dojindo Molecular Technologies, Kumanoto, Japan. Protein assay dye reagent was purchased from Bio-Rad Laboratories,
Hercules, CA, USA. All materials were of analytical grade and used as received without further purification.

**Preparation of aluminium silicate nanotube (ASNT)**
ASNT, i.e. imogolite, was prepared according to the procedure described in a previous report (Fig. 1).\(^{26,27}\) Sodium silicate (18.47 g, Na\(_2\)SiO\(_4\)) and aluminium chloride (9.20 g, AlCl\(_3\)·6H\(_2\)O) were dissolved in 500 g of distilled water, respectively. After mixing, the solution was titrated with 1 M sodium hydroxide (NaOH) up to pH 6.8. The resulting samples were collected by centrifugation, and the obtained precipitates were washed three times with distilled water. Adding 12 L of distilled water that was acidified with 5 M hydrochloric acid was added to the solution, and the mixture was stirred for 10 min and then kept standing for 1 h at 4 °C. The produced ASNT was collected by centrifugation at 6,000 rpm for 15 min. The amount of ASNT obtained was ~100 mg.

The precursor solution was aged at 95 °C for 1, 2, 4 and 7 days, which controlled the length of ASNTs. Finally, aqueous solutions of 0.087 wt% of dispersed ASNTs were prepared in distilled water that was acidified with 5 M hydrochloric acid and then a small aliquot was taken from a pH 7.0 with 0.4 M NaOH. Laccase solution (100 µL) was added to the solution, and the mixture was stirred for 10 min and then kept standing for 1 h at 4 °C. The produced laccase-ASNT hydrogel was collected by centrifugation at 6,000 rpm for 15 min. The amount of ASNT hydrogel obtained was ~100 mg.

**Laccase encapsulation in ASNT hydrogel**
Each heated ASNT dispersed solution (3.0 g) was adjusted to pH 7.0 with 0.4 M NaOH. Laccase solution (100 µL, 0.5 mg mL\(^{-1}\)) was added to the solution, and the mixture was stirred for 10 min and then kept standing for 1 h at 4 °C. The produced laccase-encapsulated ASNT hydrogel was collected by centrifugation at 6,000 rpm for 15 min. The amount of ASNT hydrogel obtained was ~100 mg.

**Characterization of laccase-ASNT hydrogel composite**
The morphology of the products was characterized using a field-emission scanning electron microscope (FESEM, Hitachi S-4700, Hitachi, Tokyo, Japan). Transmission electron microscopy (TEM) images were acquired using a JEOL JEM 2010 (JOEL, Tokyo, Japan) model equipped with a CuKa source. The 2θ scanning range was set between 3° and 60°; generator settings were 40 kV and 30 mA. Zeta potentials of ASNT suspension and free laccase were determined in a solution at pH 7.0 using a zeta potential and particle size analyser (ELSZ-2, Otsuka Electronics Co., Japan).

**Catalytic activity of laccase encapsulated in ASNT hydrogel**
The potential for applying the ASNT hydrogel as biocatalyst and biosensor based on catalytic activity was investigated using laccase. The peroxidase activity of laccase was measured using ABTS and O\(_2\). ABTS is water soluble and has a strong absorption band at 340 nm,\(^{33}\) while its oxidized derivative is blue–green in colour and its absorption band is shifted to 420 nm. For TEM studies, the synthesized ASNT suspension was diluted 10 times by distilled water, and then a small aliquot was taken from a suspension and placed in a lacy carbon-coated TEM grid that was pulled through suspension and allowed to dry in air. Fourier transform infrared spectroscopy (FT-IR) spectra were obtained using an MFT-2000, JASCO, Tokyo, Japan. For FT-IR analyses, the samples were pelletized with KBr (sample/KBr = 1:100) using a hydraulic press. Thermogravimetry and differential thermal analysis (TG and DTA) were performed on an SSC-5200, SEIKO Instrument, Tokyo, Japan. Peaks were collected from room temperature to 1050 °C (heating rate = 10 °C min\(^{-1}\)). Atomic force microscope (AFM, scanning probe microscope, SPA-400, SII Nano Technology Inc., Tokyo, Japan) was also used to investigate the morphological characteristics of ASNT. An area of 5 µm × 5 µm was scanned using a 110 µm scanner and an SI-D2F-20 cantilever (SII Nano Technology Inc.). Rheological properties of the ASNT hydrogel were measured using a rheometer (MCR101, Anton Paar Japan, Tokyo, Japan). A 500 µL sample of the ASNT hydrogel was placed on a stainless parallel plate of 25 mm in diameter, and the geometric gap was set to 1 mm. The storage modulus (G\(^′\)) and loss modulus (G\(^′′\)) were measured at various strain amplitudes at f =1 Hz. In addition, the frequency dependence of both moduli of the samples was measured from 0.1 to 10.0 Hz at 25 °C. Powder X-ray diffraction (XRD) analysis was performed using a RINT2000/PC (Rigaku Co., Tokyo, Japan) model equipped with a CuKa source. The 2θ scanning range was set between 3° and 60°; generator settings were 40 kV and 30 mA. Zeta potentials of ASNT suspension and free laccase were determined in a solution at pH 7.0 using a zeta potential and particle size analyser (ELSZ-2, Otsuka Electronics Co., Japan).

