Mendeleev Communications

*Mendeleev Commun.*, 2025, **35**, 592–594

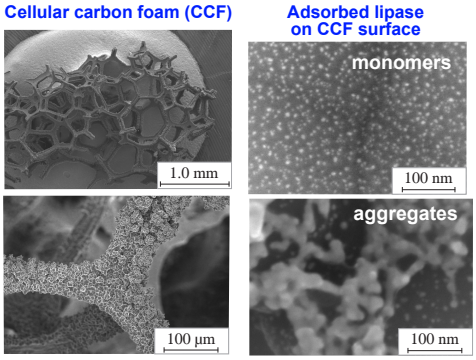
# SEM and SAXS characterization of recombinant lipase immobilized by adsorption on the surface of cellular carbon foam

**Galina A. Kovalenko,* Larisa V. Perminova, Alexandra N. Serkova, Aleksei N. Salanov and Yurii V. Larichev**

*G. K. Boreskov Institute of Catalysis, Siberian Branch of the Russian Academy of Sciences, 630090 Novosibirsk, Russian Federation. E-mail: galina@catalysis.ru*

DOI: 10.71267/mencom.7757

**Using modern scanning electron microscopy (SEM), a research was conducted on the supramolecular aggregation of protein globules of recombinant *Thermomyces lanuginosus* lipase (TLL) adsorbed on the surface of cellular carbon foam (CCF) by the two methods of physical adsorption: spontaneous and forced (impregnation–drying). After the spontaneous adsorption, more than half of the entire surface of CCF was uniformly occupied by 10–50 nm nanoparticles, which corresponded in size to the monomers and dimers of TLL globules. After the forced adsorption, protein films were formed between and around pyrolytic carbon deposits protruding beyond these films.**




*Keywords*: scanning electron microscopy, cellular carbon foam, surface morphology, recombinant lipase, adsorption.

Modern scanning electron microscopy (SEM) opens new possibilities for visualizing the processes of adsorption and supramolecular aggregation of various biological nano-objects, such as proteins, enzymes, antibodies and bacteria.[1] This technique provides a new insight into the distribution of enzymatically active molecules on the surface of solid supports used for immobilization, and the forms of their existence (monomer or aggregated into oligomers). This information has both fundamental scientific importance and applied significance for practical implementation, particularly in heterogeneous biocatalysis, as well as in analytical sensorics for specific, reliable and accurate determination of the metabolite concentration in multicomponent biological fluids. Commercial biosensors are of great demand in medical diagnostics (for example, glucose control in blood), production of beverages (mainly beer) and pharmaceutical industry at the stages of the technological process control.

Biocatalysis is an interdisciplinary area of research and implementation of the green one-stage processes of highly selective single-enzyme conversion of target reagents (substrates) into valuable products demanded in markets.[2–6] Biocatalytic processes having all the unique features of enzymatic catalysis (chemo-, regio- and stereospecificity) are energy-saving and environmentally friendly ones. The world market for industrially relevant enzymes for biocatalysis was expected to reach $7.0 billion by 2023 at a remarkable compound annual growth rate of 4.9% for the period of 2018–2023.[7] Among the most used enzymes, lipases (triacylglycerol hydrolase, E.C. 3.1.1.3) clearly predominate, especially due to their unique ability to catalyze reactions (esterification, interesterification) in non-aqueous reaction media, including organic solvents. Reviews[7–11] describe in detail the biocatalytic properties of both soluble and immobilized microbial lipases, their kinetics parameters and latest trends in lipase-catalyzed synthesis of highly valuable commercial products, particularly esters of carbohydrate (glucose, fructose, galactose) with fatty acids.[11] The possibilities and prospects for industrial application of various lipase-active biocatalysts are also assessed.[7–10]

Since 2007, the practical direction of 'biocatalytic engineering'[12] has been intensively developing, aimed at finding approaches to modulating the functional (catalytic) properties of enzymes by immobilizing them on solid supports. For the effectiveness of such 'engineering', it is of great importance to know the distribution of immobilized enzymes over the surface of supports and whether aggregation processes occur. These processes undoubtedly determine the properties, such as enzymatic activity and substrate specificity, of the prepared heterogeneous biocatalysts. Some examples from the literature can be given. When immobilized on hydrophobic supports, hyperactivation of lipase was observed;[13–16] its mechanism was suggested to be the correct orientation of a lipase molecule on the surface in an 'open' conformation of the active site, accessible for the formation of a productive enzyme–substrate complex. Biocatalyst engineering was carried out by modulation of catalytic properties by means of careful variation of the chemical composition of the multi-walled carbon nanotubes (MWCNT)–silica adsorbents for recombinant *T. lanuginosus* lipase (TLL) catalyzing the esterification reaction in organic solvents.[17] Undoubtedly, research of the distribution, aggregation and orientation of enzymes on different solid supports helps formulate practical approaches to preparing commercially attractive highly active and stable biocatalysts.

