Biocatalysis

Deutsche Ausgabe: DOI: 10.1002/ange.201605609
Internationale Ausgabe: DOI: 10.1002/anie.201605609

Bicontinuous Nanoporous Frameworks: Caged Longevity for Enzymes

Jae-Sung Bae, Eunkyung Jeon, Su-Young Moon, Wangsuk Oh, Sun-Young Han, Jeong Hun Lee, Sung Yun Yang, Dong-Myung Kim, and Ji-Woong Park**

Abstract: *The preparation of bicontinuous nanoporous covalent frameworks, which are promising for caging active enzymes, is demonstrated. The frameworks have three-dimensionally continuous, hydrophilic pores with widths varying between 5 and 30 nm. Enzymes were infiltrated into the bicontinuous pore by applying a pressured enzyme solution. The new materials and methods allowed the amount of caged proteins to be controlled precisely. The resulting enzyme-loaded framework films could be recycled many times with nearly no loss of catalytic activity. Entropic trapping of proteins by a bicontinuous pore with the right size distribution is an unprecedented strategy toward facile in vitro utilization of biocatalysts.*

Active enzymes isolated and immobilized inside a nanoscale volume are promising for advanced in vitro applications of enzymatic cascade reactions, such as enzymatic fuel cells and enzymatic nanoreactors.[1–3] Although various porous materials have been employed for immobilization of enzymes by different methods,[4] challenges remain for the development of simple, efficient, and reproducible methods to isolate and immobilize enzymes in a small volume while allowing the reactants and products to move in and out freely.

A bicontinuous nanoporous structure[5] is of particular interest because it allows enzymes to be accessed by reactant molecules through three-dimensionally reticulated channels. Additionally, if the channel contains constricted necks narrower than the size of the enzyme, these can act as entropic traps that would protect the proteins from leaching or aggregation.[6,7] For these unique aspects of the bicontinuous pore to be realized, the frameworks must have high dimensional stability against swelling by reactants or solvents and their nanopore surface must be sufficiently hydrophilic to facilitate the flow of aqueous solutions.

Herein we report the preparation of new hydrophilic bicontinuous nanoporous frameworks and show their promising potential as an efficient cage for enzymes. The frameworks are built upon a covalent organic network that is cross-linked on the molecular level, and have pore widths varying with a sigmoidal distribution between 5 and 30 nm, allowing proteins in the range of the framework pore size to be infiltrated in a quantitative manner into the bicontinuous pore by a simple pressurized flow method. The caged enzymes were efficiently used for running a biocatalytic reaction and could be recycled many times with nearly no loss of catalytic activity.

For the pore size and topology to be sufficiently resistant to chemical swelling, it is necessary that the materials constituting the bicontinuous framework be built upon chemically resistant covalent molecular networks.[8–11] We reported previously on the organic sol-gel (OSG) method, which enabled solution-based synthesis of covalent organic molecular networks.[12] The first step of the OSG process was the preparation of a dispersion (sol) of molecularly cross-linked urea network nanoparticles (UNNs) grown to a few tens of nanometers by polymerization of a tetra-functional amine and a diisocyanate. Because the gelation time (t_g: the time it takes for the UNN sol to become a gel at a fixed concentration) is usually tens of hours, or days, the sol may be combined with a polymer to derive microstructures by phase separation.[13,14]

In the present work, we mixed polyethylene glycol (PEG) with the UNN sol, anticipating that the hydroxy end-groups of PEG chains would react slowly with the UNN surface and that the grafted PEG chains would reduce interfacial energy so as to suppress macroscopic aggregation of either component. We added PEG with an average molecular weight of 35 kg mol^{-1} to the UNN sol, which was prepared by polymerizing tetrakis(4-aminophenyl)methane (TAPM) and hexamethylene diisocyanate (HDI) at a concentration of 4 wt % in DMF (Figure 1a), as reported previously,[12–15] to obtain a homogeneous UNN/PEG mixed-sol (Figure 1b; see the Supporting Information for experimental details). The mixed UNN/PEG sol was cast on a glass plate and dried to obtain a microphase-separated blend-film (Figure 1c).