Oxidative activity of Mb was measured by a procedure similar to that for laccase. Free Mb (0.05 mg) or Mb in ASNT hydrogel was added to a solution of 100 µL of ABTS, 20 µL of a 8.8 mM hydrogen peroxide solution (H\(_2\)O\(_2\) as an oxidant) and 780 µL of a 10 mM phosphate buffer solution (pH 7.0), and then laccase in ASNT hydrogel was allowed to react for 10 min at 30 °C. Subsequently, the absorbance at 420 nm for each reaction mixture was measured using a UV-vis spectrophotometer (V-560, Jasco Co., Tokyo, Japan). In addition, an ASNT hydrogel reaction mixture without laccase was prepared as a blank, and its absorbance was measured at 420 nm. The activity of free laccase was expressed as 100%. The recyclability of laccase in hydrogel was also determined using a similar procedure.

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Conformational changes of lactase in ASNT hydrogel

The experiments comprised monitoring the fluorescence of tryptophan residues, which gives information on the tertiary state of the protein. For analysis, lactase in hydrogel (~100 mg) was suspended in 3 mL of a 10 mM phosphate buffer solution (pH 7.0). After stirring in the dark at 25 °C for 30 min, fluorescence emission spectra of lactase were recorded at excitation and fluorescence wavelengths of 280 nm and from 300 to 450 nm, respectively.

Drop-coating of Laccase-ASNT hydrogel composites

Lactase was conjugated with fluorescein isothiocyanate (FITC) according to the procedure provided from supplier (Dojindo Molecular Technologies). FITC-conjugate lactase (100 µL; 1 mg 100 µL⁻¹) was mixed in 3 mL of 1-day ASNT solution adjusted to pH 7.0, and the mixture was stirred at 4 °C for 10 min. The resulting solution (150 µL) was drop-coated on 15 mm-diameter cover glass cover slip (cleaned by acetone), and the film was dried at room temperature for 24 h. In addition, the dot coating of lactase in 1-day ASNT solution was performed using 1 µL of the above solution on the cover slip. The obtained samples were immediately observed using a fluorescent microscope (BX51, Olympus Co., Tokyo, Japan) with a band-pass filter.

Results and discussion

Encapsulation of lactase in ASNT hydrogels and their characterization

With this AFM specimen preparation method, direct observation of individual ASNT with definite dimensions could be achieved. It also makes the investigation of the growth process of the nanotubes possible because the concentration and the exact length of the nanotubes can be quantitatively analysed through our controlled experiments. Figure 2 shows AFM images of the ASNT obtained after heating periods of 1, 2, 4 and 7 days. As shown in Fig. 2, a small number of the nanotubes could be discerned out of the amorphous product, indicating the formation of ASNT as early as after a one day reaction. The lengths of the nanotubes are below ~50 nm. For the rest of the images in Fig. 2, the concentration of the ASNT increases with reaction time, especially after two days, and the length of the nanotubes falls into a fairly large range. The lengths of ASNTs were increased when the reaction periods were longer, and over 1 µm and 3 µm of ASNTs were obtained after a heating period of 4 days and 7 days, respectively. Moreover, it should be noted that the diameter of the nanotubes remains almost unchanged throughout the synthesis. According to the paper described previously, the isoelectric point of free lactase is approximately pH 3.5, and the value of the zeta potential of free lactase in a solution at pH 7.0 was ~22.5 mV. In addition, the value of ASNT suspension was +33 mV, suggesting that the lactase protein molecules with negatively charge will be encapsulated inside the ASNT hydrogel with positively charge.

