

tumor cells in eliciting an immune response that can eradicate an established metastatic disease. In this regard, *in situ* tumor destruction by radio frequency ablation in combination with the administration of a blocking cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-specific monoclonal antibody elicited a long-lasting tumor protection immunity in melanoma-bearing mice⁹. Similarly, if the killing of tumor cells by readily available approaches such as chemotherapy and radiotherapy were at least as effective as the killing of normal cells, one might ask whether the gene therapy strategy of Vile and coworkers is really needed.

Third, Vile and coworkers have shown that administration of hsp70 in conjunction with tissue-specific killing of melanocytes improved the therapy for established tumors. However, no comparisons have been made with other molecules, such as antibodies specific for costimulatory molecules CD40 and 4-1BB^{10,11} or antibodies that specifically block CTLA-4¹², which are known to augment an immune response to self-antigens expressed by tumor cells.

Fourth, most of the tumors eradicated in the present study are subcutaneous lesions. Therefore one might wonder whether the immunity elicited by killing of normal melanocytes is also able to eradicate lesions located in visceral organs such as brain and liver.

Lastly, it remains to be determined how effective the immunity elicited by the killing of normal cells with the coadministration of an immunostimulatory agent will be in counteracting the escape mechanisms that are frequently used by tumor cells to avoid the host's immune attack¹³. Answers to these questions will contribute to defining the relevance of the strategy described by Vile and coworkers and its potential impact on the treatment of malignant diseases.

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Finding enzymatic gold on silver surfaces

Uwe T Bornscheuer

Surface-enhanced resonance Raman scattering enables rapid and sensitive identification of lipase activity and enantioselectivity on dispersed silver nanoparticles.

The application of enzymes in biocatalysis plays an increasing role in industrial processes, especially in the development of novel approaches for the synthesis of fine chemicals^{1–3}. This requires a priori the availability of a suitable biocatalyst, which traditionally has been identified by methods, such as spectrophotometry, conventional gas chromatography or high-performance liquid chromatography (HPLC), that are limited in sensitivity, portability and speed. Now, Graham and colleagues⁴ have developed a principle based on the use of surface-enhanced resonance Raman scattering (SERRS) to measure lipase activity on silver nanoparticles at ultra-low levels. Their concept has the potential to significantly facilitate enzyme characterization and optimization.

Lipases and hydrolases are among the most important enzymes in biotechnology today⁵. They are capable of functioning in aqueous and nonaqueous media, are active against a broad range of substrates and are highly stable at extremes of temperature and pH. As well as providing biocatalysts for fine chemical synthesis, their enantioselective and regioselective properties have been exploited in such diverse industrial applications as chiral drug separations, the modification of fats in foods and even in cosmetic treatments for the personal care sector.

In the past few years, tremendous progress has been made in discovering new activities and optimizing existing lipases and other industrially relevant enzymes. This progress has been spurred by the application of directed evolution approaches (that is, error-prone PCR or DNA shuffling⁶), which enable the creation of large mutant libraries of a given enzyme. Two approaches for library creation are phage display⁷, in which enzyme variants are pre-

sented on the surface of phage, and *in vitro* (cell-free) compartmentalization (IVC)⁸, in which aqueous droplets in water-in-oil emulsions serve as 'cell-like' compartments in which a gene, its encoded enzyme and the products of enzyme activity remain isolated. Both approaches in principle allow screening of huge libraries (>10⁸ variants), but phage display can rarely be used when assaying catalytic turnover, as this is much more difficult to implement for enzymes compared to antibodies, and IVC has not yet been demonstrated to permit the determination of enzyme enantioselectivity.

One of the most significant challenges in enzyme engineering is identifying the optimal enzyme in a pool of 10⁴ to >10⁸ enzyme variants. In all high-throughput systems for screening enzyme activity, compromises have to be made with respect to sensitivity, simplicity and availability of assay substances. It is always advantageous to use an enzyme assay compound that closely resembles the substrate of an enzyme's chemical reaction—for a lipase, for example, an acetate of a secondary alcohol is preferable over a chromogenic derivative (or surrogate substrate)—because the 'first law of directed evolution' states 'you get only what you screen for.' In addition, the assay should enable the determination of an enzyme's kinetics and intrinsic stereoselectivity. Thus, although spectrophotometric detection can be used to detect NADH derived by an enzyme cascade from acetic acid released as a result of lipase hydrolysis⁹, detection is of insufficient sensitivity and the determination of true enzyme enantioselectivity is also not possible. One alternative is to use mass spectrometry to measure the products of catalytic breakdown of a pseudo-racemic mixture of a deuterated and a nondeuterated enantiomer of an acetic acid ester; however, this requires investment in high-throughput electrospray ionization mass spectrometry devices¹⁰.

