Bacterial Outer Membrane Vesicles as Nano-Scale Bioreactors: A Fatty Acid Conversion Case Study

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Bacterial outer membrane vesicles (OMVs) are small unilamellar proteoliposomes, involved in various functions including cell-to-cell signalling and protein excretion. We have engineered the OMVs of *Escherichia coli* to nano-scaled bioreactors for the biotransformation of fatty acids by targeting a fatty acid double bond hydratase of *Stentrophomonas maltophilia* (*Sm*OhyA) and/or a photoactivated fatty acid decarboxylase from *Chlorella variabilis* NC64 A (*Cv*FAP) into OMVs. Engineered OMVs containing both *Sm*OhyA and *Cv*FAP were able to catalyse the transformation of oleic acid ((*Z*)-octadec-9-enoic acid) into 9-hydroxyheptadecane via (*R*)-10-hydroxyoctadecanoic acid. The specific biotransformation rates of oleic acid reached 8.0×10^{-12} μmol/min per OMV.

Introduction

Lipid-based micellar and liposomal nanoparticles have been studied intensively as carriers for proteins, genes and other biomolecules. Liposomes, for example, have been used as model systems for plasma membranes, for drug delivery or imaging purposes.[1] Liposomes have also been explored as nano-scaled reaction vessels,[2] their small diameters between 50 and 250 nm allow for rapid diffusional mixing thereby enabling fast reaction kinetics.[3] Next to liposomes, bacterial outer membrane vesicles (OMVs), spherical, bilayered proteolipids with an average diameter of 20 to 200 nm are gaining increased interest.[4] OMVs can contain both, periplasmic and outer membrane proteins and therefore have been proposed to play a role in the delivery of toxins to cells and in the transfer of proteins between bacterial cells.[5] Bacterial OMVs have also been investigated as vaccine carriers, because they are structurally stable and may protect the encapsulated proteins and biomolecules from a variety of stresses.[6] In addition, bacterial OMVs have been reported as nano-scaled bioreactors. For example, an OMV loaded with an organophosphate hydrolase from *Brevundimonas diminuta* was used for the remediation of organophosphate pesticides.[7] In another example, cellulases encapsulated in OMVs enabled the 23-fold accelerated hydrolysis of cellulose as compared to the non-complexed enzymes.[8]

Preparation of synthetic liposomes can be a tedious task including not only the heterologous expression of the biocatalysts and their purification but also the liposome preparation comprises various synthesis and purification steps.[9] In contrast, bacterial OMVs can readily be produced by a single synthesis and purification step. Furthermore, the composition of the vesicles can be engineered:[6a,10] protein engineering and synthetic biology tools enable to construct diverse protein-OMV complexes.[7–8,11] Overall, OMVs represent a versatile nano-scaled bioreactor platform and we became interested in evaluating OVMs for the conversion of fatty acids.

Fatty acids, one of the most abundant renewable resources in nature, serve as starting materials in the synthesis of biofuels and oleochemicals but also for the synthesis of signalling molecules and bioactive metabolites.[12] For instance, unsaturated fatty acids have been converted into hydroxy fatty acids by fatty acid double bond hydratases[12c,13] or into hydrocarbons through decarboxylation of the terminal carboxyl group by a photoactivated decarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP) (Scheme 1).[14] Moreover, functionalisation of fatty acids[15] sets the stage for the preparation of further products such as ω-amino fatty acids and α,ω-dicarboxylic acids.[15a,c, 16] The majority of the aforementioned reactions relies on whole-cell biocatalysis. The major limitations here reside with the poor solubility and dispersibility of fatty acids in aqueous reaction media and with transport issues into the living cell.[17]

