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Smart Trypsin Adsorption into *N*-(2-Aminoethyl)-3-aminopropyl-Modified Mesoporous Silica for Ultra Fast Protein Digestion

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Comprehension of protein networks and expression patterns in cells, tissues, and organisms is essential for technological applications of modern life sciences. Due to the recent developments over the past few years, mass-spectrometry-based proteomics has expanded its interface role to broad and diverse research areas of science and technology.[1–3] Even so, to satisfy the demand for high-speed analysis, new flexible and efficient tools are an urgent priority.

Peptide mass fingerprinting remains the most powerful and straightforward method for protein mass identification. Specifically, proteolytic digestion generates characteristic peptide fragments that are typically acquired by matrix-assisted, laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF MS) and matched to theoretical digestion patterns in a database.[4] However, conventional protein digestion protocols need exceedingly long digestion times that limit high-throughput protein identification. The use of trypsin immobilized onto solid support has recently captured the attention of many research groups, because these systems can significantly speed-up digestion.[5]

Herein, we report for the first time the application of trypsin adsorbed on *N*-(2-aminoethyl)-3-aminopropyl-derivatized mesoporous silica particles (MPs) used as an exceptional enzymatic bio-reactor for protein digestion. Proteolytic fragments of myoglobin were obtained as quickly as 1 min from the addition of the protein to the trypsin-immobilized mesoporous support, giving a 100% sequence coverage. Furthermore, the substantial contribution of *N*-(2-aminoethyl)-3-aminopropyl and aminopropyl (indicated as AAPTES and APTES, respectively) in SBA-15-functionalized materials to proteolysis was evaluated in comparison to nonfunctionalized SBA-15. This reactor system has great potential for significantly increasing proteolytic efficiency compared to similar biocatalysts, as well as providing a flexible and rapid approach to the field of digestion techniques for MS-based proteomics. The main goal of this study was to design a simple and fast approach for protein digestion as a part of an on going project aimed to the development of convenient and sensitive procedures for proteomics applications.[6–8]

MPs, characterized by a well-ordered porous structure and extremely high surface area, are excellent candidates for biomolecules and enzyme immobilization.[9] While trypsin-immobilization strategies mostly rely on covalently linked trypsin to the support,[5] only very few examples of physical adsorption of trypsin onto a porous support have been reported for MS-based protein analysis.[10,11] The use of covalent bonding between the enzyme and the support, while reducing enzyme leaching from the support, has been reported to modify the conformation of the enzyme with reduction of catalytic activity.[9,12] Moreover recent studies suggest that mesoporous structures with a pore size close to the enzyme dimension provide stable enzyme adsorption with high efficiency.[13–15]

Starting from the above considerations, MP SBA-15 and its amino-functionalized derivatives were prepared with pore dimensions of about 4.3 nm, slightly larger than the diameter of trypsin (3.8 nm),[16] to obtain a well-fitting physical entrapment. In fact, enhanced stability and activity were reported when the pore entrance was large enough to allow the protein to enter the channel.[17] This should ensure not only stable trypsin adsorption/efficiency, but also reduction of enzyme leaching, which takes place when pore dimensions are much larger than enzyme. As a control experiment, we also prepared MP SBA-15 with a pore dimension of 6.3 nm (from this point, we will refer to these materials as shown in Table S1 of Supporting information). Trypsin immobilization onto four different MP SBA-15 samples was carried out by physical adsorption in mild conditions. This simple and fast approach used to immobilize enzymes onto

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MPs is essential to maintain enzymatic activity. The enzyme solution was gently stirred at room temperature with the MPs, followed by centrifugation and washing to remove the excess of unbound enzyme. The amount of enzyme adsorbed was measured by UV/Vis analysis of the supernatant for residual protein content. To address the suitability of this protocol for practical and daily implementation in proteomics applications, we set up a procedure in which a tiny amount (mg scale) of MPs was used to adsorb the amount of trypsin required for several digestions (low μg scale) in just a few minutes. This procedure provides good daily lab convenience, because from a single-loading step, many aliquots of biocatalyst can be obtained for a ready and prompt use, otherwise they can be stored for future experiments. The well-ordered porous array of SBA-15 (6 nm) demonstrated the greatest adsorption capacity by allowing the fastest diffusion of trypsin in the host frameworks (Figure 1 a), which correlates with the observation that this material has the largest pore diameter and volume (Supporting Information). Also SBA-15 (4 nm) showed greater adsorption capacity and faster diffusion of trypsin in comparison to APTES and AAPTES modified SBA-15 (Figure 1 a). This is mainly due to the fact that, despite similar pore size, the surface area as well as the pore volume of the amino functionalized derivatives are respectively 40 % and 60 % of SBA-15 (4 nm) (Supporting Information). APTES and AAPTES SBA-15, with same pore size distribution and structural texture properties, showed a similar trend in trypsin adsorption (Supporting Information). It is important to emphasize that the effect of pH value and initial concentration play a crucial role on the loading of proteins.[17] Hence, solutions of trypsin with different initial concentration were tested to obtain the desired loading suitable for proteomic purposes. Regarding pH value, we worked at pH 6.5 in order to allow a favorable electrostatic interaction between positively charged trypsin ($IP = 8.23$) and negatively charged silicate walls. In our experimental conditions, a proteomic scale amount of roughly 0.3 nmol of trypsin were adsorbed per mg of MP for both SBA-15 amino derivatives in 10 min (Figure 1 b); the same amount was loaded onto SBA-15 (4 nm) in 2 min and onto SBA-15 (6 nm) in only 1 min.

