Assembly-Controlled Biocompatible Interface on a Microchip: Strategy to Highly Efficient Proteolysis


Abstract: A biocompatible interface was constructed on a microchip by using the layer-by-layer (LBL) assembly of charged polysaccharides incorporating proteases for highly efficient proteolysis. The controlled assembly of natural polyelectrolytes and the enzyme-adsorption step were monitored by using a quartz-crystal microbalance and atomic force microscopy (AFM). Such a multilayer-assembled membrane provides a biocompatible interconnected network with high enzyme-loading capacity. The maximum digestion rate of the adsorbed trypsin in a microchannel was significantly accelerated to $1600 \text{ mm} \text{ min}^{-1} \text{ mg}^{-1}$, compared with the tryptic digestion in solution. Based on the Langmuir isotherm model, the thermodynamic constant of adsorption $K$ was calculated to be $1.6 \times 10^5 \text{ m}^{-1}$ and the maximum adsorption loading $I_{\text{max}}$ was $3.6 \times 10^{-6} \text{ mol m}^{-2}$, 30 times more than a monolayer of trypsin on the native surface. The tunable interface containing trypsin was employed to construct a microchip reactor for digestion of femtomoles of proteins and the produced peptides were analyzed by MALDI-TOF mass spectroscopy. The efficient on-chip proteolysis was obtained within a few seconds, and the identification of biological samples was feasible.

Keywords: interfaces · layer-by-layer assembly · microchip reactors · proteomics · trypsin

Introduction

The construction of a functionalized surface on various substrates is critical for biological analyses, including clinical and cellular assays, and enzymatic reactions.[1-3] Appropriate functional groups that have a high affinity for biomolecules offer alternative opportunities for high-throughput and sensitive identification of specific binding sites of biological interaction events. The main challenge is to find a general approach to engineer surfaces with targeted specific properties, such as tunable reactivity, biocompatibility, or wettability.[4]

Microfluidic devices are becoming powerful tools for performing chemical or biological assays, due to the increased speed and reliability at reduced sample consumption. The continuing progress in microchip-based bioanalysis will depend on the development of novel surface-modification technologies in a simple and reliable fashion. The chemical patterning of a biocompatible interface within microfluidic channels must be efficient to immobilize domains of antibodies, enzymes, and other important biologically active compounds for a highly sensitive detection. In particular, as protein analysis continues to push the limits, the availability of new strategies will become more critical. Proteolysis is the key step for positive protein sequencing in proteomics research integrated with MALDI-TOF MS. The conventional techniques of in-solution digestion of proteins offer limited sensitivity and are time-consuming procedures, affecting severely the determination of comprehensive proteomic profiles.[5]

One of the promising solutions is the incorporation by patterning processes of enzymes, such as proteases, within a microchannel to form a microbioreactor to carry out highly efficient and low-level protein digestion. Relative to free en-
zymes in bulk solution, the enzymes immobilized in a micro-
channel are more stable and resistant to environmental
changes, and provide molecular-level interactions with flow-
ing substrates. The heterogeneity of the immobilized
enzyme systems allows multiple reuses of the enzymes, con-
tinuous operation of enzymatic processes, and a greater vari-
ety of bioreactor designs. Several approaches, such as physi-
cal adsorption, sol–gel encapsulation, and covalent linking,
have been explored to immobilize enzymes onto the walls of
microfluidic channels to design reactors for proteolysis.[6,7]
Another alternative and facile option is the multilayer-as-
sembly technique established by the layer-by-layer deposi-
tion in a controlled way.[8] The layer-assembled microstruct-
ures with tailored composition and architecture can be used
to incorporate functional biomolecules.[9] Biomolecules em-
bedded into the polyelectrolyte multilayers are close to their
native form, so that the biological films have many potential
applications, such as biosensors and immunoassays.

Here, a biocompatible interface was constructed on a
poly(ethylene terephthalate) (PET) microfluidic chip pro-
viding a miniaturizing and viable platform for the immobili-
zation of biomolecules. The PET microchannel is functional-
ized by the alternating deposition of two naturally occurring
polysaccharides, the cationic chitosan (CS) and anionic hya-
luronic acid (HA). In view of their biocompatibility, nontox-
icity, and hydrophilicity, the CS/HA-assembled networks
offer the unique characteristic of affinity to enzymes or pro-
teins. So far, they have been usually used for tissue engi-
eering or drug delivery.[10] We have proposed a simple
method with this self-assembly for imbedding enzymes on
microchips for protein identification.[7c] The present investi-
gation is focused on the characterization of enzyme-loading

multilayer assemblies within a microchannel for the sensi-
tive proteolysis of standard proteins at lower detection
limits and also of real biological samples. The constructed
microreactor provides a large surface-area-to-volume ratio
and a confined microenvironment, resulting in an increased
reaction rate. Within a few seconds, the peptides derived
from digestion of proteins are detected with high sequence
coverage. Biological samples, such as casein extracted from
bovine milk and attenuated hepatitis A virus vaccine, were
identified by using this proposed microchip reactor coupled
with MALDI-TOF MS. This strategy could be applied po-
tentially as an on-chip microreactor for challenging other
techniques for low-abundance proteome analysis, as well as
the rapid analysis of real-world samples.