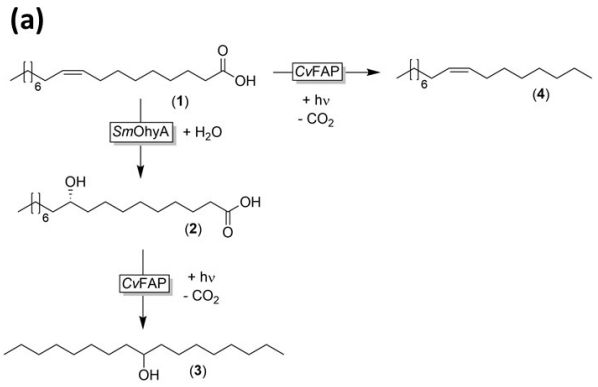
In the present study, we have engineered *E. coli* OMVs to form nano-scale bioreactors for the biotransformation of unsaturated long chain fatty acids. The biocatalysts used were the fatty acid double bond hydratase of *Stentrophomonas*

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202100778

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Scheme 1. Biotransformation of oleic acid by a fatty acid double bond hydratase (*Sm*OhyA) and/or a photoactivated decarboxylase (*Cv*FAP). (a) the reaction scheme and (b) schematic representation of the OMVs. The circles indicate enzymes.

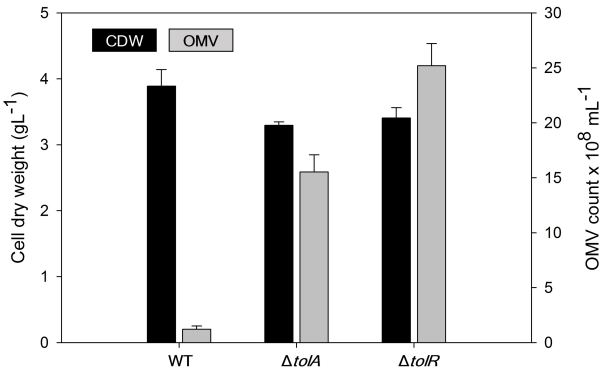


Figure 1. Cell dry weight (CDW, in g L^{-1}), (black bars) and number of outer membrane vesicles (OMVs) (grey bars), which were determined at the stationary growth phase of recombinant *Escherichia coli* BL21(DE3) pACYC-PelBSS-OhyA (WT), recombinant *E. coli* BL21(DE3) Δ*tolA* pACYC-PelBSS-OhyA (Δ*tolA*), and recombinant *E. coli* BL21(DE3) Δ*tolR* pACYC-PelBSS-OhyA (Δ*tolR*) in Terrific Broth (TB) medium. OMV suspensions were prepared from recombinant cell cultures by removing cells, purification via ultracentrifugation, and resuspension into 50 mM Tris buffer (pH 8.0) (see Experimental section for details). The experiments were performed in triplicate. Error bars show standard deviations.

maltophilia (*Sm*OhyA)[13a,b] and *Cv*FAP.[14] Engineered OMVs, encapsulating the *Sm*OhyA and/or *Cv*FAP, were investigated for the biotransformation of oleic acid ((*Z*)-octadec-9-enoic acid, **1**) into (*R*)-10-hydroxyoctadecanoic acid (**2**) and 9-hydroxyheptadecane (**3**) (Scheme 1). Also, the direct decarboxylation of oleic acid yielding (*Z*)-heptadec-8-ene (**4**) was investigated.

Results and Discussion

Preparation and characterisation of the engineered OMVs

Hyper-vesiculating *E. coli* BL21(DE3) mutants were constructed by deletion of *tolA* or *tolR* (as parts of the Tol-Pal gram-negative bacteria[18]). To target *Sm*OhyA into the periplasm of the *E. coli* cells its gene sequence was fused with the signal sequence of pectate lyase B from *Erwinia carotovora* (PelBSS)[19] obtaining the plasmid pACYC-PelBSS-*Sm*OhyA. Using this construct in wild type *E. coli* (BL21(DE3)) and its *tolA* and *tolR* deficient derivates expression of *Sm*OhyA was achieved. The resulting OMVs were isolated from the cultivation broths via ultracentrifugation. As shown in Figure 1, the OMV concentrations in the Δ*tolA* and Δ*tolR* broths were 1.6×10^{9} mL^{-1} and 2.5×10^{9} mL^{-1}, respectively and thereby 13- and 21-fold higher than from wt *E. coli*.

