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Chemical Modifications of Electrospun Non-woven Hydroxypropyl Cellulose Fabrics for Immobilization of Aminoacylase-I

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Abstract

Because of its wide solubility range, Hydroxypropyl Cellulose (HPC) is suitable for fabricating fine-fiber materials *via* electrospinning. The resulting electrospun non-woven fabric (HPC-ESNW) requires an appropriate post-spinning treatment for applications in aqueous environments. In the present study, we examined the insolubilization of HPC-ESNW *via* cross-linking using bifunctional isocyanates. Modification of the fine-fiber surfaces with NCO groups enables introduction of cationic functionalities; we found that cationic Diethylaminoethyl (DEAE) groups are suitable for immobilization of aminoacylase-I onto these fine-fiber surfaces under mild conditions. The NCO groups can be also converted to amino groups, followed by activation with bifunctional *N*-hydroxysuccinimide (NHS) esters. The NHS-ESNW can chemically bind the aminoacylase-I. Two kinds of immobilized enzyme were tested for stereospecific recognition of a substrate and for immobilized activity yields. The results suggest that NCO-ESNW is a multi-purpose intermediate for chemical modification of ESNW and that the hydrophilic HPC-ESNW is a promising material for use as a matrix for enzyme immobilization.

Keywords: Aminoacylase; Chemical modification; Electrospinning; Hydroxypropyl cellulose; Immobilization

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1 Introduction

Electrospinning is a versatile method for fabrication of fine fibers directly from polymer solution and has been widely studied on spinning mechanisms and applications [1]. The fibers are typically recovered as non-woven sheets and often called as electrospun non-woven fabrics (ESNWs), which are composed of fine fibers having diameters of submicro- (200–600 nm) to nano- (> 100 nm) scales [2]. Applied research of the ESNWs encompasses biomaterial, filtration, catalyst support, composite, and so on [3]. Most of these focus the available greater surface on the ESNWs for material functions, as compared with other shapes such as films, membranes, or gels [4].

In a series of previous studies, we have described preparative methods of ESNWs using polysaccharides, chitosan [5, 6] and cellulose [7]. We also have reported several applied research works on the ESNWs prepared from polysaccharide [7, 8] and synthetic poly(amino acid)s under watery environments [9, 10]. Hydroxypropyl cellulose (HPC) is a soluble derivative of cellulose, which has propylene oxide units bound to the hydroxyl groups of the parent cellulose molecules (Scheme 1). Electrospinning of HPC has been already described by Shukla [11]. Due to widely ranged solubility of HPC, on the other hands, a post-spun treatment is required for insolubilization of the HPC-ESNWs for further usage, namely, insolubilization of HPC-ESNW allows it to be utilized for application in watery environments. One of appropriate post-spun treatments is chemical cross-linking using bifunctional cross-linker molecules, which is reactive towards the side chain hydroxyl groups of HPC.

The cross-linked HPC-ESNW has still hydrophilic fiber surfaces, which can be adopted for biological molecules to interact with the large surface area. In order to design a reasonable application associated with the material properties of HPC-ESNWs, here, we concern to evaluate the HPC-ESNW as a support matrix for biological catalysts, *i.e.*, enzymes. Aminoacylases and lipases have been studied as immobilized enzymes for a prolonged period [12-17] and in the present study, aminoacylase-I (E.C. 3.5.1.14) was selected as the first attempt for immobilization onto the HPC-ESNW, since the enzyme is the earliest catalyst for industrial production of optically pure amino acids [12]. The aminoacylase-I catalyses hydrolysis of N^{α}-acetyl-L-amino acids to yield free L-amino acids and acetates. In the case of aminoacylas-I from hog kidney, the enzyme has stereo-specificity for N^{α}-acetyl-L-amino acids and does not hydrolyze N^{α}-acetyl-D-amino acid [17] Methods for immobilization of aminoacylases have been already described mainly by Chibata *et al*, utilizing electrostatic interaction between the enzyme and cationic diethylamino ethyl (DEAE-) cellulose solids [12] and DEAE-Sephadex hydrated beads [13]. In order to adopt their concepts to the application of HPC-ESNW, requirements for efficient immobilization of aminoacylase-I is to find out a chemical modification procedure to give a cationic HPC-ESNW matrix, which should well coincide with the chemical cross-linking method of HPC-ESNW. In the present study, hexamethylene diisocyante (HMDI) was used for cross-linking the HPC molecules in as-spun ESNW, resulting in insolubilization, and the unreacted NCO groups were further available for addition reaction with N, N-(diethyl)ethylene diamine to introduce DEAE groups on the HPC-ESNW matrix (Scheme 2).

In addition to that, there have been reported the immobilization procedures using chemically activated matrix, which allows condensation reaction between the carboxyl groups on matrix and the side chain amino groups of enzyme molecules, leading to covalent bond formations between the matrix surfaces and enzyme molecules [16, 17]. The chemical binding for enzyme immobilization can be applied for the enzymes which does not adsorb onto the cationic matrix, and thus the matrix is used for immobilizing more wide range of enzyme species. As described above,

after cross-linking treatment with HMDI, the excess NCO groups remained unreacted on the HPC-ESNW matrix, and these NCO groups can be converted to amino groups *via* hydrolysis, which undergoes the chemical activation of the HPC-ESNW matrix using excess amount of *N*-hydroxysuccinimide (NHS-) diester derivatives, resulting in introduction of NHS groups on the matrix surfaces. The enzyme molecules, thus, can be immobilized *via* condensation reaction between the active NHS esters on the matrix and the side chain amino groups of the enzymes. This approach utilizing chemically activated HPC-ESNW matrix was also examined in the present study (Scheme 2). The enzyme-immobilized HPC-ESNWs were subjected to (i) quantification of immobilized enzyme, (ii) evaluation of catalytic activity and stereo-specificity, and (iii) comparisons of immobilized activity yields.

2 Experimental Part

2.1 Materials

HPC was purchased from Wako Pure Chem. Ind., Japan (Manufactures specification: M_w, 250 $\times$ 10^3 – 400 $\times$ 10^3; Molecular substitution, ca. 0.65). All chemicals were in the reagent grades and used without purification otherwise mentioned.

