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Enzyme Immobilization on Porous Silicon Surfaces**

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Immobilization of enzymes on various solid platforms, such as glass,[1] silicon,[2–4] polymers,[5–7] and electrode materials,[8] is of great interest for applications as diverse as enzyme reactor-based decontamination,[9,10] enzyme microreactor-based healthcare,[11] biological fuel cells, and chemical and biological sensing.[12–14] In the past, the solid platform has mainly been used as a passive support for the enzymes,[15] but never as a direct transducer allowing in-situ and real-time detection of enzymatic activity. In this article, we present a new immobilization technique that allows the covalent anchoring of enzymes on an active silicon platform while retaining enzymatic activity. We will show that the luminescence of the silicon matrix constitutes a direct transduction mode for the detection of enzymatic activity, because the silicon nanocrystals are able to transfer energy with the products of the enzymatic breakdown. This result opens the way for a new class of extremely compact and inexpensive chemical sensors based on silicon technology.

The main techniques reported to immobilize enzymes on silicon platforms are silanization, metal linking, gel entrapment, and poly(L-lysine) attachment.[2] Their main disadvantage is that they require a preliminary oxidation of the silicon surface, and therefore drastically reduce the probability of any energy coupling between the silicon platform and the products of the enzymatic breakdown.[16] Instead of using the classic silane route,[17] we adapted a silicon–carbon surface attachment technique.[18]

Luminescent porous silicon samples were formed by light-assisted electrochemical etching of n-type, <100> oriented silicon wafers, in a mixture of hydrofluoric acid, de-ionized water, and ethanol (see Experimental section for details). The composition of the electrolyte leads to a clean hydride-terminated porous silicon surface (see Fig. 1), which was chemically

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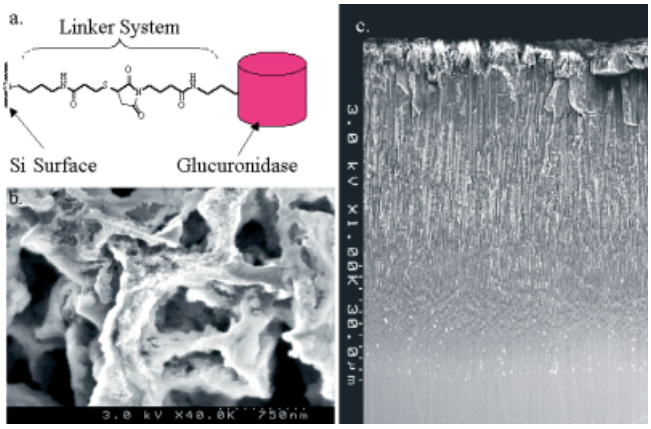
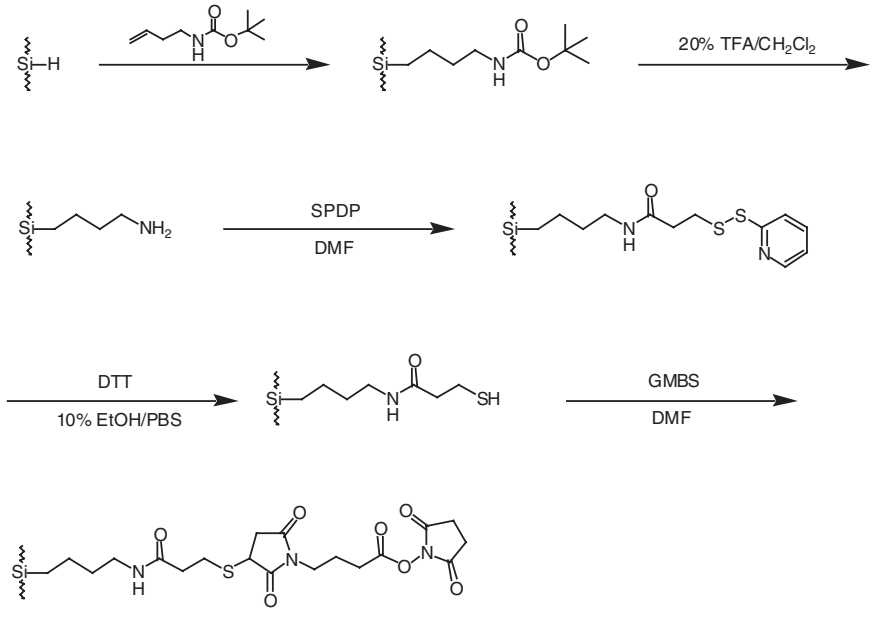