The morphological properties of the engineered OMVs were investigated by transmission electron microscopy (TEM) and dynamic light scattering (DLS). Although the number of OMVs produced per recombinant cells was significantly different, the morphology of the OMVs appeared to be similar (Figure S1). The OMVs were mostly spherical and ranged in diameter between 40 and 50 nm (Figure S1B). This is in accordance with previous findings with OMVs isolated from *E. coli* DH5α.[20]

The protein composition of the engineered OMVs was examined by SDS-PAGE and Western-Blot analysis (Figure S2). Next to the typical OMV protein OmpF[11b,21] also the heterologous protein *Sm*OhyA was found as a major protein in the OMVs (Figure S2). Notably, the overall concentration of *Sm*OhyA linearly correlated with the number of OMVs irrespective of which expression system was used (Figure S3), indicating that the *Sm*OhyA expression level did not vary significantly between the three strains.

Compared to the free enzyme, the OMV-confined *Sm*OhyA exhibited a significantly higher apparent K_M value indicating some diffusion limitation of the reagents over the OMV membrane.

Fatty acid biotransformation activity of the OMVs containing *Sm*OhyA

To evaluate the fatty acid hydration activity of the *Sm*OhyA-encapsulating OMVs, the biotransformation of oleic acid (**1**) (Scheme 1) was used as model reaction.

The biotransformations were initiated by adding oleic acid to a reaction medium containing the OMVs obtained from *E. coli* fermentations of wt, Δ*tolA* or Δ*tolR* *E. coli* (the resulting

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OMV concentrations were 0.12×10^{10} mL^{-1}, 1.6×10^{10} mL^{-1} and 2.5×10^{10} mL^{-1}, respectively, according to nanoparticle tracking analysis).[22] In all cases oleic acid was converted into (*R*)-10-hydroxyoctadecanoic acid (**2**) (Figure 2a–c).

The initial reaction rates were 0.6 mM h^{-1}, 9.6 mM h^{-1} and 10.8 mM h^{-1} (10, 160 and 180 U L^{-1}) and thereby linearly correlated with the OMV concentration in the reaction mixture (Figure 2d).

Similar reaction profiles were also observed with ricinoleic acid as starting material (Figures S5 and S6).

*Sm*OhyA activity was almost not detectable in the supernatant of the biotransformations indicating that surfactants (such as 0.1% (v/v) Tween 80) did not significantly affect structural stability of the OMVs. It is interesting to note that the thermal stability of the OMV-confined *Sm*OhyA was somewhat higher than of the purified enzyme. While the purified enzyme lost approx. 90% of its activity already after 4 h incubation at 20 °C, the OMV-confined enzyme exhibited more than 40% of residual activity (Figure S8A). Similar observations were made incubating the enzyme at 4 °C (Figure S8B).

Decarboxylation of oleic acid by the engineered OMVs

Next, we drew our attention to the *Cv*FAP-catalysed light-dependent decarboxylation of fatty acids. Similar to the *Sm*OhyA strategy outlined above, OMVs encapsulating *Cv*FAP were constructed by fusion of *Cv*FAP with the PelB signal