2.2 Electrospinning of Hydroxypropyl Cellulose

Hydroxypropyl Cellulose (HPC) (Fig. 1) was dissolved in 2-propanol [11] at the concentration of 3.0 wt% and 7.0 mL of the solution was placed in a plastic syringe (12 mL). The syringe was set on the electrospinning apparatus (a MECC, NANON3, Fukuoka, Japan). Electrospinning procedure was continued for 15 hr under the following processing parameters: voltage, 30 kV; tip-to-collector distance, 14.6 cm; pump rate, 0.1–0.5 mL/h. The as-spun HPC-ESNWs were recovered on an aluminum sheet and dried under reduced pressure.

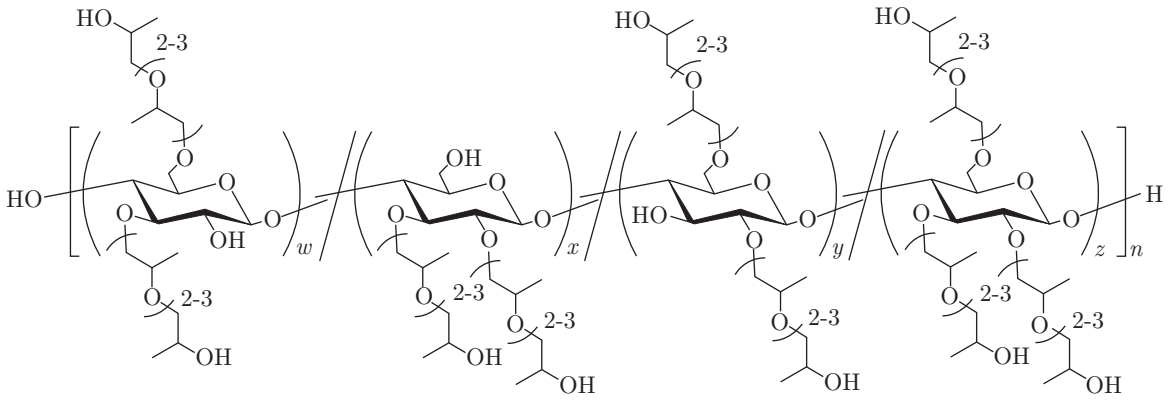


Fig. 1: Structure of hydroxypropyl cellulose

2.3 Insolubilization of As-spun HPC-ESNW

A bifunctional isocyanate, Hexamethylene Diisocyanate (HMDI), was used for cross-linking the hydroxyl groups of HPC in order to insolubilize the as-spun ESNW. Stoichiometry between the hydroxyl and isocyanate groups were fixed at 4.0 equivalent molar excess for isocyanate group, which means that the HMDI were added in 2.0 equivalent molar excess than the hydroxyl groups. In a typical procedure, a small piece (ca. 1.0 x 1.0 cm^2) of the as-spun HPC-ESNW was prepared and the weight was determined to estimate the contents of the hydroxyl groups. The specified amount of HMDI was dispensed into 40 μL of absolute hexane (distilled from sodium), and then the solution was added dropwise onto the HPC-ESNW. The solvent hexane was immediately evaporated and the HPC-ESNW was incubated at 50 ℃ for 2 hr, then the excess HMDI was washed with absolute hexane repeatedly. The dried ESNW should be kept in vacuum desiccator until used for the next reaction. The HMDI-treated ESNW is designated as the NCO-ESNW, because the material has reactive NCO groups on the surface of fine fibers.

2.4 Preparation of Cationic HPC-ESNW Matrix

Four equivalent molar of *N*, *N*-(diethyl)ethylene diamine towards hydroxyl groups on the as-spun HPC-ESNW was dispensed into absolute hexane (200 μL), and the solution was added dropwise onto the NCO-ESNW, followed by incubation for 2 hr at 50 ℃. The excess reagent was removed by repetitive immersion of the sample into absolute hexane and finally the sample was dried up. The cationic HPC-ESNW was designated as the diethylamino ethyl (DEAE)-ESNW.

2.5 Fourier-transform Infrared Spectroscopy

The Fourier-transform infrared (FT-IR) spectra were recorded using a Horiba FT-720 apparatus. The samples, as-spun HPC-ESNW, NCO-ESNW, or DEAE-ESNW, was placed on a holder, and the record of the spectra was performed by means of transmission mode.

2.6 Immobilization of Aminoacylase-I by Electrostatic Adsorption on DEAE-ESNW

Aminoacylase-I (E.C. 3.5.1.14, N^{α}-acetyl-L-amino acid hydrolase, from hog kidney) was purchased from Wako Pure Chemical Ind., Inc (Osaka, Japan). Aminoacylase-I was dissolved in a 10 mM phosphate buffer (pH 8.0) at the concentration of 1.0 mg/mL. The cationic DEAE-ESNW (ca. 1.0 x 1.0 cm^2) was placed on a small piece of polyamide mesh (200 mesh), and then the aminoacylase-I solution was dispensed onto the DEAE-ESNW and the preparation was allowed to stand for 8 hr at 4 ℃. The ESNW was briefly washed with the phosphate buffer and the adsorbed aminoacylase-I was fixed on the matrix with diluted glutaraldehyde (0.05%) solution for 1 hr at 35 ℃. The aminoacylase-I-immobilized ESNW (AI-DEAE-ESNW) was washed with the phosphate buffer repeatedly then kept in a phosphate buffer (pH 8.0) at 4 ℃ until use, since storage in frozen state will drain the catalytic activity of the immobilized aminoacylase-I.