The UNNs aggregated slightly upon mixing with PEG, however aggregate size could be kept below 100 nm by adjusting the PEG weight fraction (f_p) to be smaller than 0.6 with respect to the total solid weight, as indicated by the dynamic light scattering (DLS) data (Supporting Information, Figure S1). Transparent films were obtained by casting and subsequent drying of the mixture with $f_p \leq 0.6$. The scanning (SEM) and transmission (TEM) electron microscopy images

[*] J.-S. Bae, E. Jeon, S.-Y. Moon, W. Oh, S.-Y. Han, Prof. J.-W. Park
Department School of Materials Science and Engineering and Research Institute for Solar and Sustainable Energies
Gwangju Institute of Science and Technology
123 Cheomdan-gwagiro, Buk-gu, Gwangju, 61005 (Korea)
E-mail: jiwoong@gist.ac.kr
J.-H. Lee, Prof. S. Y. Yang
Department of Polymer Science and Engineering
Chungnam National University
299 Daehak-Ro, Yuseong-Gu, Daejeon, 34134 (Korea)
E-mail: sungyun@cnu.ac.kr
Prof. D.-M. Kim
Department of Fine Chemical Engineering and Applied Chemistry
Chungnam National University
299 Daehak-Ro, Yuseong-Gu, Daejeon, 34134 (Korea)
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201605609.

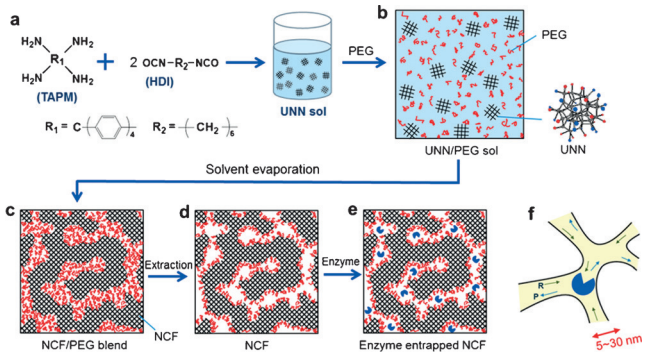

Figure 1. Preparation of a bicontinuous nanoporous covalent framework (NCF) with caged enzyme. a) Cross-linking polymerization of TAPM and HDI to obtain the UNN sol. b) A UNN/PEG sol mixture was formed by adding PEG to the UNN sol. c) A bicontinuous phase-separated NCF/PEG blend is obtained after complete evaporation of solvent. d) A bicontinuous NCF is obtained by extraction of PEG. PEG brushes remain coated on the pore surface. e) Enzymes are entrapped in the bicontinuous pore by applying pressure. f) The enzyme is physically fixed in the three-dimensional pore trap, while the reactant (R) and product (P) move in and out freely.

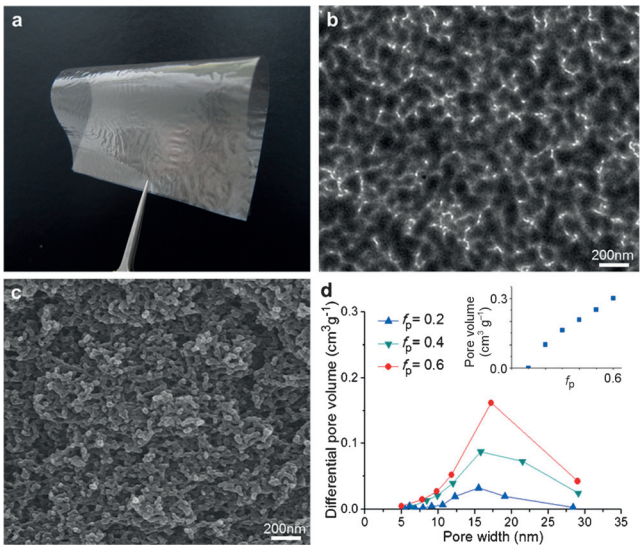

Figure 2. The structure of a bicontinuous NCF film. a) Optical photograph of an 8×12 cm^2 NCF film (f_p = 0.6) with a thickness of 40 μm. b) TEM image of an ultrathin cross-section. The brighter regions correspond to the pores through the film. c) SEM image of an NCF film fractured after being immersed in liquid nitrogen. d) BJH pore size distributions of NCFs prepared with f_p = 0.2, 0.4, and 0.6, respectively. Inset: pore volume of the NCF as a function of f_p.

(Supporting Information, Figure S2) of the films clearly showed the formation of bicontinuous microdomain structures for the nearly symmetric compositions (that is, $f_p = 0.4$ and 0.6). Isolated domains of the minority component appeared for relatively unbalanced compositions ($f_p = 0.1$ and 0.8).