**Results and Discussion**

The PET substrate, a commonly used polymer material, has
been employed to fabricate microfluidic chips by laser abla-
tion for practical proteomics purposes interfaced to mass
spectroscopy, electrochemistry, and fluorescence detec-
tion.[11] However, the PET surface properties of hydropho-
bicity and poor biocompatibility are serious obstacles for bi-
oanalysis. Thus, the chemical modification of PET micro-
channel surfaces is essential. In the present study, a simple
modification protocol is proposed for an enzyme multilayer
adsorption on microchips to obtain a microreactor for on-
line proteolysis. Scheme 1 describes the process of enzyme
immobilization and protein identification in the multilayer-
assembled microchip.
Several bilayers of CS and HA were prepared to produce a stable supramolecular thin film by electrostatic interaction. Atomic force microscopy (AFM) images present topographical features of the multilayer-assembled PET surface containing the enzyme. As shown in Figure 1a, the surface of native PET is rather flat and smooth with few defects, and the RMS roughness is about 87 Å. In comparison, after deposition of CS/HA multilayer films on the PET surface, the image reveals uniform protrusive structures with a roughness of 318 Å (Figure 1b), among which pores with a size of several tens to hundreds of nanometers are quite evenly distributed on the surface. Such a well-interconnected porous network is suitable for incorporating enzymes whilst maintaining easy pathways for protein access and peptide diffusion.[12] Trypsin, used here as the protease, has an approximate crystallographic size of 4.0–5.0 nm.[13] Figure 1c displays the surface morphology of trypsin-loaded CS/HA multilayers, with pores of a few nanometers and smoother than that exhibited in Figure 1b. This illustrates that the trypsin was embedded into the microstructured network of CS/HA multilayers, and adsorbed onto the CS/HA-modified PET surface.

The adsorbed mass of CS/HA and trypsin was monitored by using the quartz-crystal microbalance (QCM) frequency shift according to the Sauerbrey relation. Experimental data obtained by using a gold electrode show that the gradual CS/HA multilayer growth is a linear function of the number of layers (Figure 2, inset). Layer-by-layer (LBL) deposition driven by electrostatic forces can easily take place on a model surface, which will provide the enzyme with a suitable microenvironment. The first layers (CS1/HA2/CS3/HA4/CS5) offer a supporting precursor film for better adsorption of the enzymes on the surface. As shown in Figure 2, a continuous decrease of the QCM frequency shift as the number of enzyme layers increases confirms the successive adsorption of enzymes on the functional scaffold by means of CS/HA multilayer build-up. After deposition of the second HA/CS–trypsin layer, the frequency decrease reaches a plateau. We speculate that the first enzyme layer is within an incompact microenvironment, and subsequently, the enzymes adsorb mainly onto the surface of the multilayer and do not penetrate the multilayer fully as the number of layers increases. Nevertheless, the amount of adsorbed enzyme increases as the deposition of multilayers (HA/CS–trypsin) continues.[14] Four trypsin layers correspond to an enzyme loading of about 107 mg m^-2, higher than with other polyelectrolytes.[14]

Adsorption and transport processes were modeled mathematically to gain a better understanding of the behavior of enzyme adsorption onto the multilayer-assembled substrates. The Langmuir isotherm model has a wide application for the adsorption of proteins on substrates, which is useful in practice.[15] According to the Langmuir adsorption isotherm equation:

\[
\Gamma_{eq} / \Gamma_{max} = \frac{KC^0}{1 + KC^0}
\]

in which \(\Gamma_{eq}\) is the surface concentration at equilibrium, \(\Gamma_{max}\) is the initial concentration of the active sites, \(K\) is the thermodynamic constant of adsorption, and \(C^0\) is the initial concentration of the solution. The experimental isotherm of
trypsin adsorption is shown in Figure 3a, and the linearized
isotherm is the inset line. By plotting $C^o/C_{eq}$ versus $C^o$, $I_{max}$
and $K$ are provided as the respective reciprocals of the
slope and the intercept, giving the fitted values $I_{max} = 3.6 \times 10^{-6}$ mol m$^{-2}$ and $K = 1.6 \times 10^{5}$ m$^{-1}$. Furthermore, we suppose