2.7 Immobilization of Aminoacylase-I by Chemical Binding with SADS-ESNW

The NCO- ESNW (ca. 1.0 x 1.0 cm^2) was immersed in distilled water and incubated for 18 hr at 50 ℃. This procedure converts the excess isocyanate groups of the NCO-ESNW to primary amino groups (H_2N-ESNW) *via* an addition reaction between H_2O and NCO to form carbamic acid, followed by rapid decarboxylation. The amino group content in the H_2N-ESNW was quantified according to Ngo [18]. Suberic acid bis(3, 5-sulfo-*N*- hydroxysuccinimide ester) disodium salt (SADS) was dissolved in distilled water at 1.0 mM and 20 equivalent molar of SADS was added onto the H_2N-ESNW, followed by incubation for 10 hr at 40 ℃. The free SADS was removed by repetitive rinsing with distilled water. The resulting material is designated as *N*-hydroxysuccinimide-activated (NHS-)ESNW. Aminoacylase-I in phosphate buffer solution (pH 9.0) was prepared at the concentration of 20 mg/mL, and 30 μL of the aminoacylase-I solution was added onto the NHS-ESNW. After incubation at 4 ℃ for 8 hr, free aminoacylase-I was removed by washing the material repeatedly with the phosphate buffer. The aminoacylase-I-immobilized NHS-ESNW was designated as AI-SADS-ESNW.

2.8 Scanning Electron Microscopy

The fibrous structures of as-spun ESNW, HMDI-ESNW, H_2N-ESNW, enzyme immobilized AI-DEAE-ESNW, and AI-SADS-ESNW were observed under scanning electron microscope (SEM) (a Hitachi S3000, Japan), in order to compare the morphological changes of the ESNWs during the chemical modification procedures.

2.9 Quantification of Immobilized Aminoacylase-I on ESNWs

Typically 0.5-1.0 mg of the AI-DEAE-ESNW or the AI-SADS-ESNW was taken for the complete hydrolysis, followed by automated amino acid composition analysis [19]. The AI-DEAE-ESNW, the AI-SADS-ESNW, or free aminoacylase-I sample was placed in an ampoule bottle, and then 260 μL of 6 N aqueous hydrogen chloride was added. The ampoule was sealed with oxygen torch under the reduced pressure, and the samples were heated at 110 ℃ for 24 hr to ensure complete hydrolysis. The ampoule was opened and the reaction mixture was dried up in a vacuum desiccator. The residue was dissolved in a acidic citrate buffer (pH 2.2) (Wako Pure Chemical., Ind., Osaka, Japan) and set on the automated sampler, which is attached to a Shimadzu LCVp10 amino acid composition analyzer system. The detection of the eluted amino acids was performed by a post-column labeling with *o*-phthalaldehyde chemistry. The peak areas of amino acids were calculated as the amino acid found in μmol per 1.0 mg of ESNWs or per 1.0 mg of free aminoacylase-I, on the basis of a standard curve and sample weight. Finally, the amino acids found in μmol/mg were calculated as the amount of aminoacylase-I immobilized on ESNW.

2.10 Preparation of Substrates

L-Methionine (L-Met) was suspended in acetic anhydride and the suspension was heated at 50 ℃ with stirring for 1 hr. Crystalline powder of L-Met was finally dissolved to give clear and transparent reaction mixture. Excess amount of water was then poured and the mixture was cooled below

4 ℃. The product, N^{α}-acetyl-L-Met (Ac-L-Met), was collected by filtration and re-crystallized from ethyl acetate and hexane. Other substrates, Ac-L-Met, Ac-D, L-Met, Ac-L-Phe, Ac-D-Phe, and Ac-D, L-Phe were synthesized by a similar manner.

2.11 Activity Assay of Immobilized Aminoacylase-I

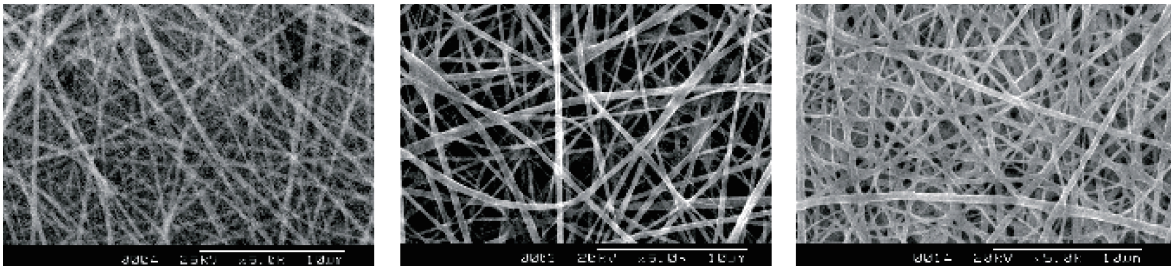
Each of the substrates described above was dissolved in a phosphate buffer (pH 8.5) at the concentration of 15 mM. The substrate solution (90 μL) was dispensed into a polypropylene tubes. Cobalt dichloride was dissolved in the same buffer at the concentration of 0.15 M and this stock solution was used for activating *aminoacylase-I* with dispensing 10 μL to the substrate solution. The control experiment was also performed using the same amounts of the phosphate buffer in the absence of $CoCl_2$. The AI-DEAE- and AI-SADS-ESNWs were cut into small pieces of which weights were typically 80-120 μg. The pieces were then immersed into the substrate solutions both in the presence and absence of $CoCl_2$.

The reaction mixtures were incubated at 25 ℃. After pre-determined incubation time, the supernatant (20 μL) was analyzed by means of the high performance liquid chromatography using a chiral column (OA4700, Sumika Chem, Osaka, Japan: mobile phase, methanol containing 20 mM ammonium acetate; flow rate, 0.5 mL/min). The eluate was continuously monitored (a Shimadzu SPA20M, Kyoto, Japan) by absorbance at 235 nm for Ac-L-Met, Ac-D-Met, and Ac-D, L-Met, and at 254 nm for Ac-L-Phe, Ac-D-Phe, and Ac-D, L-Phe. The remaining substrate was quantified from the peak area reduced upon catalytic deacetylation of the substrate by the immobilized aminoacylase-I. The specific activity of the immobilized aminoacylase-I was represented as nano-moles (nmol) of substrate hydrolyzed per minute (min) per 1.0 mg of the immobilized aminoacylase-I. Physical or electrostatic adsorption of the substrate onto the ESNW matrices affects the specific activity assay, so that the adsorbed amount was determined using the DEAE- and SADS-ESNWs, which were prepared in the absence of aminoacylase-I.