Because it was co-continuous with the UN domain in three dimensions, the PEG domain of the bicontinuous NCF/PEG microstructure could be extracted with water to generate three-dimensionally continuous channels through the film (Figure 1 d). (We denote the resulting film as a "nanoporous covalent framework (NCF) film" below.) An enzyme was loaded into the NCF pores by passing an enzyme-containing solution through a film under pressure (Figure 1 e). Applied pressure enables the macromolecules to migrate against the entropic penalty of elastic deformation across the constricted barriers present along the continuous channel. It was anticipated that, in a reaction flask with no applied pressure, the enzyme would be physically fixed in the three-dimensional pore trap while the reactant and product move in and out freely (Figure 1 f).

Cross-sectional TEM images of the NCF film (Figure 2; Supporting Information, Figures S3 and S4) clearly showed that bicontinuous nanochannels do indeed form throughout the matrix. The tortuosity factor for the film estimated using the Hagen–Poiseuille equation was about 1.65 (Supporting Information, Figure S5), confirming the bicontinuous pore structure.[16,17] The BJH pore size[18] was in the range of 5–30 nm (Figure 2 d; Supporting Information, Figure S6), consistent with the range observed by TEM (Figure 2 b). Pore volume varied linearly with f_p in the range of 0.10–0.30 cm^3 g^{-1} (Figure 2 d, inset).

The procedure to prepare the bicontinuous NCF was easily reproducible and scalable because it consists of a straightforward sequence of procedures: solution mixing, film casting, and evaporation, followed by solvent extraction. Rectangular films that were 40 μm thick and 12 × 8 cm were prepared in a laboratory flask, as shown in Figure 2 a. As expected, the NCF films were insoluble in many solvents, and their porosities remained nearly unchanged before and after treatment in common organic solvents, including water.

While the PEG added to the gelling UNN sol led to the formation of the microstructure, it also formed hydrophilic covalent grafts at the UNN/PEG interface. As the mixed UNN/PEG sol was concentrated by solvent evaporation, the hydroxy ends of PEG were able to react with isocyanate moieties pendent to the surface of the UNN domains. Both the FTIR spectra and measurements of the change of the sample weight with extraction time indicated that about 40 % of the initial PEG remained in the NCF (Supporting Information, Figure S7). The water contact-angle of the NCF film approached that known for the purely PEG-coated surface[19] as f_p was increased (Supporting Information, Figure S8). The films also exhibited anti-biofouling activity (Supporting Information, Figure S9). All these data appeared to confirm that the nanopores of the NCF film were coated with hydrophilic PEG brushes, as shown in Figure 1. The hydrophilic polymer, such as PEG[20] or polyelectrolytes,[21] grafted on the nanopore surface is expected to enhance the activity and stability of the enzyme when entrapped in the pore.

The PEG brushes coated on the continuous pore surface made the NCF films we prepared sufficiently hydrophilic for transporting aqueous solutions. The NCF films quickly absorbed aqueous solutions up to about 60 % of its dry weight (Figure 3 a) by capillary action through the bicontinuous channel. The weight fraction of the absorbed water in a wet NCF film was approximately 38 % of the total weight,