The advanced materials, cellular carbon foams (CCFs), have attracted much attention due to their high ability to adsorb (on surface) or absorb (in volume) various pollutants, in particular petroleum contaminants, and effectively purify waste waters.[18] A unique method for the synthesis of CCFs by high-temperature pyrolysis of $C_3$–$C_4$ alkanes was developed, and a mechanism for the formation of an openwork 3D macrostructure was

proposed.[19,20] The CCFs seem to be the excellent supports for enzyme immobilization in order to prepare attractive heterogeneous biocatalysts. Due to the cellular 3D macrostructure, open porosity (up to 98%) and the presence of super-macropores, diffusion restrictions on the mass transfer of high-molecular reagents (enzymes and substrates) are completely overcome. High mechanical strength and low hydrodynamic resistance of CCFs allow one to use the prepared biocatalysts in continuous biocatalytic processes using packed bed reactors.

Previously, Kovalenko and co-workers comprehensively explored the properties of heterogeneous biocatalysts prepared by adsorptive immobilization of recombinant *T. lanuginosus* lipase on supports based on nanostructured carbon, in particular, MWCNT in the form of macroporous carbon aerogel[21,22] and MWCNT–silica composites,[17] in the reaction of esterification of fatty $C_4$–$C_{18}$ acids with aliphatic $C_2$–$C_{16}$ alcohols.

In this communication, the results of SEM visualization of supramolecular aggregation of adsorbed recombinant TLL on the surface of a unique CCF are presented. The confidence that the obtained data on the distribution and aggregation of enzymes on solid supports will help find correlations with their functional properties and formulate practical approaches to the preparation of highly active and stable, commercially attractive biocatalysts is expressed.

Immobilization of recombinant TLL, specially designed using genetic engineering manipulations, was carried out by the two methods of adsorption: spontaneous and forced (impregnation–drying) as described in Online Supplementary Materials. It has been shown that the high mechanical strength, openwork three-dimensional macrostructure and surface morphology did not change during the prolonged contact of CCF with aqueous protein solutions (Figure S1, Online Supplementary Materials). The surface was uniformly covered by round-shaped pyro-carbon deposits more than 5 μm in size [Figures S1(*c*),(*d*)]. In the case of spontaneous adsorption of lipase, a monodisperse distribution of nanoparticles 3–5 nm in size over the surface of pyro-carbon deposits was observed (Figure 1). This size is quite consistent with the size of the protein globule (monomer) of TLL [Protein Data Base (PDB) ID: 4ZGB], whose crystallographic size is $3.5 \times 4.5 \times 5.0$ nm$^3$.[7–9] An overview analysis of numerous SEM images allowed us to estimate that half of the entire CCF surface was occupied by monomers and/or dimers of lipase. It is well known that TLL molecules can easily form dimers especially in the region of hydrophobic lids over the catalytically active center.[9,11] The formation of larger aggregates was possible due to the hydrophobic interactions between enzyme globules. Really, the branched fibril-like aggregates of different sizes and shapes were formed among and over the pyro-carbon deposits (Figure 2). The additional evidence was obtained by SEM regarding the supramolecular aggregation of immobilized lipase globules on a flat surface of polished aluminum (Figure S2, Online Supplementary Materials). As can be seen, molecules of lipase associated into the large aggregates up to 1 μm in size, sometimes looking like a 'butterfly' or a 'rice grain', evenly covering the aluminum surface. At high magnification, one can examine the fine structure of the aggregates, consisting of smaller nanoparticles of apparently protein origin, possibly monomers or dimers of TLL [Figures S2(*b*)–(*d*)]. Surely, as mentioned above, this information is of great importance for heterogeneous biocatalysis and analytical sensorics.

