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Chemo-enzymatic synthesis of valrubicin using Pluronic conjugated lipase with temperature responsiveness in organic media†

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A novel synthetic route for valrubicin, an anti-cancer drug, was proposed using a temperature-responsive lipase–Pluronic conjugate which gave an overall yield of 82% and a purity of 98% under mild conditions (50 °C, 6–8 h). The high activity, stability, selectivity and reusability of this new lipase catalyst hold great promise for practical applications.

Valrubicin (*N*-trifluoroacetyladriamycin-14-valerate), a FDA approved anti-cancer drug for the treatment of refractory carcinoma in the bladder[1] can be produced by a three-step chemical synthesis including trifluoroacetylation, iodination and nucleophilic displacement with sodium valerate. The low reactivity of the second and third steps results in a low overall yield of 24% (ref. 2 and 3) and consequently a heavy burden of downstream processing including product purification and waste treatment. Enzymatic catalysis with high catalytic activity offers great opportunities for the synthesis of therapeutic molecules with complex structures.[4] Cotterill and Rich firstly reported the enzymatic synthesis of valrubicin, in which the first step was the *N*-trifluoroacetylation of doxorubicin, followed by the esterification at the C-14 site using immobilized *Candida antarctica* lipase, Chirazyme L2 and C3 with an optimized overall yield up to 79%.[5] However, the apparent activity of this heterogeneous catalysis was scarified due to the mass transfer resistance generated by the supporting matrix. Thus the reaction temperature was chosen as high as 60–120 °C in order to achieve a reasonable reaction rate (*e.g.*, 117 °C in MIBK with 82% conversation). Unfortunately, the high reaction temperature led to the formation of diacylated product and undetermined side-products. Another problem is the irreversible adsorption of doxorubicin, which reduces the yield significantly.[6] Nanostructured enzyme catalysts[7–9] such as enzyme nanogels[10–13] and flower-like enzyme–inorganic hybrid crystals[14] have demonstrated their capacities in enhancing enzyme stability at less compromise of enzymatic activity in either aqueous or non-aqueous media. Very recently, we described the first example of enzyme–polymer nanoconjugates which show temperature responsiveness in organic media. The solubilized enzyme nanoconjugates appeared an increased apparent activity by 60–600 fold compared to their native counterparts existing in the form of insoluble aggregates in organic media.[15] The recovery of the solubilized enzyme nanoconjugates can be readily accomplished by a temperature-induced precipitation. The high activity and ease of recycle make the enzyme conjugate appealing for chemical synthesis in organic solvents. This encourages us to examine the possibility of using this enzyme–Pluronic conjugate to achieve simultaneously a high selectivity, high yield and high reaction rate for the synthesis of valrubicin at a relatively low temperature which is more convenient for practical operation.

A two-step chemo-enzymatic synthesis route was employed (Fig. 1), in which the first step was the trifluoroacetylation of doxorubicin (Dox), followed by the lipase–Pluronic nanoconjugate catalyzed transesterification of the *N*-trifluoroacetyl

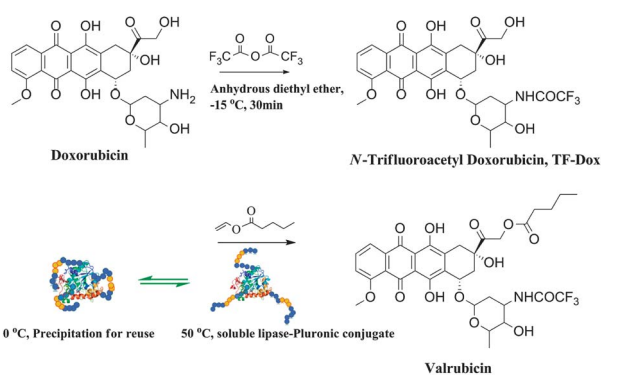

Fig. 1 The chemo-enzymatic synthesis of valrubicin.

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† Electronic supplementary information (ESI) available: Experimental details, TEM image of lipase conjugate in MIBK, HPLC of TF-Dox and valrubicin, mass spectrum, ^{1}H-NMR and ^{13}C-NMR of TF-Dox and valrubicin. See DOI: 10.1039/c3ra44879g

doxorubicin (TF-Dox) with vinyl valerate. For the synthesis of TF-Dox, 800 mg (1.3 mmol) of doxorubicin hydrochloride was dissolved in 8 mL of dry pyridine at −20 °C. Then, 2.8 mL of trifluoroacetic anhydride dissolved in 10 mL of anhydrous ester was added dropwise over a period of 10 min. After 20 min, 10 mL of water was added to quench the reaction, followed by the extraction with ethyl acetate for 3 times, washing with deionized water, and drying with Na_2SO_4. After removing the excess ethyl acetate by the vacuum evaporation, the crude product was obtained by precipitating from $CHCl_3$–petroleum ether (98% yield). The further purification was carried out using a silica gel column chromatography (elution with $CHCl_3$–CH_3OH). After purification, the purity of TF-Dox reached over 99% with a yield of 91% (see NMR and mass spectrum in ESI†).