3 Results and Discussion

3.1 Insolubilization of HPC-ESNW

Fig. 2 (a)-(c) represents the SEM morphologies for a series of HPC-ESNW samples. The as-spun ESNW (Fig. 2 (a)) was composed of fine fibers of which average diameter was 186 ± 96 nm. A fine fiber network was observed, however, this material was completely soluble in water so that the as-spun HPC-ESNW cannot be directly used for the immobilization of enzyme in an aqueous media. The as-spun ESNW was treated with excess amounts of HMDI for insolubilization *via* cross-linking of HPC molecules. The resulting material (NCO-ESNW, Fig. 2 (b)) was also a fine fiber non-woven, and the average fiber diameter slightly increased to 226 ± 111 nm. The NCO-ESNW was immersed in warm water at 50 ℃ and dried to observe under a SEM (Fig. 2 (c)). The observation revealed that cross-linking with HMDI successfully insolubilized the material, which can retain the fibrous network structure upon immersion in water to give the H_2N-ESNW. The average diameter of fine fibers in the H_2N-ESNW was 345 ± 151 nm.



(a) (b) (c)

Fig. 2: (a) As spun HPC-ESNW; (b) NCO-ESNW; (c) H_2N-ESNW

3.2 Chemical Modification of ESNWs

Cross-linking reaction using excess amount of HMDI yielded the NCO-ESNW, which has active NCO groups on the fiber surfaces. This chemical conversion can be proven by means of FT-IR spectral changes before and after cross-linking reaction. The FT-IR spectrum of the as-spun HPC-ESNW exhibits the O-H stretching vibration at 3467 cm^{-1}, as well as a complex coupling of the C-H bending vibrations ranging from 1000 to 1200 cm^{-1} (Fig. 3, curve 1).

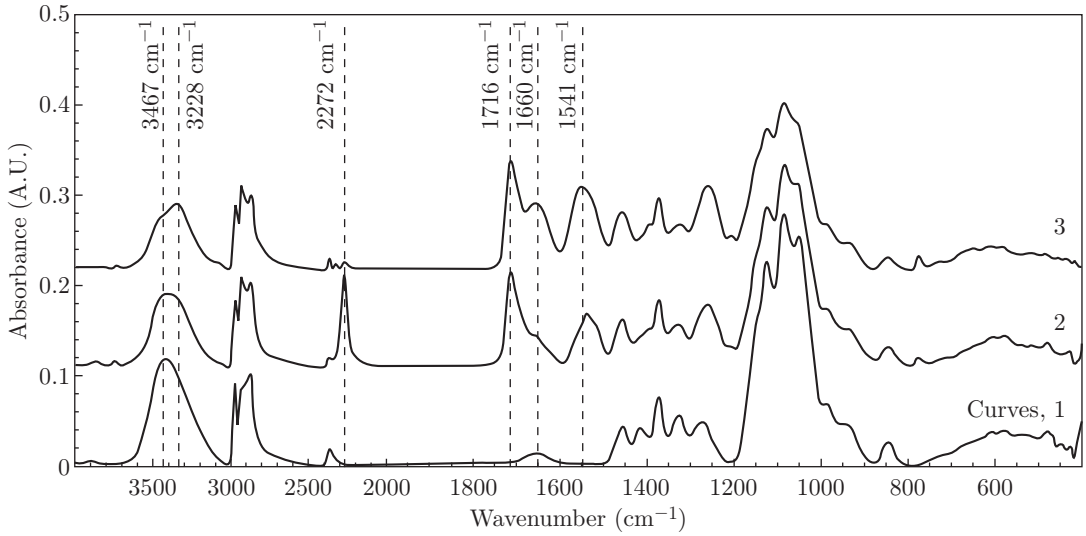


Fig. 3: FT-IR spectra of (Curves, 1) as-spun HPC-ESNW, (2) NCO-ESNW, and (3) DEAE-ESNW

The chemical cross-linking reaction with HMDI induces development of a new adsorption band at 1716 cm^{-1} for the C=O stretching vibration, indicating the presence of urethane bond, which is the adduct formed from the NCO group of HMDI and the OH group of HPC (Fig. 3, curve 2). The adsorption band at 2272 cm^{-1} found in the same spectrum can be assigned as the N=C=O stretching vibration, which indicates that excess NCO groups were left unreacted on the fiber surfaces. The reactive NCO groups are available for further modification of the NCO-ESNW. When *N*, *N*-diethyl(ethylene diamine) was added to the NCO-ESNW, remarkable spectral changes were observed as follows (Fig. 3, curve 3): the stretching vibration of NCO at 2272 cm^{-1} was mostly disappeared, while relative intensities of adsorption bands at 3228 cm^{-1} (N-H stretching), 1660 cm^{-1} (C=O stretching plus N-H bending), and 1541 cm^{-1} (C-N stretching plus N-H bending) increased. These spectral changes indicate successful conversion of the NCO groups to the urea derivative, which is an adduct from the NCO group on ESNW and the NH_2 group of *N*, *N*-diethyl

(ethylene diamine). Based on these results, conversion process and resulting chemical structure of the cationic DEAE-ESNW can be illustrated in Fig. 4. Conversion of the NCO-ESNW to the H_2N-ESNW, followed by NHS-ESNW (Fig. 5), was also confirmed by means of FT-IR in a similar manner (data not shown).