Figure 1. Attachment of β-glucuronidase enzyme on a linker-functionalized porous silicon device. a) Chemical structure of the linker covalently bound to the silicon surface and to the β-glucuronidase (the attachment site to the enzyme is not specified). b) SEM top view of an etched device with enzyme attached. c) SEM cross-section of an etched silicon device with enzyme attached.

functionalized according to a process detailed in the Experimental section and illustrated in Scheme 1. The hydride-terminated porous silicon surface was covalently functionalized with *t*-butyloxycarbonyl- (BOC)-protected amine using a photochemical process.[19] The construction of the rest of the linker was characterized and described in detail elsewhere.[18] Briefly, following modification of the surface, the BOC group was removed and the amine was reacted with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). The resulting terminal 2-pyridyldithio group was removed using dithiothrietol (DTT) to give a terminal thiol. This surface was subsequently reacted with *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS) to give the final linker with terminal *N*-hydroxysuccinimide (NHS) activated ester. Finally, β-glucuronidase was reacted with this linker to give the enzyme-functionalized silicon surface (see Fig. 1a). Methods and verification of attachment of various biomolecules on silicon surfaces with a similar linker system have been described elsewhere.[18] The attachment point of the β-glucuronidase has not been determined, but is thought to be through lysine residues. Removal of noncovalently bound enzyme was achieved by washing the sample extensively with copious amounts of 1 M sodium chloride (NaCl) in phosphate-buffered saline (PBS, pH 7.4). Scanning electron microscopy (SEM) images of the enzyme-functionalized surface (Fig. 1b,c) show pore diameters between 500 and 750 nm in the first 5 μm of the porous silicon surface.

The glucuronidase-functionalized porous silicon samples were mounted in a Teflon flow cell fitted with a quartz window and connected to a UV-vis quartz cell in a closed loop. Samples were first equilibrated in PBS buffer, and then in a solution of the substrate *p*-nitrophenyl-β-D-glucuronide in PBS in order to assay the activity of the immobilized β-glucuronidase. Each porous silicon sample was tested with substrate concentrations ranging from 25 to 250 μM. The enzymatic activity was monitored in situ in real time by passing the solution through a UV-vis spectrophotometer. The increase in *p*-nitrophenol concentration due to the enzymatic breakdown of the substrate *p*-nitrophenyl-β-D-glucuronide was measured as an increase in absorbance of the solution at 405 nm versus time (see Fig. 2, insert). Activity assays were run for a minimum of 30 min for each substrate concentration. Each experiment was repeated twice and a 10 min PBS buffer rinse was performed between two consecutive substrate concentrations. The absorbance slopes for each substrate concentration were converted into velocities by using an extinction coefficient of 9000 M^{-1} cm^{-1} for *p*-nitrophenol at 405 nm and pH 7.4.[20] The data were then fitted with the Michaelis–Menten equation for a first-order enzyme kinetic system (see Fig. 2):[21] $V = (V_{max}[S])/(K_m + [S])$, where V is the



Scheme 1. Successive steps involved in the surface functionalization of silicon hydride terminated porous silicon: light-promoted hydrosilylation, removal of BOC protecting group, SPDP attachment, reductive cleavage of pyridyl disulfide protecting group, and GMBS attachment.