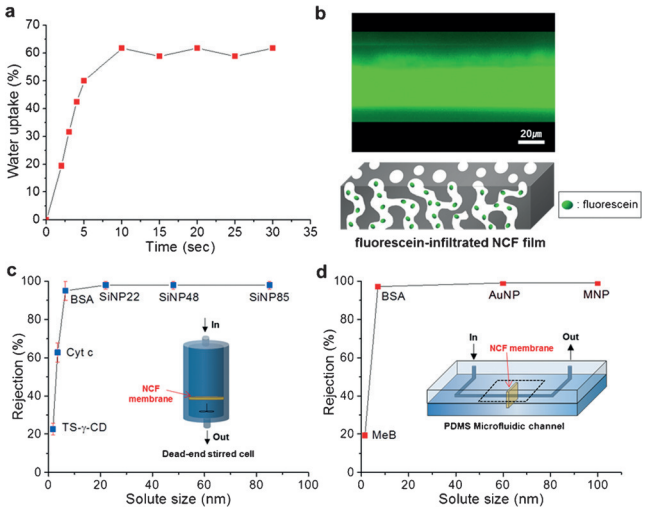

Figure 3. Transport of water and solutes through NCF films. a) The weight of the water taken up by an initially dry NCF film ($f_p = 0.6$) as a percent of the weight of the dry film. b) A confocal microscope tomographic image of the fluorescein-infiltrated NCF film with $f_p = 0.6$. c) and d) Filtration of different solutes dissolved in aqueous solutions by the NCF ($f_p = 0.6$) film using (c) a dead-end stirred cell and (d) an integrated microfluidic device. The solute rejection (%) was plotted as a function of average solute size: MeB, 1.5 nm; Ts-γ-CD, 1.7 nm; cytochrome *c*, 3.5 nm; BSA, 6 nm; SiNP22, 22 nm; SiNP48, 48 nm; SiNP85, 85 nm; AuNP, 60 nm; MNP, 100 nm (see the Supporting Information).

which is approximately the same as that of the PEG removed from the as-prepared NCF/PEG blend. An initially dry NCF strip expanded by about 3% laterally after being immersed in water overnight. The small lateral expansion was indicative of the resistance of the framework to swelling. The NCF was also resistant to organic solvents, such as methanol, THF, and acetone, expanding only 1–3%.

A confocal microscopy study (Figure 3b) showed that a solution of fluorescein could be absorbed into the NCF film immersed in the solution. In contrast, fluorescein-labeled bovine serum albumin (FITC-BSA) did not infiltrate the film. The results indicate that, although small solutes could be diffused into the bicontinuous water channel, proteins could not pass through pores of similar size by the simple immersion method. The diffusional resistance of proteins may be accounted for by entropic trapping effects exerted by the pores.[6,7,22] This suggests that once the proteins are captured within the pores by applying an external stimulus such as pressure, they can be kept physically entrapped as long as the applied pressure is lower than the loading pressure, as shown by the proceeding results.

The molecular weight (size) cut-off (MWCO) of the NCF film, estimated by macro- and micro-fluidic filtration tests (Figure 3) corresponded to the lower limit of the BJH pore size distribution, indicating three-dimensional pore continuity. The NCF films nearly completely prevented solutes greater than 5 nm in size from passing through in a stirred dead-end filtration cell (Figure 3c). While BSA and the larger silica particles could not traverse the film, mono-6-*O*-(*p*-toluenesulfonyl)-γ-cyclodextrin (Ts-γ-CD) did pass through. Cytochrome *c* was partially retained. A small piece of the NCF film could be easily bonded across the microfluidic channel;[23] the molecular separation performances from the aqueous solutions (Figure 3d) were observed to be similar to those observed using the dead-end stirred cell. The hydrophilicity and the chemical and mechanical robustness of the NCF film make it suitable as a modular membrane for microfluidic devices.

Particles larger than 5 nm but smaller than 30 nm (the upper limit of NCF pore width) should be entrapped inside the pores as the enzyme solution starts to permeate through the film. We presumed that the entrapment could be performed in a controlled manner by using a sufficiently dilute solution. A dilute aqueous solution of *Candida rugosa* lipase at a concentration of 90 mg L^{-1} was passed through an NCF film by applying a pressure of 5 bar, as shown in Figure 4. The molecular volume of the lipase was 5.0 × 4.2 ×

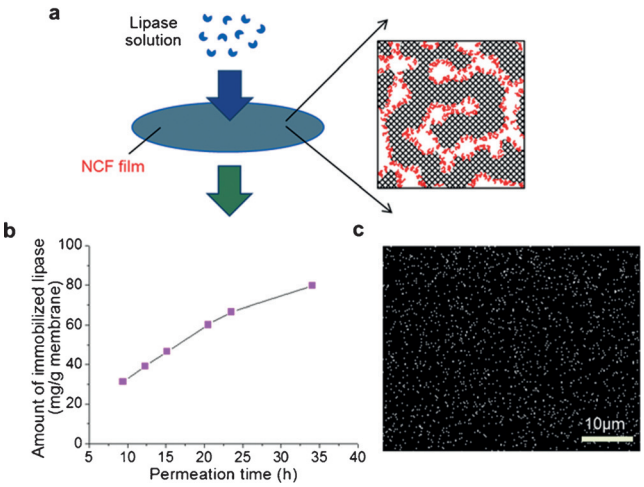

Figure 4. Pressure loading of enzyme into an NCF film. a) Lipase was loaded into the NCF ($f_p = 0.6$) film by passing an aqueous enzyme-containing solution (16 mg L^{-1}) through a 40 μm thick film by applying a pressure of 5 bar. b) The plot of the amount of the entrapped enzyme in the NCF film as a function of the permeation time of the lipase solution. c) EDX mapping of lipase-loaded NCF film for sulfur.