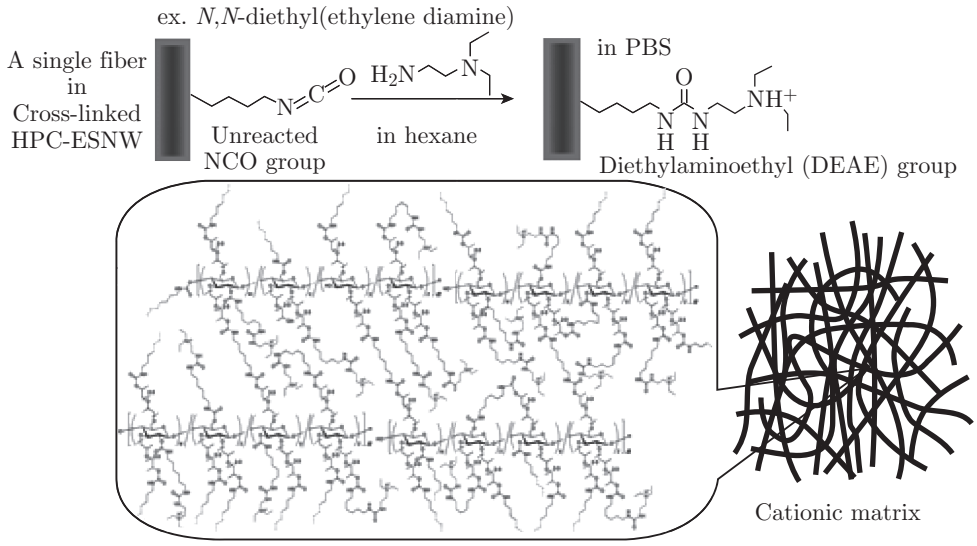


Fig. 4: Preparation of DEAE-ESNW

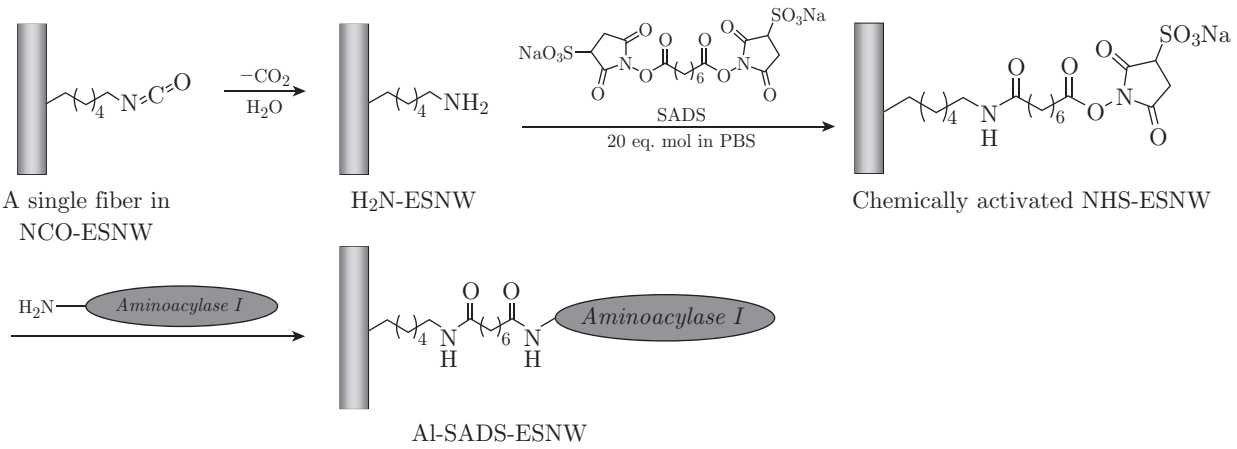


Fig. 5: Preparation of NHS-ESNW and AI-SADS-ESNW

3.3 Comparison of Immobilization Efficiency between DEAE- and NHS-ESNWs

Fig. 6 represents the elution profile of the automated amino acid composition analysis, and the amino acids found for free aminoacylase-I (Fig. 6, trace 1) were used for quantification of the amount of aminoacylase-I immobilized on the DEAE- (trace 2) and the NHS- (trace 3) ESNWs. During acidic hydrolysis, glutamine (Gln) and aspargine (Asn) residues in the aminoacylase-I molecules were converted to glutamic acid (Glu) and aspartic acid (Asp), respectively, so that the amount of Glu in the hydrolysate was a sum of Glu and Gln, which is reasonable for a

representation as "Glx (= Glu + Gln)", as well as the similar manner for "Asx (= Asp + Asn)". The hydrolysate of free aminoacylase-I exhibited five kinds of enriched amino acids, Glx, Leu, Asx, Ala, and Gly (Fig. 6, trace 1), and the elution patterns of these major amino acids seemed mostly identical to the hydrolysates of the AI-DEAE- (trace 2) and the AI-SADS-ESNWs (trace 3).

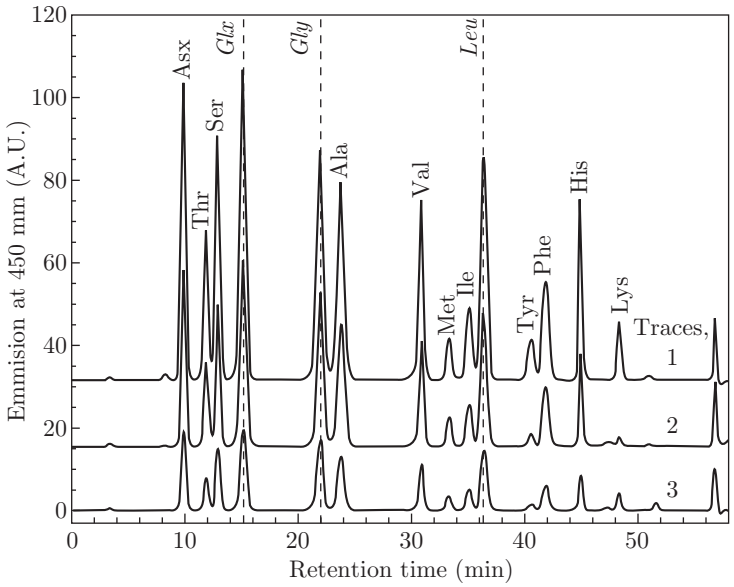


Fig. 6: Elution profiles of hydrolysates from (1) free aminoacylase-I, (2) AI-DEAE-ESNW, and (3) AI-SADS-ESNW. Elution peaks used for quantification of immobilized aminoacylase-I are indicated by broken lines on Glx (= Glu + Gln), Gly, and Leu