The lipase–polymer conjugate was synthesized according to ref. 15. Typically, Pluronic-F127 was first functionalized with aldehyde groups by Dess-Martin periodinane. A solution of the aldehyde-functionalized Pluronic (the molar ratio of aldehyde groups to amine groups of protein being 1.1 : 1) was added to the lipase solution (5–10 mg mL^{-1} in borate buffer, 50 mM, pH 8.0). After 2 h reaction, $NaCNBH_3$ (10% weight of Pluronic) was added to the mixture to reduce the Schiff base for 4 h, followed by the dialysis against the phosphate buffer (10 mM, pH 7.0). After the conjugation, the hydrolytic activities of lipase from *Candida rugosa* (CRL), *Candida antarctic* lipase B (CALB), lipase from *Thermomyces lanuginosus* (LTL) were measured as 42.0%, 98.5%, 91.4% (Fig. 2a) with reference to free lipase. As described in ref. 10, the conjugates can be solubilized and thus well dispersed in commonly used organic solvents such as toluene, methyl isobutyl ketone (MIBK), acetonitrile, and *etc.* Transmission electron microscopy (TEM) images of the CALB–, LTL–, and CRL–Pluronic conjugates dried from MIBK solution showed that the lipase–Pluronic conjugates self-assembled to nanospheres with diameters of 20–40 nm (Fig. 2b and S1†).

The high dispersion of the lipase nanoconjugate in organic solvents facilitates both the uptake of the substrate and the discharge of the product. This is favorable for an enhanced apparent activity for the transesterfication of *N*-trifluoroacetyl doxorubicin with vinyl valerate. In a typical experiment, 100 mg of *N*-triflutoacetyl doxorubicin (0.156 mmol) and 180 mg (1.4 mmol) of vinyl valerate were dissolved in organic solvent, *e.g.* 1.0 mL of MIBK, followed by the addition of 20 mg of native CALB (or CALB–Pluronic nanoconjugate containing the same amount of protein). The mixture was stirred at 50 °C for 8 h. The reaction was monitored by the thin-layer-chromatography (TLC, CH_3OH–$CHCl_3$, 1 : 10) and HPLC analysis. After reaction, the mixture was cooled to 0 °C, the catalyst was precipitated and collected by centrifugation. The supernatant was evaporated and the product was precipitated from $CHCl_3$–petroleum ether and purified by the silica gel column chromatography (elution with $CHCl_3$–CH_3OH). The conversions of TF-Dox calculated from HPLC analysis (Fig. 2c) showed that the LTL and CALB were the appropriate catalysts for the reaction. The conjugates exhibited remarkably increased conversions (2.1 times for LTL and 6.0 times for CALB) compared to that obtained by the native lipase at the same condition. However, neither native CRL nor its conjugate showed any activity in the reaction. Fig. 1d indicated that within a period of 10 h at 50 °C in MIBK, the reaction at saturated substrate condition catalyzed by native CALB showed a consistent rate, indicating there was no loss of activity for CALB in MIBK. After 10 h reaction, only a conversion of ~20% was achieved. In contrast, the CALB–Pluronic conjugate

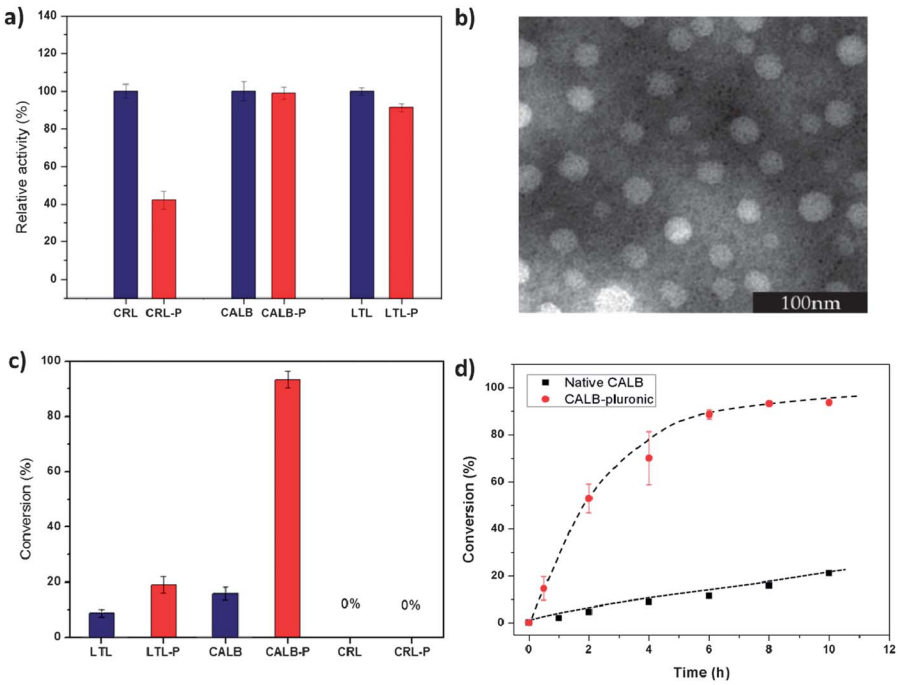

Fig. 2 (a) The hydrolytic activities of lipase conjugates; (b) TEM image of CALB–Pluronic conjugate in MIBK; (c) the conversations of TF-Dox catalyzed by lipase and their conjugates in MIBK at 50 °C; (d) conversions of TF-Dox in the enzymatic synthesis using free CALB and CALB–Pluronic conjugate.