3.3 nm.[24] DLS of the 90 mg L^{-1} solution used for infiltration showed an average particle size of 15 nm. The amount of entrapped lipase varied with the permeation time of the solution (Figure 4b). Energy dispersive X-ray spectroscopy (EDX) mapping of sulfur indicated that the enzyme was uniformly distributed over the NCF matrix (Figure 4c). Surprisingly, none of the infiltrated enzyme was leached away by the flow of water after one day, even under 15 bar, when tested with the filtration cell used for enzyme loading. The simple and quantitative nature of the technique for pressure loading of enzyme through the NCF film is distinct from any other method. The untrapped enzymes can be collected easily from reservoirs.

The lipase-loaded NCF films were then tested for esterification of oleic acid with *n*-butanol in isooctane (Figure 5a).[25,26] Although the reaction with the entrapped lipase was slower, no leaching was observed even after reaction for

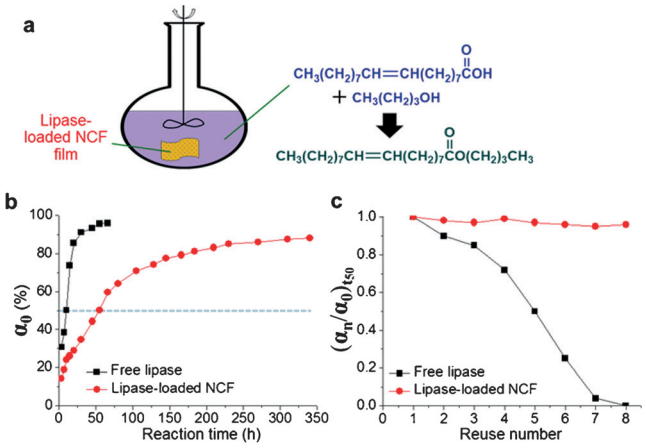

Figure 5. Catalytic reaction on the lipase-loaded NCF film. a) The esterification of oleic acid with *n*-butanol is performed in a flask with free lipase and lipase-loaded NCF film, respectively. b) The conversion (α_0) of the oleic acid into butyl oleate as a function of reaction time. The oleic acid concentrations were determined at regular time intervals by titration of a sample solution with potassium hydroxide. c) The ratio of the conversion (α_n) by reused enzyme (α_0) to that by the initial enzyme at t_{50}; the reaction time that gives 50% conversion in the first run for each system was plotted against the reuse number (n) of free lipase and the NCF lipase, respectively.

350 h (Figure 5b). The films in the flask could be picked up and reused for reaction with fresh substrates. Figure 5c shows that the NCF-loaded lipase was stable with nearly no loss of catalytic activity over multiple reuse cycles, for which free enzyme lost its entire activity. Relatively low initial activity of the entrapped enzyme, which is most likely caused by diffusional resistance of nanopores, can be overcome simply by using a large amount of enzyme-loaded film, which can be reused many times.

Exceptional stability in the activity of the enzyme caged in the bicontinuous pore may be accounted for by the spatial confinement of protein molecules by the entropic trapping effect. Since the migration of proteins across channels is prohibited, their three-dimensional distribution over the reticulated nanochannel remains intact during the progress of chemical reactions. The materials and methods presented herein, which facilitate caging of enzymes in the three-dimensionally continuous pore structure of a framework with pore widths close to the size of the introduced enzyme, are promising for in vitro utilization of enzymes in various areas of chemistry. Studies on the preparation of the NCF film loaded with multiple enzymes or the application of the enzyme-loaded membrane for continuous flow reactions are underway.

Acknowledgements

This work was supported by Basic Science Research (NRF-2015R1A2A1A15052067 and 2014R1A1A3A04050950) through the National Research Foundation (NRF) funded by the Korean government (Ministry of Science, ICT & Future Planning) and the grant from the GIST research institute (GRI).

Keywords: bicontinuous nanoporous structures · enzyme nanoreactors · microfluidics · nanoporous films · organic sol-gel synthesis

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 11495–11498
Angew. Chem. **2016**, *128*, 11667–11670

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Received: June 17, 2016
Published online: August 11, 2016