The peak area for each amino acid eluted was represented as the amino acids found in μmol per 1.0 mg of ESNW or aminoacylase-I, and also as residues per thousand (RPT), latter of which means the ratio of amino acid found per 1000 residues (Table 1). In the case of free aminoacylase-I, the RPT values were compared with theoretical RPTs (TRPTs), which are deduced from primary amino acid sequence of aminoacylase-I with referencing the UniProt gene database #P37111(ACY1–PIG). The RPT values for all amino acid found in free aminoacylase-I were coincident with corresponding TRPT values, particularly for the Glx (= Gln + Glu), Gly, and Leu, indicating that free aminoacylase-I was completely hydrolyzed under the present condition. Among 14 kinds of amino acids, Glx, Gly, and Leu, were chosen for calculating the amount of immobilized aminoacylase-I using the Eq. (1) as follows,

$$W_{im} = \frac{1}{3}\left(\frac{F^{Glx}_{ESNW}}{F^{Glx}_{fAI}} + \frac{F^{Gly}_{ESNW}}{F^{Gly}_{fAI}} + \frac{F^{Leu}_{ESNW}}{F^{Leu}_{fAI}}\right) \tag{1}$$

here, W_{im} is weight of immobilized *aminoacylase-I* per 1.0 mg of ESNW. The F^{Glx}, F^{Gly}, and F^{Leu} are amino acid found in μmol for these residues per 1.0 mg of ESNW or free *aminoacylase-I*, and the subscripts *ESNW* and *fAI* indicate the values for ESNW and free aminoacylase-I, respectively. Based on the calculation, immobilized amount of the *aminoacylase-I* were estimated as 25 μg/mg for AI-DEAE-ESNW and 11 μg/mg for AI-SADS-ESNW. The reason why the DEAE-ESNW exhibited a higher value than the SADS-ESNW is that the DEAE-ESNW can condense aminoacylase-I molecules by an electrostatic adsorption onto the cationic ENSW matrix at pH

8.0. The NHS-ESNW, on the other hand, still has an advantage to bind any enzymes which do not adsorb on the ESNW at a given pH condition for immobilization.

Morphologies of the aminoacylase-I-immobilized ESNWs were observed under SEM (Fig. 7). Both in the cases of the AI-DEAE- (Fig. 7 (a)) and the AI-SADS-ESNWs (Fig. 7 (b)), the fiber net works were retained, and all fibers in the microscopic fields seemed hydrated, suggesting the presence of the enzymes on the surfaces of the fiber. The average fiber diameters are 422 ± 221 nm for AI-DEAE-ESNW and 448 ± 190 nm for AI-SADS-ESNW. These values are 2.27 folds and 2.41 folds thicker than that of the as-spun ESNW (186 ± 96 nm), respectively for the AI-DEAE- and the AI-SADS-ESNWs.

Table 1: Amino acid composition analysis

	free AI			AI-DEAE-ESNW		AI-SADS-ESNW	
	Found[a)]	RPT[b)]	TRPT[c)]	Found	RPT	Found	RPT
Asx	641	89.9	73.2	15.1	85.9	6.69	80.3
Thr	385	54.0	65.0	9.9	56.6	3.81	45.7
Ser	531	74.4	70.5	12.3	70.0	5.24	62.9
Glx	947	133	130	22.7	129	9.63	116
Gly	615	86.3	78.6	16.8	96.1	7.65	91.7
Ala	639	89.6	97.6	15.7	89.6	6.66	80.0
Val	634	88.9	100	14.7	83.9	5.87	70.5
Met	161	22.6	24.4	4.6	26.1	1.94	23.2
Ile	277	38.9	29.8	6.3	36.2	3.12	37.4
Leu	704	98.7	97.6	16.6	94.9	7.32	87.8
Tyr	220	30.9	24.4	2.5	14.4	1.23	14.8
Phe	370	51.9	62.3	8.8	50.1	3.78	45.4
His	225	31.6	40.7	5.0	28.8	2.04	24.4
Lys	402	56.4	46.1	2.5	14.3	5.43	65.2
Arg	380	53.3	59.6	21.7	124	12.9	155
Total	7132	1000	1000	175	1000	83.4	1000

[a)]amino acid found in μmol per 1.0 mg free aminoacylase-I (μmol/mg AI) or ESNW (μmol/mg ESNW). [b)]residues per thousand. [c)]theoretical RPT deduced from primary sequence of aminoacylase-I.

3.4 Stereo-specific Recognition of Substrate

Fig. 8 represents the elution profiles of the chiral column chromatographies. The activity test was performed in turns with Ac-L-Met for the first time, then Ac-D-Met for the second time, and finally Ac-D, L-Met for the third time, using the same AI-DEAE-ESNW sample in the presence or absence of Co^{2+} ion as an activator of *aminoacylase-I*. The unhydrolyzed peak area of the substrate Ac-L-Met was defined as 100 %, and upon progress of hydrolytic removal of N^{α}-acetyl groups from the substrate molecules, the peak area was reduced. The remaining amount of the substrate was thus estimated from the reduced magnitudes of the substrate peak area before and after enzymatic hydrolysis. In the absence of Co^{2+}, the peak area of the unhydrolyzed Ac-L-Met (Fig. 8 (a), trace 2) was decreased by 38 % after incubation time for 72 hr, indicating that 62 % of

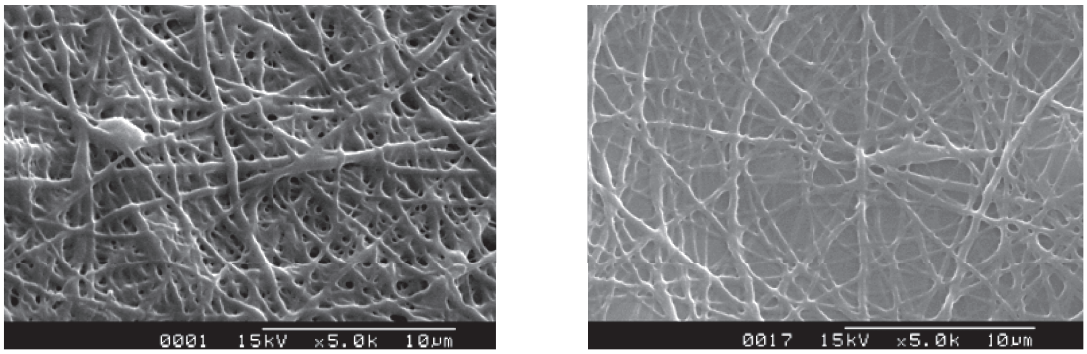


Fig. 7: Morphologies of aminoaclyase-I-immobilized ESNWs: (a) AI-DEAE-ESNW and (b) AI-SDAD-ESNW