![Graph showing trypsin adsorption isotherm]

Figure 3. a) Isotherm of trypsin adsorption on the multilayer-assembled
PET obtained from the QCM experimental results. Inset line: lineariza-
tion of the adsorption isotherm. b) The maximum tryptic rate as a func-
tion of the number of HA/CS–trypsin layers.

a compact monolayer of trypsin molecules on the native sur-
fact. The maximum adsorption $I_{max}$ can be calculated by
taking into account the cross-section area of a trypsin mole-
cule (a trypsin molecule is globular\cite{16} with a diameter of
about 4–5 nm\cite{13}), resulting in $1.0 \times 10^{-7}$ mol m$^{-2}$. The fitted value ($I_{max} = 3.6 \times 10^{-6}$ mol m$^{-2}$) obtained from the multilay-
er-assembled system is 30 times more than for the monolay-
er of trypsin on the unmodified surface. These results dem-
strate that the HA/CS network on the wall of the micro-
channel provides a porous medium for enzyme loading.

This interface provides a well-defined scaffold for enzyme
immobilization, in which the bioactivity is assumed not to be compromised. Accordingly, the adsorbed trypsin activity is characterized by the values of the Michaelis constant and maximum velocity, which are usually derived from the Mi-
chaelis–Menten equation. As shown in Figure 3b, enzyme activity increases steeply from one layer to three layers. However, after the deposition of a third HA/CS–trypsin
layer, the activity reaches a plateau, because the diffusion and permeation of substrate into the membrane is more lim-
itted as the number of layers increases.\cite{14} Four enzyme-loading
layers showed an activity of 7.85 mm s$^{-1}$, which is of a
similar order of magnitude to those reported for a trypsin-
encapsulated reactor on porous polymer monoliths.\cite{10} The
value of maximum velocity ($V_{max}$) per unit trypsin was about
1600 mm min$^{-1} \mu$g$^{-1}$, thousands of times faster than in bulk solution. Both a high surface-to-volume ratio and micro-
structured confinement within the microchannel induce order-of-magnitude enhancement of catalytic efficiency. In addition, the significantly increased velocity in the function-
alized microchannel may also be attributed to the fact that the well-defined interface assembled by CS/HA could pro-
vide not only a large surface area for enzyme immobiliza-
tion, but also a biocompatible microenvironment for pre-
serving the bioactivity and reducing the autolysis, which is promising for sensitive analysis.

The CS/HA-assembled microchip serving as an on-chip
enzyme reactor coupled to MALDI-TOF MS could be a
facile strategy for efficient digestion. Proteins, such as
bovine serum albumin (BSA), myoglobin, cytochrome c
(Cyt-c), casein extracted from bovine milk, and attenuated
hepatitis A virus vaccine, are exemplified for the feasibility
of this approach. The trypptic efficiencies were evaluated on
the basis of the protein identification results with the detect-
ed peptide mass fingerprinting (PMF) and signal-to-noise
ratios.

Figure 4a displays the PMF spectra of tryptic digests of
200 ng mL$^{-1}$ BSA with the as-prepared microreactor, and in
solution as a control. At a flow rate of 120 $\mu$L h$^{-1}$, a total of
28 tryptic peptides from BSA were assigned and correspond
to an amino-acid sequence coverage of BSA as high as
43%. Relative to proteolysis in solution (Figure 4b), both
the signal-to-noise ratio and sequence coverage are clearly
enhanced, and the reaction time is reduced to a few seconds.
The results show that trypsin entrapped in the PET micro-
channels can act as a good biocatalyst and can achieve more
rapid reaction.

This strategy has potential in the identification of protein
at low abundance. As the substrate concentration is re-
duced, the kinetics of the reaction are reduced to a point at
which a longer digestion time is required. To decrease the
digestion time, the concentration of enzyme can be in-
creased. However, the presence of a high enzyme-to-sample
ratio often produces a severe enzyme autolysis, which can
occlude the signals of the sample peptides in the spec-
trum.\cite{17} There has been speculation that the enzymes immo-
obilized into a microfluidic channel are more stable than
their soluble forms because of the suppression of autolysis
by the matrix. Naturally expressed proteins at low abun-
dance play key roles in cells and tissues. In the interest of
achieving highly specific activity, while significantly minimiz-
ing reaction time with the microchip reactor, the digestion
efficiency of proteins at a low level will be enhanced. The
mass spectrum of digests of 1 ng mL$^{-1}$ myoglobin is shown in
Figure 5a. The amino-acid sequences of 34–40% and protein
scores of 119–145 are detected, and no enzyme autodiges-
tion signals are observed in the spectra. The lower trypptic
autolysis in the microchip reactor is favorable for the positive identification of low-abundancy proteins. Additionally, Cyt-c at a low level of 0.1 ng mL⁻¹ was digested by using the reactor, as shown in Figure 5b. Three tryptic peptides from Cyt-c are assigned. The detection limit can reach a few femtomoles for proteins and the sample size is as low as 0.05 ng per analysis. This property minimizes the sample size, compared with the common trypsin reactions.