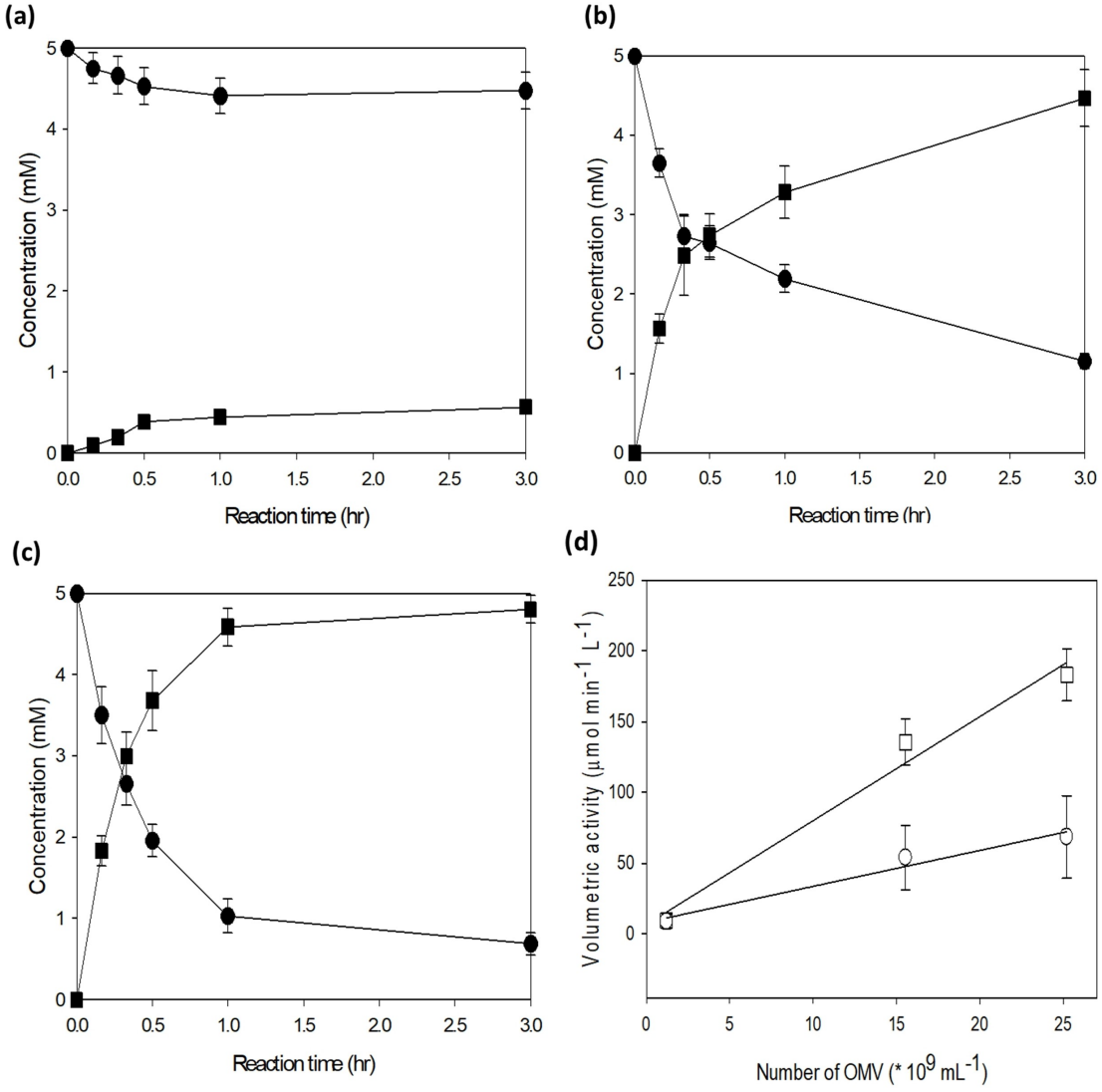


Figure 2. Time courses of the oleic acid hydratation by the OMVs prepared from the recombinant *E. coli* pACYC-PelBSS-OhyA (a), *E. coli* ΔtolA pACYC-PelBSS-OhyA (b), and *E. coli* ΔtolR pACYC-PelBSS-OhyA (c) cultures. Relationship between the initial biotransformation rates of oleic acid or ricinoleic acid and the number of OMVs (d). Biotransformations were initiated by adding 5 mM oleic acid and 0.1% (v/v) Tween 80 into the 50 mM Tris buffer (pH 8.0) containing the OMVs (the OMV densities were 0.12, 1.6, and 2.5×10^{10} OMV/mL, respectively) (35 °C and 200 rpm). The OMVs were prepared as described in the caption of Figure 1. The experiments were performed in triplicate. Error bars indicate the standard deviations. The symbols indicate the concentrations of oleic acid (**1**) (closed circle), 10-hydroxyoctadecanoic acid (**2**) (closed square), and volumetric biotransformation rates of oleic acid (1) (open square), and volumetric biotransformation rates of ricinoleic acid (**3**) (open circle).