The format described by Graham and colleagues clearly overcomes limitations associated with previous (hydrolase) assays.

Uwe T. Bornscheuer is at the Institute of Chemistry and Biochemistry, University of Greifswald, 17489 Greifswald, Germany.
e-mail: uwe.bornscheuer@uni-greifswald.de

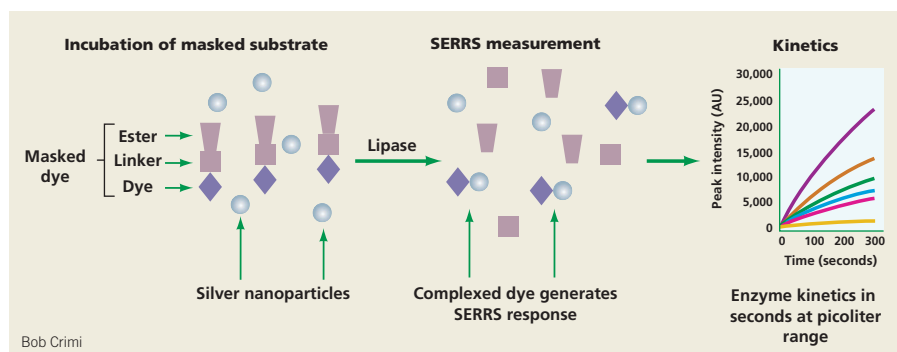


Figure 1 A diagram of the surface-enhanced resonance Raman scattering (SERRS) measurement of enzyme activity. A masked dye consisting of the substrate (3-phenylbutyric acid) linked via a spacer group to a dye (a naphthalene- or aniline-based azo-benzotriazole) is cleaved by a lipase. This turnover leads to the release of the surface-targeting dye, which complexes with citrate-reduced silver nanoparticles dispersed in the aqueous reaction system. This causes a characteristic change in the vibrational spectrum, which is quantified by SERRS enabling the determination of reaction kinetics within seconds in the picoliter range. Measurement of the hydrolysis rate for both enantiomers of the chiral substrate in parallel experiments allows the calculation of the enantioselectivity of the biocatalyst.

The principle is based on the use of a benzotriazole dye, which is masked via a linker to the lipase substrate. Lipase-catalyzed turnover of the substrate releases the dye, which complexes to dispersed silver nanoparticles. Rather than using spectrophotometry or mass spectrometry to detect enzyme activity, the authors measure dye deposition on the nanoparticles using SERRS. Detection via SERRS relies on the very high enhancement of the Raman intensity of molecules adsorbed to a surface when illuminated by an infrared laser; thus in the nanoparticle assays, adsorbed dye molecules generate a SERRS response that is proportional to the enzymatic reaction (Fig. 1).

The authors demonstrate the assay's sensitivity to be in the picoliter (and possibly even femtoliter) range. Thus, enzyme concentrations similar to *in vivo* levels can be detected. In addition, the data depicting the use of 14 different lipases in comparison with conventional HPLC spectroscopy (which required a 100 times higher dye concentration and longer incubation times: 6 h versus 300 s) shows that enantioselectivity values determined for the hydrolysis of the model compound 3-phenyl butyric acid are almost identical. Thus, despite the use of surrogate substrates, not only activity but also enantioselectivity can be quantified at extremely low concentrations (although some scattering of data can be observed at high enzyme concentrations).

One drawback of the authors' assay format is the requirement for the specific instrumentation for the Raman measurements and the need to adapt the system for

microtiter plate-based formats. In addition, calibration might be necessary to compensate for changes in surface with hot and cold spots. Other aspects to be addressed in future work include the design of dye substrates for the measurement of enzyme activity for further lipase substrate groups, such as chiral alcohols. The development of specific masked substrates for other enzyme classes should significantly broaden its versatility and applicability.

The prospect of adapting this assay to living cells, as suggested by the authors, is intriguing but likely to present significant challenges. Problems with measurements in the presence of cell components as well as access of substrate or binding of product to the silver nanoparticles need to be addressed. If these hurdles can be overcome, the assay could find wide application in functional protein analysis and might even allow the simultaneous detection of multiple enzyme activities at very low concentrations.

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