Furthermore, the advantage of this method is the identification of complex proteins. For example, casein extracted from bovine milk consists of four principal primary subtypes, αS1-, αS2-, β-, and κ-casein. The four main proteins differ not only in amino-acid sequence and length, but also vary in the degree of phosphorylation and glycosylation, disulfide-linked polymerization, and genetic polymorphism. Casein was tryptically digested by using this on-chip micro-

subunit contains four kinds of peptides. In consideration of safety, an attenuated hepatitis A vaccine was used in this research. To avoid being affected by its surrounding environment, the HAV vaccine contains several chemical and biological additives that usually deactivate the free trypsin. In this study, the vaccine was digested by using this microchip reactor and the resulting peptides were identified by using MALDI-TOF MS and the Mascot search-engine software of virus database. The results are summarized in Table 1. Six possible proteins were detected. This is a general and effective on-chip proteolysis approach for identifying proteins, and can be applied in rapid and high-throughput biological and clinical analyses.
Conclusion

We have constructed a stable biocompatible interface for trypsin immobilization on the basis of CS/HA multilayer assembly to yield high enzyme loading with well-preserved bioactivity. Digestion of proteins was performed within the proposed on-chip microreactor prior to identification by MALDI-TOF MS. The remarkable enhancement of proteolytic efficiency is attributed to the biocompatible surface and microstructure confinement. The advantage of this method lies in its flexibility, which implies that the approach can be applied in automated high-throughput analysis by using a parallel channel microchip platform integrated with the liquid chromatography or two-dimensional-gel separation procedures for original complex protein mapping. Besides chitosan and hyaluronic acid, other natural polyelectrolytes, such as collagen, alginate, and different glycosaminoglycans, may enhance biocompatible multilayer architecture for the functionalization of substrates, so that various biomolecules of interest can be encapsulated and stabilized in the designed scaffold. It can be anticipated that the layer-by-layer assembly is a facile and versatile approach for microchip modification and biomolecule stationary-phase patterning, which would expand potential applications in the fields of chemical biology and biotechnology.

Experimental Section

The microchip was fabricated by UV laser photoablation and thermal lamination as previously reported.[9] Briefly, the PET sheet (100 μm thick) was placed on computer-controlled XY translation stages and scanned to be photoablated by a UV excimer laser (ArF 193 nm) for generating a 2-cm long channel with a trapezoidal cross-section shape of depth 42 μm and width 100 μm. The straight-line channel was terminated on both sides by photoablated reservoirs. The PET microchannel was thermally sealed with a PET film by using a lamination machine (Morane, UK).

Firstly, the PET microchannel was hydrolyzed with NaOH (1 M) for 20 min at 60°C followed by rinsing with HCl (0.1 M), water, and ethanol, and parts of the surface ester groups were converted to free OH or COO groups. Next, positively charged chitosan and negatively charged hyaluronic acid were deposited successively onto the hydrolyzed PET surface by incubating the latter in aqueous solutions of chitosan (1.0 wt%, medical grade, Qingdao Heppe Biotechnology Co.) and hyaluronic acid (1.0 mg mL⁻¹, CPN, Czech Republic), respectively, each for 30 min. This procedure was repeated five times until a stable film was obtained. After the five layers (PET/CS1/HA2/CS3/HA4/CS5) had been built, an further enzyme-loading step was performed. On top of this stable precursor system, several multilayers of HA/CS–trypsin were deposited by immersing the microchip alternately in trypsin solution (5 mg mL⁻¹, in tris-HCl buffer (50 mM) containing CaCl₂ (20 mM), pH 8.0, Sigma Company, USA) for 3 h at 4°C, and aqueous solutions of hyaluronic acid and chitosan, respectively, each for 30 min. This procedure was repeated five times until a stable film was obtained. After the five layers (PET/CS1/HA2/CS3/HA4/CS5) had been built, an further enzyme-loading step was performed. On top of this stable precursor system, several multilayers of HA/CS–trypsin were deposited by immersing the microchip alternately in trypsin solution (5 mg mL⁻¹, in tris-HCl buffer (50 mM) containing CaCl₂ (20 mM), pH 8.0, Sigma Company, USA) for 3 h at 4°C, and aqueous solutions of hyaluronic acid and chitosan, respectively, followed by sufficient washing with water. On top of the HA/CS–trypsin layers, a final layer of trypsin was adsorbed. This multilayer system was fabricated on the PET microchip as a bioreactor.