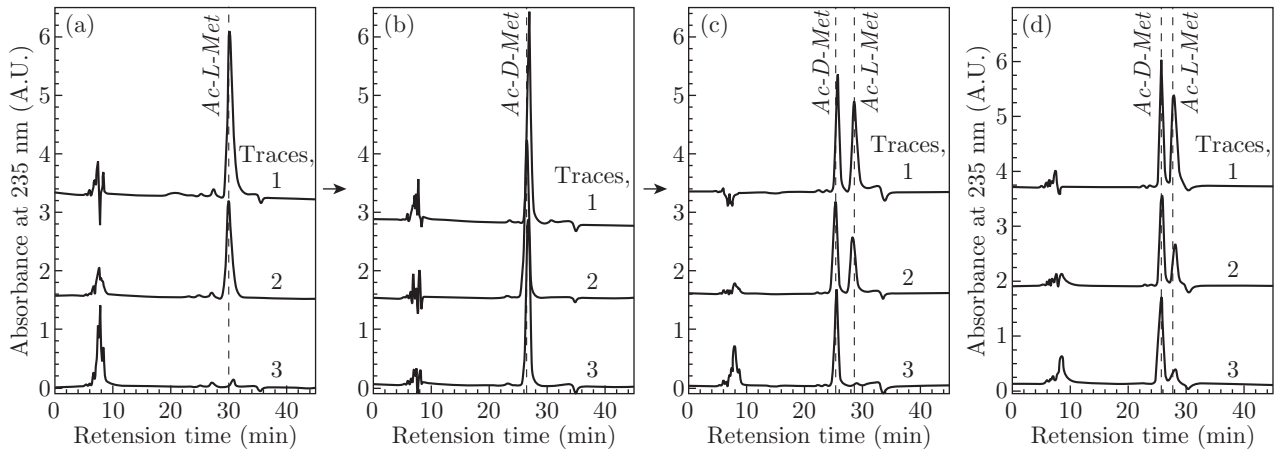


Fig. 8: Chiral column chromatography profiles of enzyme reaction mixtures containing aminoacylase-I-immobilized ESNW and substrates: (a) AI-DEAE-ESNW and Ac-L-Met, (b) AI-DEAE-ESNW and Ac-D-Met, and (c) AI-DEAE-ESNW and Ac-D, L-Met, after reaction for 72 h. Arrowheads indicate that these three experiments were performed using the same ESNW sample in turns. Panel (d) is a profile of AI-SADS-ESNW and Ac-D, L-Met after reaction for 140 h. Trace 1 is unhydrolyzed substrate, and traces 2 and 3 are profiles of enzyme reactions in the absence and presence of Co^{2+} ions, respectively

the substrate was left unhydrolyzed. In the presence of Co^{2+} ions, the most remarkable decrease of the peak area was found as 96 %, implying that the remaining substrate was only 4 % (Fig. 8 (a), trace 3).

At the second experiment using Ac-D-Met as the substrate, after the incubation for 72 hr, the original peak area was decreased by 16 % and 17 % in the absence and presence of Co^{2+} ion, respectively (Fig. 8 (b), traces 2 and 3). The decrease of the peak areas, however, are not due to hydrolysis of the substrate, but probably the physical adsorption of Ac-D-Met on the cationic matrix of the AI-DEAE-ESNW, because of the fact that, in the case of the DEAE-ESNW on which the enzyme was not immobilized, the peak area of the Ac-D-Met decreased by the similar magnitude (data not shown), suggesting physical adsorption of the substrate by an electrostatic interaction between the cationic DEAE group and the α-carboxyl groups of Ac-D-Met.

At the third experiment using the racemic mixture of the substrate, Ac-D, L-Met, the peak areas of Ac-L-Met and Ac-D-Met were decreased by 32 % and 13 %, respectively in the absence

of Co^{2+} ion (Fig. 8 (c), trace 2). In the presence of Co^{2+} ion, 98% of peak area for Ac-L-Met was reduced, while 12 % for Ac-D-Met (Fig. 8 (c), trace 3). Together with the results in the first and second experiments, these observations clearly indicate that the immobilized aminoacylase-I on the DEAE-ESNW recognizes configuration of the substrate, namely, it retains the substrate specificity for Ac-L-Met identical to that of free enzyme.

In the case of the AI-SADS-ESNW, the immobilized aminoacylase-I also recognizes only the Ac-L-Met for hydrolysis (Fig. 8 (d), traces 2 and 3). The reduced peak areas for Ac-L-Met and Ac-D-Met were 86 % and 16 %, respectively after incubation in the presence of Co^{2+} ions for 120 hr. Due to less amount of immobilized enzyme on the AI-SADS-ESNW as compared with AI-DEAE-ESNW, complete hydrolysis of the substrate by AI-SADS-ESNW required longer incubation time.

3.5 Comparisons of Immobilized Activity Yields

Table 2 summarizes specific activity values of AI-DEAE-ESNW, AI-SADS-ESNW and free aminoacylase-I towards six kinds of substrates, including Ac-L-Met, Ac-D-Met, Ac-D, L-Met, Ac-L-Phe, Ac-D-Phe, and Ac-D, L-Phe. The specific activity values were presented in units of substrate hydrolyzed (nmol) per minute (min) per 1.0 mg of immobilized aminoacylase-I or free aminoacylase-I. The specific activity of free aminoacylase-I was used for calculation of the immobilized activity yield E (%) by the following equation 2, (2) where A_{ESNW} and A_{fAI} are specific activity values towards a specified substrate. Because the specific activity values were normalized with the weight of aminoacylase-I, the value means the hydrolyzing rate of a substrate, and hence, the immobilized activityyield gives a measure for estimating the degree of recovery of the aminoacylse-I activity upon immobilization onto the ESNW matrices.