Table 1 Effect of solvents on CALB–Pluronic conjugate catalyzed reaction[a]

Entry	Solvent	V_0[b] (mM h^{-1})	Conversion[b] (%)
1	Acetonitrile	10.5	89.9
2	MIBK	4.7	94.3
3	Chloroform	0.3	6.6
4	Acetone	3.0	27.4
5	Methyl *tert*-butyl ether	0.9	21.4

[a] *The reaction condition*: 10 mg *N*-trifluoroacetyl doxorubicin, 10 μL vinyl valerate in 1 mL solvent, 25 mg CALB–Pluronic nanoconjugate (amount to 2 mg protein), 50 °C, 8 h. [b] Conversions were determined by HPLC analysis, V_0 was calculated from the conversion in the first 30 min. All data were obtained from three parallel experiments.

achieved an over 90% conversion after 6 h reaction. Calculated from the initial reaction rate, the catalytic activity of the CALB–Pluronic conjugate is 11 times higher than that of the native lipase.

Table 1 gives the initial reaction rates and the final conversions (50 °C, 8 h) obtained by the CALB–Pluronic conjugate in commonly used organic solvents. Here acetonitrile and MIBK are the favorable solvents for the enzymatic reaction. Although acetonitrile gave a faster initial rate, the final conversion was lower than that in MIBK. In addition, a more complete precipitation of the CALB–Pluronic conjugate is achieved in MIBK than acetonitrile when subjected to a low temperature, which facilitates the reuse of the conjugate. Thus MIBK is selected as the solvent for the enzymatic catalysis. For the optimized enzymatic process (10 mg mL^{-1} *N*-trifluoroacetyl doxorubicin, 10 μL mL^{-1} vinyl valerate, 25 mg mL^{-1} conjugate, MIBK, 50 °C, 10 h), the purity of valrubicin reached 98% (determined by HPLC) with a yield of 90%. No diacylated product was found (see NMR data, MS spectrum and HPLC in ESI†).

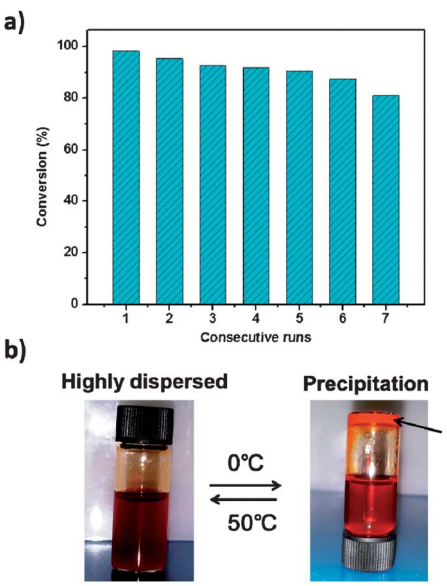

Fig. 3 (a) Conversions of TF-Dox for the consecutive batches of the synthesis catalyzed by CALB–Pluronic conjugate. After each batch (in MIBK, 50 °C, 6 h) the mixture was cooled from 50 °C to 0 °C, and centrifuged at 7000 rpm for 10 min to separate the catalyst. Then new reactants and solvent were added to the precipitated catalyst for the subsequent batch; (b) photos showing CALB–Pluronic nanoconjugate dispersed in the reaction mixture (TF-Dox and vinyl valerate in MIBK) and precipitated from the mixture after reaction.

Fig. 3 shows that the CALB–Pluronic nanoconjugate is well dispersed in MIBK at 50 °C and thus enables a homogenous catalysis. After the reaction, the conjugate precipitates from the catalysis system at 0 °C and thus facilitates the recovery and reuse of the catalyst. In the present study, 50 mg of CALB–Pluronic conjugate (containing 4 mg protein) was applied to catalyze the conversion of TF-Dox to valrubicin (10 mg in every batch) for 7 batches with an average yield over 80%. The high enzyme stability and ease of recycle hold great promise for the lipase conjugates in the industrial synthesis of valrubicin.

Conclusions

In summary, we have prepared a temperature-responsive lipase–Pluronic conjugate for the chemo-enzyamtic synthesis of valrubicin. The CALB–Pluronic conjugate has a greatly increased apparent activity in organic solvent and can be reused conveniently by a temperature-induced precipitation. The reaction rate for the synthesis of valrubicin in organic media was increased by 11-fold compared to that of the native CALB at a relatively low temperature which is more convenient for practical operation. Under an optimized condition, the overall yield of valrubicin is 82% with a purity of 98%. The temperature responsiveness of the lipase conjugate offers advantages for the recovery and operation of the lipase nanoconjugates.

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