The morphology of the freeze-dried ASNT samples was characterized using FE-SEM and transmission electron microscopy (TEM). Figure 3A shows the bare ASNT hydrogel prepared according to procedure provided in the experimental section. Once the surrounding pH was brought up to pH 7.0 using 0.4 M NaOH, turbid and non-flowing hydrogel was formed after centrifugation of the mixture. The weight of ASNT hydrogel obtained was 108 mg, prepared from the 3 g of 1-day ASNT dispersed solution, and 1.6 mg of freeze-dried solid material was obtained from the ASNT hydrogel. This result indicated that 1.5% (w/w) (98.5% water) ASNT was present in the hydrogel. Subsequently, the mixture was manually mixed for 30 s, and thus the turbidity disappeared within 15–20 s and the mixture turned into a translucent...
solution. Figures 3B (low-magnification) and 3C (high-magnification) shows FE-SEM images of ASNT hydrogel. The large aggregates with 1–10 µm size were formed from the small particles (<50 nm) of ASNT bundles. Figure 3D shows ASNT bundles forming a continuous network structure, having lengths between 100 and 300 nm and widths between 30 and 40 nm.

Figure 4A shows the XRD patterns of the bare ASNT and laccase-ASNT composites. In this study, the ASNTs prepared showed typical peaks at 26° and 40° that correspond to amorphous ASNT, and a broad peak at ~9° shows that tubular units of ANSTs exist. The XRD patterns after encapsulation of laccase within ASNT hydrogel was unchanged compared to bare ASNT hydrogel, indicating that enzyme encapsulation have no effect on the structure of ASNTs.

TG-DTA curves for bare ASNT and laccase-ASNT composites are shown in Fig. 4B. The ~15% weight loss observed for both composites is observed from 100 to 120 °C due to the presence of moisture (H2O). The weight loss for samples between 350 and 450 °C is assigned to the decomposition of the enzyme’s protein component, as shown in Fig. 4B. The weight loss percentage of the laccase-ASNT composite is 2%–3%, which agrees with the value corresponding to the weight of the enzyme inside the silica (0.05 mg enzyme/~1.50 mg total mass of ASNT freeze-dried gel).

ASNT gel encapsulation of laccase was also monitored by FT-IR spectroscopy (Fig. 5). The characteristic peaks of bare ASNT hydrogel at 960 cm⁻¹, corresponding to the stretching of Al–O–Si group, and a peak at 1060 cm⁻¹ could be attributed to silica vibrations (Si–O–Si). The absorption bands observed at 3000–3600 cm⁻¹ could be assigned to the OH vibrational modes of hydroxyl groups. The H–O–H bending of water is observed at 1640 cm⁻¹. The FT-IR spectrum shown in Fig. 5 green line indicated a N–H deformation vibration band at 3350 cm⁻¹.

Fig. 4 (A) XRD patterns and (B) TG–DTA curves of 1-day ASNT hydrogel and laccase encapsulated ASNT hydrogel. Red line, bare ASNT hydrogel; blue line, laccase-ASNT hydrogel composite.

Fig. 5 FT-IR spectra of 1-day ASNT hydrogel (blue line), laccase encapsulated 1-day ASNT hydrogel (green line) and laccase only (red line).

Fig. 6 (A) Stain sweep measurement and (B) Frequency sweep measurement on 1-day ASNT hydrogel and laccase-encapsulated ASNT hydrogel. Red line, bare ASNT hydrogel; blue line, laccase-ASNT hydrogel composite.
1530 cm$^{-1}$ amides, and showed peaks for C–H stretching from 2830 to 2960 cm$^{-1}$, corresponding to the CH$_2$ of laccase proteins (Fig 5 red line), suggesting the encapsulation of laccase in the ASNT gel.

The linear viscoelasticity range was observed until $\gamma \sim 8\%$ and the crossover of $G'$ and $G''$ occurred at $\gamma = 30\%$–$40\%$ on bare and laccase-encapsulated ASNT hydrogel samples (Fig. 6A). The shear stress applied at the deformation points of both samples were measured as ~60 Pa. The onset of non-linearity could be interpreted as the onset of gel network breaking. The following frequency sweep test was conducted under an applied strain of 1%, where the samples are confirmed to be in the linear viscoelasticity range. The viscoelastic strength of the ASNT hydrogel indicated closed to 1,000 Pa of $G'$ for 1-day ASNT and small difference was observed for the hydrogel after encapsulation of enzymes (Fig. 6B). $G'$ and $G''$ moduli showed plateau regions, and $G' > G''$ for both samples throughout the frequency, suggesting that the samples behaved as a solid structure. The laccase encapsulated ASNT sample had a $G'$ of ~700 Pa, which was close to the value obtained for bare ASNT hydrogel, ~800 Pa. A gel with this stiffness is expected to be injectable using a syringe and needle, because one report showed that a peptide gel with a storage modulus of ~480 Pa was injectable.