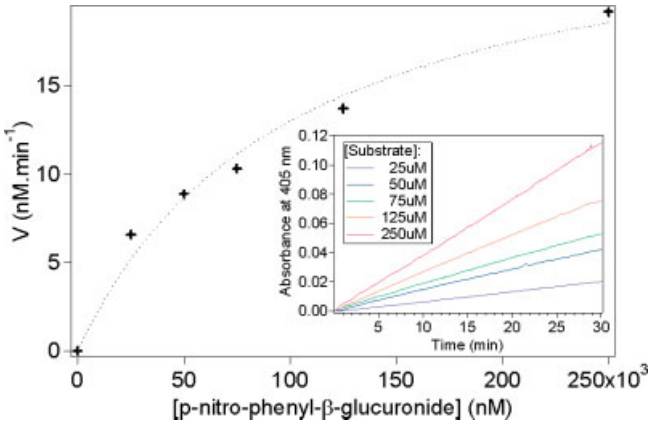


Figure 2. Plot of initial velocity versus *p*-nitro-phenyl-*β*-D-glucuronide concentration for a glucuronidase-functionalized porous silicon surface (crosses) and fit with the Michaelis–Menten equation (dashed line). The raw data (absorbance at 405 nm versus time at various substrate concentrations) are shown in insert.

velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant and $[S]$ is the substrate concentration. V_{max} and K_m were also deduced from double reciprocal plot of the Lineweaver–Burk equation:[21] $V^{-1} = (K_m/V_{max})\,(1/[S]) + (1/V_{max})$. The values obtained are summarized in Table 1. These results can be compared to the continuous spectrophotometric assay

Table 1. Activity constants for *β*-glucuronidase enzyme immobilized on porous silicon.

Technique/Constant	V_{max} [nM min^{-1}]	V_{max} [nmol min^{-1}]	K_m [mM]
Michaelis-Menten	26	0.11	0.1
Lineweaver-Burk	19	0.076	0.05

for *β*-glucuronidase conducted by Aich et al.,[20] in which the kinetic constants V_{max} and K_m for enzymes suspended in liquid phase were found to be 1.55 nmol min^{-1} and 0.078 mM, respectively. Although the values of the Michaelis constant, K_m, are in the same range for immobilized enzymes and enzymes in solution, the maximal velocity is found to be slightly lower for the immobilized enzyme. This difference in V_{max} between the free-floating enzyme and the immobilized enzyme is likely to be due to a decreased product release speed when the enzyme is bound on the silicon device.

Control experiments were performed in order to assess for non-specific binding of the *β*-glucuronidase enzymes to the functionalized porous silicon surface. In the first experiment, a porous silicon surface was functionalized with a BOC-protected amine. At this stage, the linker is capped and no direct binding site is available on the surface for the *β*-glucuronidase. This sample was then incubated in a solution of *β*-glucuronidase and rinsed, according to the procedure previously described for the full attachment. It was then mounted in the flow cell and an enzymatic activity assay was performed with substrate concentrations of 75 and 250 μM, respectively. No activity was detected, indicating that no measurable amount of *β*-glucuronidase enzyme was adsorbed on the surface of the BOC-protected porous silicon sample.

The rinse and substrate solutions used for the assays were also analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and with the bicinchoninic acid (BCA) based protein assay for the presence of enzymes. No traces of enzymes were detected (the detection limit of the assay is 0.5 μg mL^{-1}), demonstrating that no immobilized enzyme leaves the porous silicon surface under flow conditions.

Finally, enzymatic activity was controlled upon storage of the functionalized silicon devices in the refrigerator in a 1:1 (v/v) solution of PBS buffer and glycerol. The activity dropped by a factor of two after one week of storage and then remained steady for a month (the experiments are still in progress).

The photoluminescence of the functionalized porous silicon samples was recorded in situ in real time during the enzymatic activity assays. Figure 3 presents the absorbance of the solution at 405 nm, reflecting the *p*-nitrophenol concentration, and the photoluminescence emission of the functionalized