Immobilization of recombinant TLL was carried out by forced adsorption as described in Online Supplementary Materials. This method was developed in our group for enzymes that practically do not adsorb spontaneously on a solid surface, for example, lipase on mesoporous metal oxides ($Al_2O_3$, $TiO_2$). Impregnation of the support with enzyme solutions according to its moisture capacity ($0.8 \pm 0.2$ cm$^3$ g$^{-1}$) and its subsequent drying made it possible to increase the amount (mg g$^{-1}$) of immobilized enzyme by orders of magnitude, possibly due to the formation of protein films, which in turn increased the activity of the prepared biocatalysts. Really, the entire surface of CCF was covered with protein films [Figure S3(*a*), Online Supplementary Materials]. The round-shaped pyro-carbon deposits protruded beyond the TLL films [Figures 3(*a*),(*b*)]. The 'bubbles-like' structures were observed between and around the pyrolytic carbon deposits [Figure S3(*d*)]. Apparently, the electron beam in vacuum heated up the carbon surface, leading to 'boiling' water associated with the immobilized hydrated lipase molecules. Visible imaginary 'bubbles' in protein films of bovine serum albumin were observed on carbon supports earlier.[23]

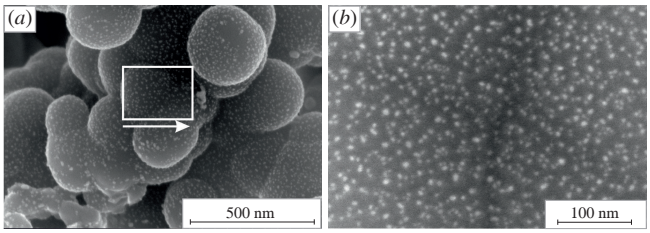



**Figure 1** SEM images of (*a*) TLL nanoparticles immobilized on the pyro-carbon surface by spontaneous adsorption. Rectangle and arrow indicate (*b*) the area with higher magnification.

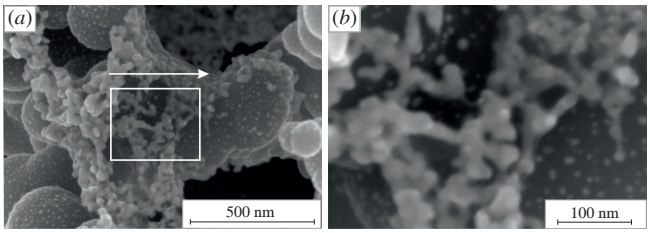



**Figure 2** SEM images of (*a*) single nanoparticles and complex aggregates of TLL immobilized by spontaneous adsorption. Rectangle and arrow indicate (*b*) the area with higher magnification.

The SAXS data confirmed the occurrence of TLL aggregation on the surface of the adsorbent formed by pyro-carbon nanoparticles similar in nature to CCF. The SAXS curves obtained using the subtraction technique demonstrated that the radius of the gyration $R_G$ value was $9.0 \pm 0.9$ nm [Figure 4(*a*)]. The weakly pronounced structural peak at 0.05 Å$^{-1}$ appeared probably due to the structuring (orientation) of lipase molecules on the carbon surface. In terms of interplanar distances, the magnitude of this peak was 11 nm. Analysis of the distance distribution $D_v(r)$ curves presented in Figure 4(*b*) showed that the distribution of TLL nanoparticles was trimodal with fractions having average sizes equal to 5.0, 9.5 and 22.0 nm. The proportional relative content of these fractions was 0.13, 0.65 and 0.22, respectively. Thus, it can be concluded that only 13% of the adsorbed lipase was present in the non-aggregated state in the form of nanoparticles close in size to the lipase molecule in the aqueous solution. The main fraction of nanoparticles of

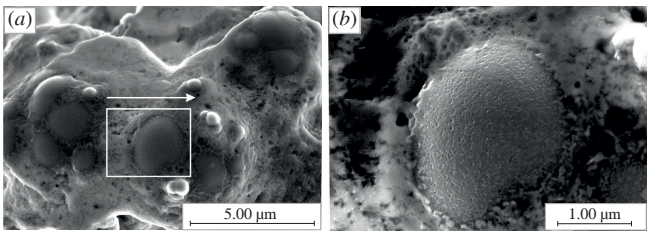



**Figure 3** SEM images of (*a*) protein films formed by TLL immobilized by forced adsorption on the pyro-carbon surface of CCF. Rectangle and arrow indicate (*b*) the area with higher magnification.