The gel forming ability of the ASNT and its properties (strength) were hardly affected by the enzyme encapsulation; thus, this ASNT hydrogel achieved sufficient viscoelastic strength and is applicable as a carrier material for an enzyme or other biomolecules for applications such as drug delivery or biocatalysis.

**Oxidative activity of laccase in ASNT hydrogels**

Figure 7A shows the oxidation activity of laccase encapsulated in the ASNT hydrogel. The activity of free laccase in solution was used as control and was considered to be 100%. Maximum oxidation activity was observed for laccase encapsulated in the 1-day ASNT hydrogel; notably, this activity (134% relative activity) was higher than free laccase in solution. The oxidation activity of laccase-ASNT hydrogel decreased when heating time in ASNT preparation was increased and was 97% for 2 days, 78% for 4 days and 61% for 7 days, respectively.

Furthermore, we explored the cycle performance of laccase encapsulated in ASNT hydrogel, as shown in Fig. 7B. During the test, the remaining activity was recorded after each cycle. The laccase-ASNT hydrogel retained ~100% of its initial activity, even after nine repetitions. This proves the recyclability of laccase encapsulated in ASNT hydrogel. This could be attributed to the decreased enzyme release in ASNT hydrogel because of the strong interaction between the enzyme and ASNT networks.

**Enzyme kinetics and conformational changes of laccase encapsulated in ASNT hydrogel**

To evaluate the difference in oxidation activity of laccase-encapsulated ASNT hydrogel, the kinetic properties of the composite prepared from ASNTs of different nanotube sizes, including the Michaelis constant, $K_m$, and the maximum rate, $V_{\text{max}}$, were determined for the enzyme reactions (Table 1). The $K_m$ values, which are given by the concentration of substrate that leads to half-maximal velocity, for all hydrogels were smaller than that of the free laccase ($K_m = 2.85$ mM). These results show that ASNT hydrogels can facilitate substrate diffusion within their matrices. Particularly, the $K_m$ value of laccase encapsulated in the 1 day ASNT hydrogel ($K_m = 1.39$ mM) was notably higher when compared with that of the other samples (1.77 mM for 2 days, 1.79 mM for 4 days and 1.91 mM for 7 days), and hence achieved enhanced apparent enzymatic activity. $V_{\text{max}}$ values, the maximum velocity or rate at which the enzyme catalysed a reaction, of laccase-ASNT hydrogel composites was slightly lower compared to that of the free laccase.

Furthermore, we analysed the changes in the tertiary structure of laccase by tryptophan fluorescence spectra to study the influence of the interactions between the enzyme and ASNT hydrogel. Fluorescence from the amino acid tryptophan has

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<th>ASNT-laccase</th>
<th>$K_m$ (mM)</th>
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<tr>
<td>1 day</td>
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<td>7 days</td>
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long been known to be sensitive to the polarity of its local environments. It is well understood that the spectral parameters of tryptophan fluorescence emission are sensitive to the environment of tryptophans and that a protein's tryptophans can give insights on environmental changes during events such as folding or unfolding. For example, the \( \lambda_{\text{max}} \) of many proteins' emission scans will red-shift upon unfolding as the tryptophans become more exposed to polar solvents. From the amino acid sequence of \textit{Trametes versicolor} laccase obtained from a protein data bank (ID: 1GTC), this enzyme has seven tryptophans inside its structure, and relatively hydrophobic amino acids (valine, leucine, etc) exist on its surface (Fig. 8A).

Figure 8B shows the fluorescence emission spectra of tryptophan residues. As evidenced from the spectra, the conformational changes of the protein were observed after thermal treatment for 30 min at 90 °C, i.e. \( \lambda_{\text{max}} \) was shifted from 338 (free laccase) to 346 nm. In addition, free laccase after thermal treatment, as described above, has no oxidation activity, indicated by an 8 mm red-shift in \( \lambda_{\text{max}} \). Laccase in dried gel prepared by freeze-drying of ASNT hydrogel has extremely low (<10%) relative activity compared with free laccase. In this case, \( \lambda_{\text{max}} \) of the laccase was red-shifted to 344 nm. However, laccase encapsulated in ASNT hydrogel generated similar tryptophan fluorescence spectral profiles to those of free laccase in solution with equivalent \( \lambda_{\text{max}} \) and emission intensity. From these results, no conformational changes to laccase were observed in ASNT hydrogel and its high oxidation activity was retained inside the hydrogel matrix.