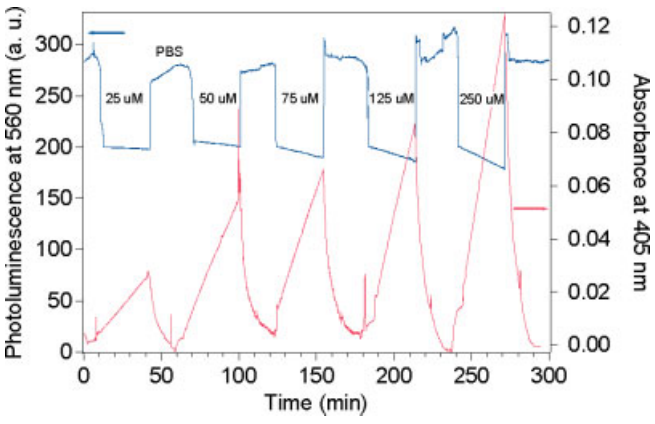


Figure 3. Absorbance of the solution at 405 nm in red (bottom trace) and photoluminescence of the functionalized porous silicon sample at 560 nm in blue (top trace), recorded simultaneously for substrate concentrations ranging from 25 to 250 μM. Sharp spikes are due to air bubbles in the flow cell.

porous silicon at 560 nm recorded simultaneously in the flow cell for *p*-nitrophenyl-*β*-D-glucuronide concentrations varying from 25 to 250 μM; PBS buffer rinses were performed between changes in concentration.

A decrease of photoluminescence was observed that was correlated with the increase of *p*-nitrophenol produced by the immobilized enzymes upon exposure to the substrate. The buffer rinses show that the photoluminescence quenching process is reversible and that it is unambiguously correlated with the increase of the *p*-nitrophenol concentration.

Aromatics such as benzene and anthracene,[22] and nitroaromatics such as DNT and TNT[23] have been reported to

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quench porous silicon photoluminescence, but the quenching mechanisms have not been studied in detail. While irreversible quenching is due to a chemical modification of the sample surface that generates efficient non-radiative surface traps, reversible quenching of photoluminescence can be induced by a variety of mechanisms such as re-absorption, charge transfer, or energy transfer. A way of ruling out the hypothesis of the re-absorption mechanism is to record photoluminescence decays. A decrease in the lifetime of the excited state of a donor species in the presence of a charge or energy acceptor is the signature of a charge or energy transfer from the donor to the acceptor. The photoluminescence decays measured in situ in liquid phase are presented in the insert of Figure 4. A 30 % decrease in the lifetime at 560 nm is measured when the porous silicon samples are immersed in a 100 M solution of *p*-nitrophenol in PBS buffer, demonstrating that the quenching of photoluminescence upon *p*-nitrophenol exposure is due to either charge or energy transfer. The fact that *p*-nitrophenol is a charge acceptor provides an indication that the quenching of photoluminescence most probably occurs via a charge transfer mechanism.

These results support the development of a new class of hybrid biosensors able to selectively detect chemical compounds. The universality of the linker designed to covalently couple the porous silicon transducer to the enzyme will allow the immobilization of numerous classes of natural enzymes as well as genetically engineered enzymes designed for high sensitivity and selectivity. Extremely compact designs can be envisioned by taking advantage of silicon-based technology, and the reversibility of the charge transfer will lead to reusable devices. Examples of possible applications are enzymatic activity assays in laboratories, enzymatic decontamination control, and chemical sensing in the field.