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sequence and expression in the hyper-vesiculating *E. coli* strains (Figures S9 and 10). The recombinant *E. coli* BL21(DE3) Δ*tolA* pACYC-PelBSS-*Cv*FAP and *E. coli* BL21(DE3) Δ*tolR* pACYC-PelBSS-*Cv*FAP were able to produce the *Cv*FAP-containing OMVs (Figure S11).

The biotransformation profiles of the *Cv*FAP-containing OMVs for the conversion of oleic acid to (*Z*)-heptadec-8-ene were comparable to those of the *Sm*OhyA-containing OMVs. Initial rates of 0.18, 5.4 and 6.6 $mM \times h^{-1}$ (3, 90, and 110 $U \times L^{-1}$) were observed with the OMVs isolated from wild-type, Δ*tolA* and Δ*tolR* mutant cultures, respectively (Figure 3).

Again, the initial rates linearly correlated with the concentration of the OMVs applied.

Cascade biotransformation of oleic acid by OMVs and whole cells containing *Sm*OhyA and *Cv*FAP

Next, we aimed at the multistep biotransformation of unsaturated fatty acids combining *Sm*OhyA-catalysed hydratation with *Cv*FAP-catalysed decarboxylation (Scheme 1). To attain this catalyst, we co-expressed *Sm*OhyA and *Cv*FAP (both containing the PelB sequence) in *E. coli* Δ*tolR*. The resulting recombinant *E. coli* Δ*tolR* pACYC-PelBSS-*Sm*OhyA, pET22b-PelBSS-*Cv*FAP produced the desired OMVs encapsulating both enzymes in soluble form (Figure S12). As model reaction we chose the transformation of oleic acid into 9-hydroxyheptadecane via (*R*)-10-hydroxyoctadecanoic acid (Scheme 1).

The two-step one-pot biotransformation (Scheme 1) was initiated by adding 3.5 mM oleic acid to the isolated OMVs (in 50 mM Tris buffer (pH 8) containing 0.1 % (v/v) Tween 80) under dark conditions. After 3 h, the second reaction was initiated by exposing the reaction setup to blue light for another 2 h (see the Experimental section for details). The first reaction proceeded to approx. 71 % conversion of oleic acid into (*R*)-10-hydroxyoctadecanoic acid (Figure 4). After 3 h, illumination was initiated which resulted in the smooth decarboxylation of both, remaining oleic acid and the intermediate (*R*)-10-hydroxyoctadecanoic acid (Figure S13).

Apparently, prolonging the dark, hydratation phase will increase the overall selectivity from oleic acid (**1**) to 9-hydroxyheptadecane and diminish accumulation of the undesired (*Z*)-heptadec-8-ene (originating from the decarboxylation of non-converted oleic acid).

Finally, we compared the catalytic performance of the OMV-confined biocatalysts with the activity of the enzymes expressed in non-hyper vesiculating *E. coli* cells (Table 1). For this, we referred the product formation rate to the cell or the OMV as catalytic unit (micro/nano reactor). Interestingly, the specific catalytic activity of whole *E. coli* cell per catalytic unit (i.e., per cell or OMV, respectively) was only 5–6 times higher than that of OMVs. Considering the size difference between OMVs ($d \sim 50$ nm, $V \sim 0.5 \times 10^{-3}$ μm^3) and *E. coli* ($d \sim 2 \times 1$ μm, $V \sim 0.6$ μm^3) [23] the volumetric productivity within the catalytic unit was more than 100-fold higher in OMVs as compared to *E. coli* cells (Table 1). This indicates that the OMVs obtained in this study contained a highly concentrated enzyme mixture compared to the non-hypervesiculating *E. coli* cells.