Coating of the laccase-ASNT hydrogel on glass and its activity

Since laccase is a dioxygen-reducing enzyme, it could be used for the cathode of a bio-fuel. For laccase-ASNT hydrogel to be used in various applications such as biofuel cell, biosensor and bioremediation, the hydrogel was coated on glass cover slips. The suspension (150 µL) was prepared by mixing FITC-labelled laccase and an ASNT dispersion solution, which was then dropped on a glass cover slip, and the resulting ASNT film was dried at room temperature for 24 h. The resulting transparent film was easily prepared and the laccase adsorbed on the nanotubes was found to be uniformly coated on the glass from fluorescence microscopy images (Figs. 9A and 9B). In addition, 1 µL of the above suspension was dot coated on the same glass cover slip. An approximately 100 µm diameter-sized dot of laccase-ASNT composite was obtained and the laccase was well distributed within the dot printed area (Fig. 9C). The enzyme activity of laccase-ASNT films were verified by ABTS oxidation with a similar procedure to the one used to test the laccase-ASNT hydrogel. Its activity (0.38 abs, as determined by absorbance of oxidized ABTS at 420 nm) was lower, about 25%, than that measured for free laccase (1.55 abs), indicating that the conformation of laccase was changed during preparation of the film, as was the case after the freeze-drying process described above. However, as enzyme reaction time was increased, activity rate was improved (1.70 abs), meaning that these laccase films are still active and are reusable (Fig. 9D). These results are very attractive for further bio-applications.

Myoglobin encapsulation in ASNT hydrogels

Fig. 8 (A) Three-dimensional structure of laccase, obtained from Protein Data Bank; ID: 1GYC. Red balls for tryptophans; blue balls for hydrophobic amino acids. (B) Conformational changes of laccase encapsulated in 1-day ASNT hydrogel, determined by tryptophan fluorescence spectra.

Fig. 9 Drop-coating of laccase in 1-day ASNT hydrogel on cover glass slip. (A) Image of laccase-ASNT on glass slip (15 mm φ). (i) laccase-ASNT film and (ii) no hydrogel on glass slip. (B) fluorescence microscopy of FITC-laccase in ASNT hydrogel coated on a slide. (C) fluorescence microscopy of FITC-laccase in ASNT hydrogel by dot-coating. (D) catalytic activity of laccase in ASNT hydrogel coated on a slide.
Laccase was encapsulated in ASNT hydrogel carefully prepared at pH 7, while maintaining protein activity and stability. The obtained ASNT hydrogels were fully characterized by FE-SEM, TEM, XRD, TG-DTA and FT-IR. In addition, rheological properties of the hydrogel with and without laccase were also determined by strain sweep and frequency sweep measurements. $G'$ and $G''$ moduli showed plateau regions, and $G'>G''$ for both samples throughout the frequency range, suggesting that the samples behaved as solid structures. Encapsulation of laccase did not affect the gel properties of the ASNT hydrogel. The performance of the laccase-ASNT hydrogel was evaluated on the basis of their enzyme activity and cycling characteristics to determine the optimal structure and length of the nanotube for enzyme encapsulation. Laccase encapsulated in ASNT hydrogel prepared from nanotube suspension heated for 1 day (1-day ASNT hydrogel), which has the shortest length of nanotubes tested, exhibited a higher activity than those of other ASNT hydrogels. Moreover, the rate of activity of laccase-ASNT hydrogel composites was 40% higher than that of free laccase activity because of an increase in substrate affinity and high conformational stability produced by the hydrogel networks. Furthermore, biocatalysts comprising laccase encapsulated in an ASNT hydrogel remained stable through repeated reaction cycles.

Finally, the laccase-ASNT hydrogel composite was easily prepared in film morphology by a drop-coating method, and the obtained enzyme films worked well on the glass cover slip. Therefore, this laccase-ASNT hydrogel system is a promising candidate for applications such as bio sensors, biofuel cells and biocatalysts.

### Acknowledgements
We thank Mr. Norito Morishita of the Aichi Institute of Technology for his technical assistance.

### Notes and references
Graphical Abstract

Laccase was encapsulated during Aluminium silicate nanotubes (ASNT), hydrogel formation. This encapsulation method has fewer negative effects on the relatively unstable enzyme because of the milder conditions used compared to sol–gel silica formation.