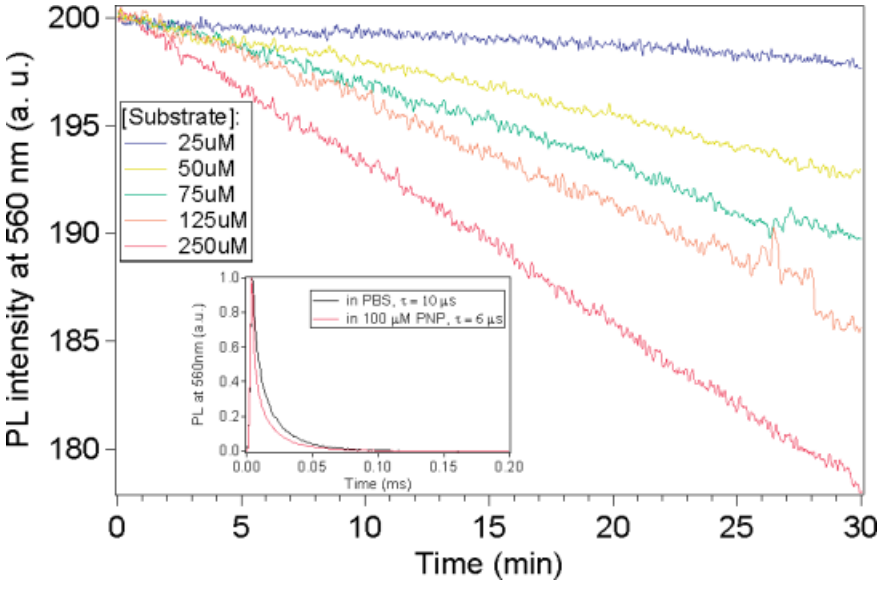

Figure 4. Time evolution of the photoluminescence signal of a functionalized porous silicon sample at 560 nm for substrate concentrations ranging between 25 and 250 µM. Photoluminescence decays at 560 nm for the same enzyme-functionalized sample immersed in PBS buffer and in a buffered solution of *p*-nitrophenol 100 µM are shown in insert.

Experimental

Porous Silicon Formation: Porous silicon samples were electrochemically etched from monocrystalline n-type silicon substrates (phosphorus doped, 1–5 Ω cm, (100) oriented, from Silicon Quest Inc.), for 5 min, at a current density of 90 mA cm^{-2}, in a 1:1 (v/v) mixture of aqueous HF (49 % hydrofluoric acid, Fisher Chemicals, Inc.) and ethanol (99.5 % absolute ACS reagent, Sigma-Aldrich). The counter electrode was a platinum ring, immersed in the electrolyte solution and the working electrode was contacted to the back of the Si wafer with In-Ga eutectic paste. All the samples used in this study were illuminated with the unfiltered light from a 150 W halogen bulb during the electrochemical etching procedure in order to photo-generate the positive charges required to dissolve silicon in the presence of HF. After etching, the eutectic back contact was removed, the samples were rinsed in three 20 mL portions of absolute ethanol, and immediately placed under vacuum to avoid airborne contamination.

Enzyme Immobilization

N-BOC-Amino-3-butene Synthesis: Amino-3-butene was prepared from 4-penteneoyl chloride using the methods described by Pfister et al. [24]. The previous step results in the free amine being partitioned in a basic aqueous solution. Di-*t*-butyl dicarbonate (5.8 g, 27 mmol) in dichloromethane (CH_2Cl_2, Sigma-Aldrich, 100 mL) was added to this solution. The reaction mixture was stirred vigorously overnight at room temperature. The aqueous phase was extracted twice with 50 mL CH_2Cl_2. The organic fractions were combined, washed twice with 100 mL 1 N hydrochloric acid (HCl, Sigma-Aldrich), and concentrated. The resulting oil was purified by flash column chromatography using 5 % ethyl acetate in hexanes.

Surface Modification: Hydrosilylation of porous silicon samples with an area of 0.95 cm^2 was carried out in a Teflon flow cell equipped with a quartz window and inlet and outlet ports for inert gas. *N*-BOC-amino-3-butene (10 µL) was placed on the porous silicon, which was then placed in the flow cell under nitrogen. Light from a 30 W halogen bulb was focused on the sample at a distance of approximately 5 cm from the silicon surface. After 2 h illumination time, the sample was removed from the flow cell and rinsed with CH_2Cl_2 and dried under nitrogen flow followed by vacuum.

Removal of BOC Protecting Group: Following surface modification, the sample was placed in a vial and exposed to 20 % trifluoroacetic acid (TFA) by volume in CH_2Cl_2 for 2 h. The sample was removed from the vial and rinsed with CH_2Cl_2 and dried under nitrogen flow followed by vacuum.

SPDP Attachment: The amino-functionalized porous silicon wafer was immersed in a solution of SPDP (2 mg, 6.4×10^{-3} mmol) in dimethyl formamide (2 mL DMF, Sigma-Aldrich) under nitrogen. The wafer was left to react for 3 h with occasional agitation. The remaining SPDP solution was removed and the silicon wafer was rinsed with DMF (3 × 5 mL) followed by ethanol (EtOH, Sigma-Aldrich, 3 × 5 mL) then dried under a nitrogen stream followed by vacuum.