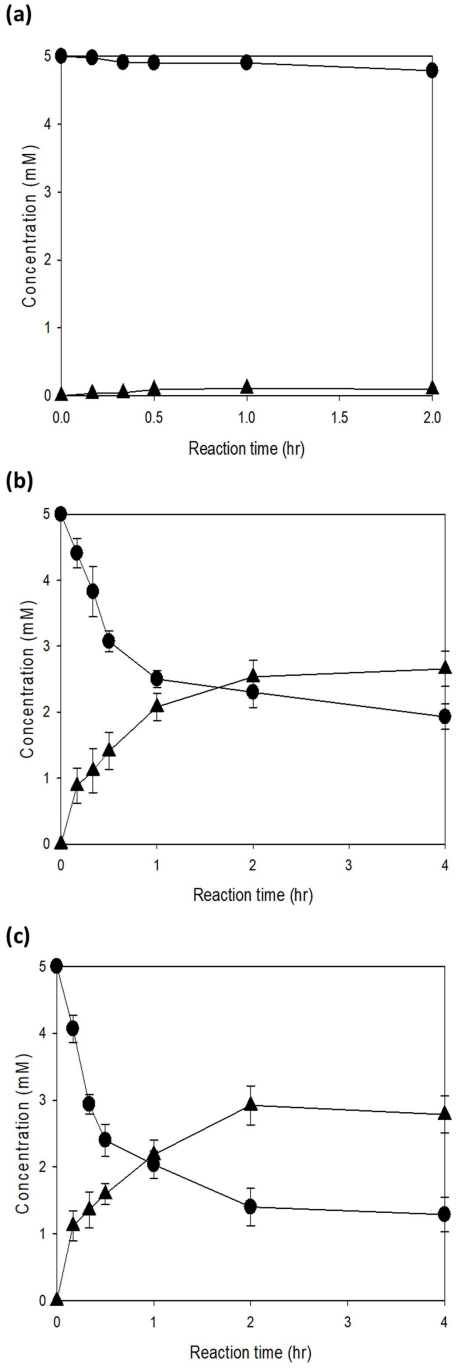


Figure 3. Time courses of the oleic acid decarboxylation reaction catalysed by the OMVs prepared from the recombinant *E. coli* pACYC-PelBSS-*Cv*FAP (a), *E. coli* ΔtolA pACYC-PelBSS-*Cv*FAP (b), and *E. coli* ΔtolR pACYC-PelBSS-*Cv*FAP (c) cultures. Biotransformations were initiated by adding 5 mM oleic acid and 0.1 % (v/v) Tween 80 into the 50 mM Tris buffer (pH 8.0) containing the OMVs under blue light ($\lambda = 450$ nm; intensity $= 13.7$ $mEL^{-1}s^{-1}$) (37 °C and 400 rpm). The OMV densities were 0.18, 4.2, and 6.7×10^{10} OMV/mL, respectively. The OMVs were prepared as described in the caption of Figure 1. The experiments were performed in triplicate. Error bars indicate the standard deviations. The symbols indicate the concentrations of oleic acid (**1**) (closed circle) and (Z)-heptadec-8-ene (**4**) (closed triangle).

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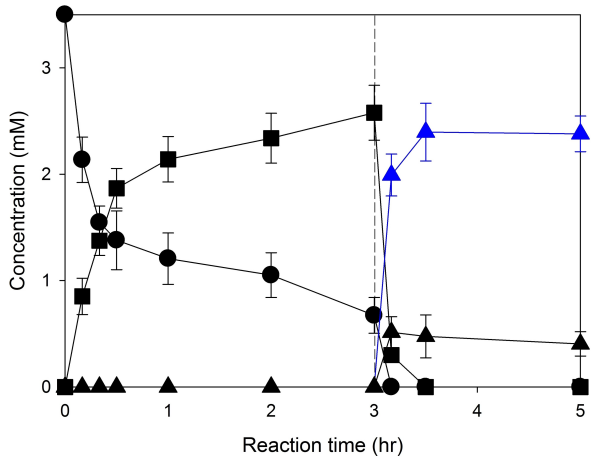


Figure 4. Time course of the oleic acid biotransformation by the OMVs prepared from the recombinant *E. coli* Δ*tolR* pACYC-PelBSS-OhyA, pET22b-PelBSS-*Cv*FAP. The biotransformation was initiated by adding 3.5 mM oleic acid and 0.1% (v/v) Tween 80 into the 50 mM Tris buffer (pH 8.0) containing the OMVs (OMV density: 4.4×10^{10} OMV/mL). The decarboxylation was initiated by illumination with blue light (λ = 450 nm; intensity = 13.7 mE L^{-1} s^{-1}) at t = 3 h. The recombinant gene expression was induced by adding 0.03 mM IPTG into the culture broth. The OMVs were prepared as described in Figure 1. The experiments were performed in triplicate. Error bars indicate the standard deviations. The symbols indicate the concentrations of oleic acid (closed circle), 10-hydroxyoctadecanoic acid (closed square), (*Z*)-heptadec-8-ene (closed triangle) and 9-hydroxyheptadecane (closed blue triangle).