The surface topography was assessed by using an atomic force microscope (EC-SPM, Picoscan 2100) in tapping mode. Micron scanning was performed with nanosensors etched silicon probes. The instrument parameters were set as follows: scan size 3.5 μm, resonance frequency 50–80 KHz. The images were flattened by using the Nanoscope software. The controlled immobilization of trypsin in well-interconnected macroporous membranes was performed in situ by using a quartz-crystal microbalance analyzer (CH4240, CH Instruments, USA) and quartz crystals (7.995 MHz) sandwiched between two Au electrodes. Chitosan, HA, and

Table 1. Identification of proteins of the HAV vaccine obtained from digestion by using the CS/HA-assembled microchip reactor.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Peptide match</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleocapsid protein</td>
<td>58096</td>
<td>10</td>
</tr>
<tr>
<td>nonstructural polyprotein</td>
<td>185108</td>
<td>16</td>
</tr>
<tr>
<td>capsid protein VP1</td>
<td>81663</td>
<td>15</td>
</tr>
<tr>
<td>RNA-directed RNA polymerase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>subunit VP1</td>
<td>124923</td>
<td>24</td>
</tr>
<tr>
<td>outer capsid protein VP2</td>
<td>123982</td>
<td>12</td>
</tr>
<tr>
<td>genome polyprotein</td>
<td>367338</td>
<td>40</td>
</tr>
</tbody>
</table>

Figure 6. MALDI-TOF mass spectrum of digests of casein obtained by using the CS/HA-modified microchip reactor. Protein concentrations are 0.20 μg mL⁻¹ in NH₄HCO₃ buffer solution (10 mmol L⁻¹, pH 8.1). Eluent/matrix ratio, 1:1.
trypsin solution were assembled onto the gold surfaces. After deposition, the gold electrode surface was rinsed thoroughly with pure water and dried under nitrogen gas. The QCM frequency change in air was measured.

The activity of the encapsulated enzyme in the microchip was examined by pumping a solution of α-N-benzyol-l-arginine ethyl ester (BAEE) in tris-HCl (50 mM) buffer (pH 8.0) through the trypsin-adsorbing PET channel at a flow rate of 100 μL h⁻¹ by using a syringe pump. The resulting products were analyzed by using a capillary electrophoresis system (P/ACE System 5000, Beckman). The capillary electrophoresis was run in phosphate buffer solution (pH 2.5) at 15 kV with UV detection at 214 nm. The trypsin activity was calculated from the flow rate and absorbance difference.

Protein solutions of BSA (Bio Basic, Toronto, Canada), cytochrome c, myoglobin, casein extracted from bovine milk (Sigma Company, USA), and hepatitis A vaccine (Changsheng Industry Co., Changchun, China) in NH₄HCO₃ (10 mM) buffer solution (pH 8.1) were driven through the LBL-modified PET microchannel by using a syringe pump at a flow rate of 120 μL h⁻¹. Effluents accumulated in the reservoir were collected and then identified by MALDI-TOF MS. All MS experiments were performed by using a 470 Proteomics Analyzer with TOF/TOF optics. Each volume of sample solution (0.5 μL) was dropped onto the MALDI plate. After the solvent was evaporated, a volume of matrix solution (0.25 μL, 4 mg mL⁻¹ α-cyano-4-hydroxycinnamic acid in acetonitrile (50%) with TFA (0.1%)) was dropped onto the dried samples. Before identification of the samples, the MS instrument was calibrated by using an internal standard. GPS Explorer software from Applied Biosystems with Mascot as a search engine and SwissProt (version of 050303) as a database was used to identify proteins. All proteins were identified by using the peptide fingerprint mass spectra combined with tandem mass spectra. The peptide mass tolerance was set to 80 ppm, and the tandem mass tolerance was set to 0.5 Da.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (20575013), the Natural Basic Research Priorities Program 2001CB510202, STCSM 0508MBH102, and EPFL through a visitor fellowship to B.H.L. The authors thank Dr. A. Lionello (EPFL) for helpful discussion and V. Devaud (EPFL) for technical help.


Received: December 23, 2005
Revised: February 20, 2006
Published online: June 26, 2006