Reductive Cleavage of Pyridyl Disulfide Protecting Group: The SPDP-functionalized porous silicon wafer was immersed in a solution of DTT (15.4 mg, 0.1 mmol) in 10 % EtOH/H_2O (10 mL). The wafer was left to react for 1 h with occasional agitation. The

remaining DTT solution was removed and the wafer was rinsed with fresh 10 % EtOH/H_2O (3 × 5 mL) then EtOH (5 × 5 mL) then dried under a nitrogen stream followed by vacuum.

GMBS Attachment: The sulfhydryl functionalized porous silicon wafer was immersed in a solution of GMBS (2.0 mg, 7.1×10^{-3} mmol) in DMF (2 mL) under nitrogen. The wafer was left to react for 3 h with occasional agitation. The remaining GMBS solution was removed and the silicon wafer was rinsed with DMF (3 × 5 mL) followed by EtOH (3 × 5 mL) then dried under a nitrogen stream followed by vacuum.

β-Glucuronidase Attachment: β-Glucuronidase was obtained as a solution in 50 % glycerol/PBS from F. Hoffmann-La Roche Ltd., with an activity of approximately 200 U mL^{-1} at 37 °C. This solution was diluted to 100 U mL^{-1} with PBS and added to the sample in a vial at room temperature. After 4 h the sample was removed and rinsed with PBS (20 mL) followed by 1 M NaCl (20 mL) and then rinsed again with PBS (20 mL).

PBS Preparation: PBS buffer solution at pH 7.4 contained 50 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl.

Scanning Electron Microscopy: The porous silicon samples were characterized by field emission scanning electron microscopy (FE-SEM) with a Hitachi S-4500 microscope. Samples were mounted on the sample holder using carbon tape and graphite paint. SEM images were recorded with an acceleration voltage of 3 KV.

Enzyme Activity Assay: The functionalized porous silicon samples were mounted in a Teflon flow cell fitted with a quartz window and connected to a UV-vis quartz cell in a closed loop. A total volume of 4 mL was re-circulated through the flow cell and the UV-vis cell with a peristaltic pump at a flow rate of 8 mL min^{-1}. The rinses were performed with PBS buffer at pH 7.4 and the activity assays were performed with various concentrations of the substrate *p*-nitrophenyl-β-D-glucuronide in PBS buffer. The β-glucuronidase activity was detected as an increase of *p*-nitrophenol concentration measured in situ and in real time in the UV-vis cell with a Cary 300 spectrophotometer (Varian Inc.) by using the kinetics recording mode at 405 nm.

Protein Assay: The BCA based protein assay (Pierce Biotechnology Inc.) was used to detect the presence of enzymes in the PBS rinse solutions used between various substrate concentrations. The MA (sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH), MB (4 % BCA in water), and MC (4 % cupric sulfate pentahydrate in water) reagents from the kit were mixed in a 50:48:2 (v/v/v) proportion, 1 mL of working reagent was added to 1 mL of sample, and the sample–reagent mixture was incubated at 60 °C for 1 h. The absorption of the solution at 562 nm was then measured with the spectrophotometer and compared to a standard (Bovin Albumin Fraction V in 0.9 % NaCl solution containing sodium azide).

Photoluminescence Spectra Measurements: The photoluminescence of the functionalized porous silicon samples was also measured in situ and in real time through the quartz window of the flow cell with a Cary Eclipse fluorescence spectrophotometer fitted with a fiber optic coupler and a remote read fiber optic probe. The photoluminescence was excited at 290 nm and recorded in the kinetics mode at 560 nm.

Photoluminescence Lifetimes Measurements: Photoluminescence decays at 560 nm were recorded in situ through the quartz window of the flow cell with the remote read fiber optic probe. The time delay was 0 ms, the time gate was 1 μs and the excitation wavelength was 290 nm.

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Patterning of Substrates Using Surface Relief Structures on an Azobenzene-Functionalized Polymer Film**

By *Suizhou Yang*,* *Ke Yang*, *Langang Niu*, *Ramaswamy Nagarajan*, *Shaoping Bian*, *Aloke K. Jain*, and *Jayant Kumar**

Azobenzene-functionalized polymers have been extensively explored for potential uses in various technological applications, such as optical information storage, optical switching, diffractive optical elements, and so on.[1–4] All of these appli-

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