Table 1. Oleic acid biotransformation rates of the OMV- and recombinant *E. coli*-based biocatalysts expressed as specific activity per micro-/nano-reactor.[a]

Enzymes	Rate [(μmol × min^{-1}) × $unit^{-1}$] × 10^{-12}			
	OMV			*E. coli* cell
	WT	Δ*tolA*	Δ*tolR*	Whole *E. coli* cells
*Sm*OhyA	8.0	4.8	7.3	42
*Cv*FAP	1.9	2.1	1.7	12
*Sm*OhyA + *Cv*FAP	–	–	4.5	22

[a] The biotransformation rates were determined based on the experiments shown in Figures 2 to 4 and Figure S7, respectively.

Conclusion

Overall, the present study demonstrates the synthetic potential of OMVs as nano-scaled reactors for biocatalytic and multi-step biocatalytic reactions. Especially if the biocatalysts are specifically targeted to the OMVs, e.g., using the signal sequence PelBSS, OMVs containing highly concentrated biocatalysts can be obtained, which especially for multi-step cascade transformations is very promising.

Experimental Section

A complete description of the experimental and analytical conditions can be found in the supporting information.

Microbial strains and cultivation conditions

Recombinant *E. coli* was cultivated at 37 °C in lysogeny broth medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with appropriate antibiotics for plasmid DNA preparation (Table S2). Terrific broth (TB) medium (24 g/L yeast extract, 12 g/L tryptone, and 4 mL/L glycerol) with the appropriate antibiotics was used for the seed and main cultivation at 37 °C. Recombinant *Sm*OhyA expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture broth at the exponential growth phase (optical density at 600 nm: 0.5) followed by a 16 h incubation at 20 °C. Coexpression of *Sm*OhyA and *Cv*FAP was induced by 0.03 mM IPTG, which allowed expression of the both enzymes to a soluble form in *E. coli* (see Fig. S12 for details).

Construction of hyper-vesiculating *E. coli* mutants and recombinant plasmids

Hyper-vesiculating *E. coli* mutants (i.e., *tolA* and *tolR* gene knockout mutants) were constructed by the method described by Datsenko and Wanner.[24] The pET22b-PelBSS-*Cv*FAP was constructed via amplification of the gene from pET28a-*Cv*FAP[14f] and insertion into pET22b vector including the *pelB* signal sequence (Merck Millipore, Darmstadt, Germany) by In-Fusion cloning protocol (Takara Bio. Inc, CA, USA). The pACYC-PelBSS-CvFAP was constructed by using the pET22b-PelBSS-CvFAP. The plasmids, strains, primers, and protein sequences used in this study are summarised in Tables S1, S2, and S3.

Isolation of outer membrane vesicles (OMVs)

The culture broth of *E. coli* cells (150 mL) was centrifuged twice at 10,000 rpm for 10 min at 4 °C, and filtered through a 0.2 μm membrane to remove intact bacteria and undesired large cellular materials and concentrated by Amicon® Stirred Cells (Millipore, Bedford, MA, USA), as previously reported.[7] The OMVs were then pelleted at 29,000 rpm (150,000 g) in a Sorvall WX Ultra 90 centrifuge using an AH-629 rotor (Thermo Fisher Scientific, Waltham, MA, USA) for 3 h at 4 °C. The culture media was decanted and the OMV pellet was suspended in 1 mL of Tris buffer (50 mM, pH 8.0).