The specific activity values of the AI-DEAE- and the AI-SADS-ESNWs towards Ac-L-Met were 15.8 and 16.8 nmol/min/mg AI-immobilized, respectively in the presence of Co^{2+} ion (Table 2). These values are almost comparable with each other. The immobilized activity yields for Ac-L-Met were 31.0 % for the AI-DEAE-ESNW and 32.9 % for the AI-SADS-ESNW, indicating that the activity recovery per immobilized enzyme weight is similar for both immobilization procedures, and also that approximately 69–67 % of free *aminoacylase-I* activity towards Ac-L-Met was suppressed during immobilization procedures in both cases for the DEAE- and the NHS-ESNWs. In the cases of Ac-D-Met, the specific activity values cannot be determined, because no hydrolysis was found for this substrate. The specific activity values of the AI-DEAE- and the AI-SADS-ESNWs towards Ac-D, L-Met were 5.83 and 4.81 nmol/min/mg AI-immobilized, respectively. These values were close to 0.5 fold to those towards Ac-L-Met, since the hydrolyzable Ac-L-Met is present in a half amount within the Ac-D, L-Met racemic mixture. This tendency was also observed for the free aminoacylase-I, comparing the specific activity values towards Ac-L-Met (51 nmol/min/mg AI) and towards Ac-D, L-Met (22.8 nmol/min/mg AI).

As for the Ac-L-Phe, the immobilized activity yields of the AI-DEAE- and the AI-SADS-ESNWs were 31.5 % and 28.6 %, respectively in the presence of Co^{2+} ion. The immobilized activity yields were found in relatively narrow range from 21.1–38.3 % throughout the all experiments, meaning that the specific activity of free aminoacylase-I towards given substrate is obviously reflected to those in the immobilized forms. This further suggests that the immobilized aminoacylase-I retains the substrate specificity of the free form.

Since there have been several preceding researches on immobilization of aminoacylase-I using

Table 2: Comparisons of specific activities

Substrates	$CoCl_2$	Free AI	AI-DEAE-ESNW	AI-SADS-ESNW
Ac-L-Met	-	27.4[a)]	10.5[b)]	9.05
			(38.3)	(33.0)
	+	51.0	15.8	16.8
			(31.0)	(32.9)
Ac-D-Met	-	0.00	0.00	0.00
			(–)[c)]	(–)
	+	0.00	0.00	0.00
			(–)	(–)
Ac-D, L-Met	-	13.8	4.27	4.89
			(34.4)	(35.3)
	+	22.8	5.83	4.81
			(25.6)	(21.1)
Ac-L-Phe	-	18.1	6.23	4.82
			(34.4)	(26.6)
	+	31.2	9.84	8.92
			(31.5)	(28.6)
Ac-D-Phe	-	0.00	0.00	0.00
			(–)	(–)
	+	0.00	0.00	0.00
			(–)	(–)
Ac-D, L-Phe	-	10.2	3.62	2.89
			(35.5)	(28.3)
	+	16.8	5.12	4.76
			(30.5)	(28.3)

DEAE-Sephadex [13] beads, carboxymethyl cellulose beads [17], poly(acyl amide) gel particles [14], and calcium alginate beads [20], and so on, and also therein the variety of experimental conditions on immobilization procedures and the activity assay, so that a direct comparison of the preceding data with the present result is rather difficult.

Alternatively, several data can be indirectly adapted to compare, for instance, Chibata *et al* described a standard enzyme assay method as follows; 100 mM phosphate buffer (pH 7.5); 100 mM Ac-D, L-Met, 1.5 mM $CoCl_2$, incubation for 30 min at 37℃ [13]. They also described that 106 g of DEAE-Shephadex-immoblized aminoacylase-I (from *Aspergillus oryzae*) was recovered from 100 g of starting DEAE-Sephadex and that the activity of the preparation is about 700 μmol/hr/g under standard assay condition (K_m = 8.7 mM) [13]. These descriptions can be compiled that the specific activity is equal to 206 nmol/min/mg (weight of immobilized AI) for the DEAE-Shephadex-immobilized *aminoacylase-I*.

The activity assay in the present work was performed with the substrate concentration at 15 mM, so that, according to the K_{m} value as 8.7 mM, the $V_{\max}$ in the present assay can be assumed

as same value as they reported, which leads to an estimation that the specific activity of the AI-DEAE-ESNW towards Ac-D, L-Met in our assay system will be correspond to 69 nmol/min/mg under the assay condition described in ref [13]. The estimation suggests that there is more room for improving the immobilization procedure for enhancing the activity, for instance, an usage of elevated equivalent molar of HMDI and N, N-diethyl (ethylene diamine) to gain the charge density on the fiber surface, which condenses the enzyme more efficiently.

According to Szajáni *et al*, [16] the immobilized activity yield of porcine kidney aminoacylase were 21–25% depending on the immobilization condition on Akrilex C-100 matrices having carboxyl groups, and the values were remarkably higher than other support matrices tested within their report. The HPC-ESNW exhibited more higher immobilized activity yield ranging from 21.1 to 38.3 %, especially for Ac-L-Met substrate, suggesting that the HPC-ESNW can be utilized for biological catalyst with suppressing activity losses of the enzyme.

4 Conclusion

In conclusion, the HPC-ESNW was characterized as a support matrix for enzyme, and the following are newly found: (i) the NCO-ESNW is considered as an useful intermediate for installing preferable functional groups to the ESNW matrix; (ii) the DEAE-ESNW can bind more amount of aminoacylase-I as compared with NHS-ESNW; (iii) the immobilized aminoacylase-I retains intact stereo-specificity; (iii) the immobilized activity yields are comparable to the preceding data [16, 17]. The present HPC-ESNW matrix can be further developed using the NCO-ESNW as a key intermediate, which can chemically bind a ligand molecules, and the resulting ESNW will be used for affinity detection of enzymes or proteins, for instances. The present research is, thus, a part of this focus for application of polysaccharide ESNW as a multi-purpose functional material.

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