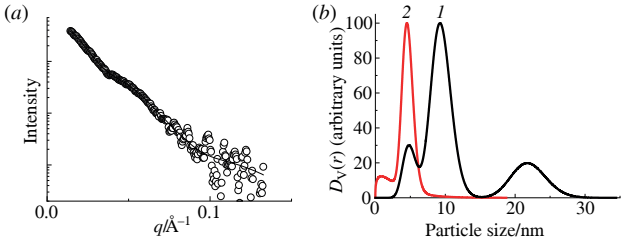



**Figure 4** (*a*) Difference SAXS curve of adsorbed lipase; (*b*) calculated distributions of nanoparticle size for (*1*) TLL immobilized by spontaneous adsorption on pyro-carbon surface and (*2*) lipase in aqueous solution.

adsorbed lipase (65%) had an average size of 9.5 nm. Considering the cubic relationship between the size of one spherical nanoparticle and its quantity in one aggregate, it can be concluded that the corresponding aggregate consisted of 8–10 molecules of spherical nanoparticles, *i.e.*, it was an octamer or decamer. Reasoning in a similar way, the third fraction with an average size of 22 nm corresponded to much larger aggregates consisting of more than a hundred TLL molecules. However, as can be seen from Figure 2, aggregates of lipase globules had a complex branched, rather than spherical, shape. Future research will be aimed at finding a correlation between the type of adsorbed enzyme molecules (single, oligomers, fibrillar, complex shaped aggregates) and the catalytic properties of immobilized enzymes.

In summary, the pioneering research on adsorption and supramolecular aggregation of immobilized enzymes on the surface of solid supports was carried out using advanced SEM. Recombinant TLL was immobilized by spontaneous and forced (impregnation–drying) adsorption onto peculiar CCF with an openwork three-dimensional macrostructure, and round-shaped pyro-carbon deposits uniformly covered the CCF surface. After spontaneous TLL adsorption, more than half of the CCF surface was covered by nanoparticles 2–5 nm in size, which corresponded to the size of the lipase monomer and/or dimer; a monodisperse distribution of such nanoparticles over the surface of pyro-carbon deposits was observed. The TLL aggregates with a complex branched structure were also visualized by SEM. After forced TLL adsorption, the dense protein films covered the entire surface of CCF, pyro-carbon deposits protruded beyond these films and imaginary 'bubbles' were observed between and around deposits under the action of a beam of high-energy electrons. The SAXS data confirmed the presence of TLL monomers and formation of large aggregates on the pyro-carbon surface. Trimodal distribution of TLL nanoparticles was observed with fractions having an average size equal to 5.0 (TLL monomer), 9.5 (TLL octamers) and 22.0 nm (aggregates of hundreds TLL globules); the relative fraction content was 13, 65 and 22%, respectively.

Undoubtedly, investigation of the distribution, aggregation and orientation of enzymes on different solid supports has both fundamental scientific importance and practical significance for the development of heterogeneous biocatalysis and analytical biosensorics. Such valuable and useful information will help relevant specialists to optimize the preparation of highly active and stable heterogeneous biocatalysts and sensitive element of biosensors.

The authors sincerely thank O. B. Belskaya for providing the samples of cellular carbon foam. This work was supported by the Ministry of Science and Higher Education of the Russian Federation within the governmental order for Boreskov Institute of Catalysis (Project FWUR-2024-0034). The studies were carried out using facilities of the shared research center 'National Center of Investigation of Catalysts' at the Boreskov Institute of Catalysis.

*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.71267/mencom.7757.