Biotransformation of fatty acids by OMVs and whole-cell biocatalysts

The hydration of oleic acid and ricinoleic acid by the isolated OMVs was conducted in the 50 mM Tris buffer (pH 8.0) at 35 °C and 400 rpm (reaction volume: 2 mL). The hydration of fatty acids was initiated by adding 5 mM oleic acid or ricinoleic acid with 0.1% (v/v) Tween 80 into the reaction medium containing the OMVs. The fatty acids were added from stock solution, which had been made by sonication with Tween80. The biotransformations were terminated by mixing with two volumes of ethyl acetate containing 5 g/L palmitic acid as internal standard.

The hydration of fatty acids by whole-cell biocatalysts was carried out based on our previous studies.[2,8] Briefly, after cultivation of the recombinant *E. coli* cells in TB medium (cell density: 3.6 g dry cells/L), the cells were harvested and washed with 50 mM Tris buffer (pH 8.0). The resulting recombinant cells were resuspended in Tris buffer (pH 8.0). The biotransformations were initiated by adding 10 mM fatty acids with 0.1% (v/v) Tween 80 into the reaction medium at 35 °C and 200 rpm (reaction volume: 10 mL).

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The decarboxylation of oleic acid by the isolated OMVs was conducted in 50 mM Tris buffer (pH 8.0) containing the OMVs at 37 °C and 400 rpm (reaction volume: 2 mL). The decarboxylation was initiated by adding 5 mM oleic acid and 0.1 % (v/v) Tween 80 into the reaction medium under blue light (λ = 450 nm). The final reaction conditions were: [oleic acid] = 5 mM, [Tween 80] = 0.1 % (v/v), [OMVs] = 0.18 to 6.7×10^{10} OMV mL^{-1}, Tris buffer pH 8.0 (50 mM, with 10 % (v/v) DMSO), blue light (intensity = 13.7 $mEL^{-1} s^{-1}$), total volume 2 mL.

The decarboxylation of whole-cell biocatalyst was carried out based on previous studies.[14f] Briefly, after cultivation of the recombinant *E. coli* cells in the TB medium (cell density: 3.6 g dry cells/L), the cells were harvested and washed with the 50 mM Tris buffer (pH 8.0). The biotransformation was initiated by 5 mM oleic acid and 0.1 % (v/v) Tween 80 into the reaction medium under blue light (λ = 450 nm). The reaction mixture was stirred at 37 °C and 400 rpm. The final reaction conditions were: [oleic acid] = 5 mM, [Tween 80] = 0.1 % (v/v), [*E. coli* cells] = 3.6 g dry cells L^{-1}, Tris buffer pH 8.0 (50 mM, with 10 % (v/v) DMSO), blue light (intensity = 13.7 $mEL^{-1} s^{-1}$), total volume 2 mL.

For serial reaction of *Sm*OhyA and *Cv*FAP in the OMVs, the decarboxylation of fatty acids was carried out under blue light (λ = 450 nm), after the hydration of oleic acids into 10-hydroxyoctadecanoic acid. The hydration of oleic acid was conducted in 50 mM Tris buffer (pH 8.0) containing the OMVs including *Sm*OhyA and *Cv*FAP at 35 °C under dark conditions (reaction volume: 2 mL). After over 70 % of the oleic acid had been converted into 10-hydroxystearic acid, illumination of the reaction medium with blue light (λ = 450 nm) was commenced at 35 °C. The final reaction conditions were: [oleic acid] = 3.5 mM, [Tween 80] = 0.1 % (v/v), [OMVs] = 4.4×10^{10} OMV mL^{-1}, Tris buffer pH 8.0 (50 mM, with 10 % (v/v) DMSO), blue light (intensity = 13.7 $mEL^{-1}s^{-1}$), total volume 2 mL. The biotransformations were terminated by mixing with two volumes of ethyl acetate containing 5 g/L palmitic acid.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2020R1A2B5B03002376) and by C1 Gas Refinery Research Center of the NRF funded by Ministry of Science and ICT, Korea (Grant Number: 2018M3D3A1A01055735). J.-W. Song was partially supported by RP-Grant of Ewha Womans University.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: outer membrane vesicles · hydratase · decarboxylase · biocatalysis · nano-scale bioreactor

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Manuscript received: May 28, 2021
Revised manuscript received: August 5, 2021
Version of record online: August 18, 2021

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