## References

1 J. J. Goldstein, D. E. Newbury, P. Echlin, D. C. Joy, C. E. Lyman, E. Lifshin, L. Sawyer and J. R. Michael, *Scanning Electron Microscopy and X-Ray Microanalysis,* Springer, New York, 2003; https://doi.org/10.1007/978-1-4615-0215-9.
2 A. S. Bommarius and B. R. Riebel-Bommarius, *Biocatalysis: Fundamentals and Applications*, Wiley-VCH, Weinheim, 2004; https://www.wiley.com/en-us/Biocatalysis%3A+Fundamentals+and+Applications-p-9783527303441.
3 *Biocatalysis for Green Chemistry and Chemical Process Development*, eds. J. Tao and R. Kazlauskas, John Wiley & Sons, Inc., 2011; https://doi.org/10.1002/9781118028308.
4 R. A. Sheldon and J. M. Woodley, *Chem. Rev.*, 2018, **118**, 801; https://doi.org/10.1021/acs.chemrev.7b00203.
5 K. Buchholz, V. Kasche and U. T. Bornscheuer, *Biocatalysts and Enzyme Technology*, 2nd edn., Wiley-VCH, 2012; https://www.wiley.com/en-us/Biocatalysts+and+Enzyme+Technology%2C+2nd+Edition-p-9783527329892.
6 C. T. Hou, *Handbook of Industrial Biocatalysis*, CRC Press, 2005; https://doi.org/10.1201/9781420027969.
7 J. López-Fernández, M. D. Benaiges and F. Valero, *Catalysts*, 2020, **10**, 1277; https://doi.org/10.3390/catal10111277.
8 R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2010, **62**, 197; https://doi.org/10.1016/j.molcatb.2009.11.010.
9 S. Naik, A. Basu, R. Saikia, B. Madan, P. Paul, R. Chaterjee, J. Brask and A. Svendsen, *J. Mol. Catal. B: Enzym.*, 2010, **65**, 18; https://doi.org/10.1016/j.molcatb.2010.01.002.
10 F. T. T. Cavalcante, F. S. Neto, I. Rafael de Aguiar Falcão, J. Erick da Silva Souza, L. S. de Moura Junior, P. da Silva Sousa, T. G. Rocha, I. G. de Sousa, P. H. de Lima Gomes, M. C. M. de Souza and J. C. S. dos Santos, *Fuel*, 2021, **288**, 119577; https://doi.org/10.1016/j.fuel.2020.119577.
11 A. Spalletta, N. Joly and P. Martin, *Int. J. Mol. Sci.*, 2024, **25**, 3727; https://doi.org/10.3390/ijms25073727.
12 C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451; https://doi.org/10.1016/j.enzmictec.2007.01.018.
13 M. Kumar, J. Mukherjee, M. Sinha, P. Kaur, S. Sharma, M. N. Gupta and T. P. Singh, *Sustainable Chem. Processes*, 2015, **3**, 14; https://doi.org/10.1186/s40508-015-0042-5.
14 E. A. Manoel, J. C. S. dos Santos, D. M. G. Freire, N. Rueda and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2015, **71**, 53; https://doi.org/10.1016/j.enzmictec.2015.02.001.
15 N. F. Mokhtar, R. N. Z. R. Abd. Rahman, N. D. Muhd Noor, F. Mohd Shariff and M. S. Mohamad Ali, *Catalysts*, 2020, **10**, 744; https://doi.org/10.3390/catal10070744.
16 J. Boudrant, J. M. Woodley and R. Fernandez-Lafuente, *Process Biochem.*, 2020, **90**, 66; https://doi.org/10.1016/j.procbio.2019.11.026.
17 G. Kovalenko, L. Perminova, A. Beklemishev, A. Serkova and A. Salanov, *J. Biotechnol.*, 2024, **389**, 13; https://doi.org/10.1016/j.jbiotec.2024.04.014.
18 E. A. Raiskaya, I. S. Sabko, O. I. Krivonos and O. B. Belskaya, *AIP Conf. Proc.*, 2019, **2143**, 020035; https://doi.org/10.1063/1.5122934.
19 E. A. Rayskaya, O. B. Belskaya and V. A. Likholobov, *Mater. Today: Proc.*, 2018, **5**, 25962; https://doi.org/10.1016/j.matpr.2018.08.012.
20 E. A. Raiskaya, O. B. Belskaya, O. I. Krivonos, M. V. Trenikhin, A. V. Babenko and V. A. Likholobov, *J. Anal. Appl. Pyrolysis*, 2023, **175**, 106189; https://doi.org/10.1016/j.jaap.2023.106189.
21 G. Kovalenko, L. Perminova and A. Beklemishev, *Catal. Today*, 2021, **379**, 36; https://doi.org/10.1016/j.cattod.2020.11.018.
22 G. Kovalenko, L. Perminova, M. Pykhtina and A. Beklemishev, *Biocatal. Agric. Biotechnol.*, 2021, **36**, 102124; https://doi.org/10.1016/j.bcab.2021.102124.
23 A. Salanov, A. Serkova, A. Zhirnova, L. Perminova and G. Kovalenko, *Micro*, 2022, **2**, 334; https://doi.org/10.3390/micro2020022.

*Received: 3rd March 2025; Com. 25/7757*