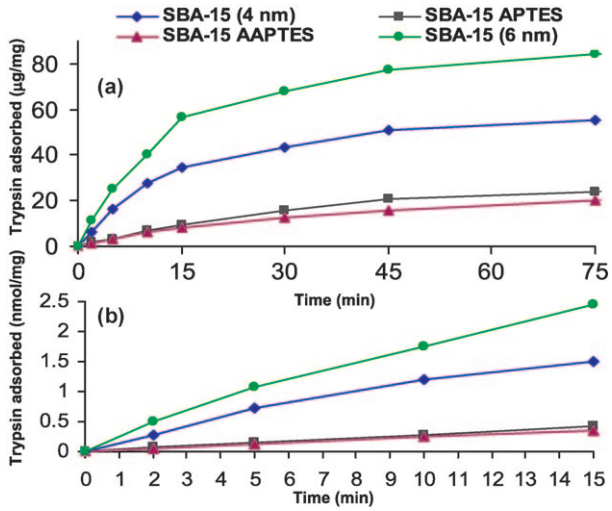

Figure 1. Amount of trypsin adsorbed in SBA-15 (4 nm), SBA-15-AAPTES, SBA-15-APTES, and SBA-15 (6 nm) as function of incubation time expressed in a) μg or b) nmol of enzyme per mg of each mesoporous material.

This controllable "trypsin-low-charging procedure" is suitable for proteomic analysis in which concentrations down to femtomoles of proteins are commonly used. It is worth mentioning that this protocol allows a quite significant reduction in the time necessary for immobilization compared to a cyano-functionalized mesoporous-based reactor (with a pore diameter of 18 nm) that requires 16 h at room temperature as reported by Quiao and co-workers.[10]

To test the proteolytic activity of trypsin in this system, we selected myoglobin, which is known to be a proteolitically resistant substrate;[18] moreover its dimensions ($3 \times 4 \times 5$ nm)[19] are compatible with the pore opening and diffusion pathways in our materials.

Myoglobin was added to trypsin entrapped in SBA-15 (6 nm), SBA-15 (4 nm), SBA-15 APTES, and SBA-15 AAPTES with a molar enzyme/substrate ratio of 1:3. Within 1 min of digestion, a rich pattern of proteolytic fragments was obtained for AAPTES (Figure 2a) and APTES, which allowed unambiguous myoglobin identification (see the Supporting Information). The best performance was achieved for trypsin adsorbed in SBA-15 AAPTES with 100 % sequence coverage obtained in just 1 min (Figures 2 and 3). Twenty-one tryptic fragments of myoglobin were identified at 1 min with digestion taking place inside the biocatalyst

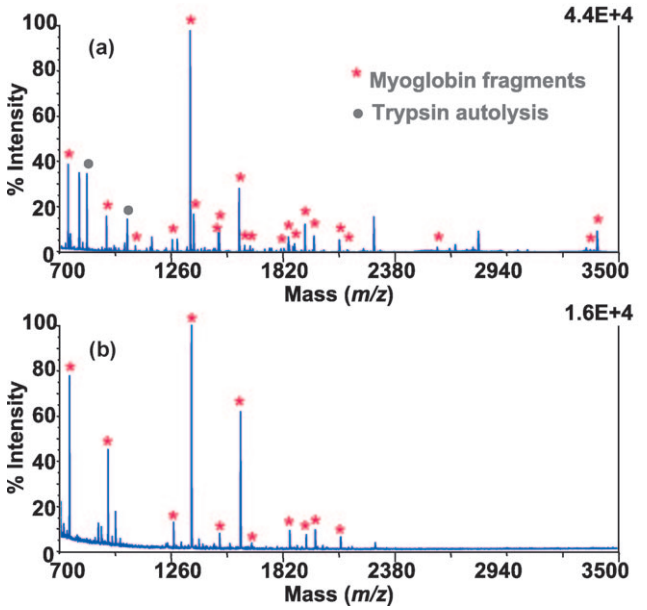

Figure 2. MALDI-TOF mass spectrum of myoglobin digested by a) trypsin-SBA-15 AAPTES reactor in 1 min, 21 peaks corresponding to 100 % sequence coverage; b) conventional in solution procedure, overnight, 11 peaks corresponding to 55 % sequence coverage.

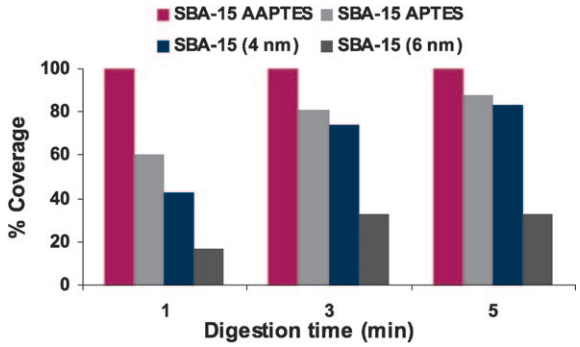

Figure 3. Sequence coverage vs digestion time for myoglobin digested by trypsin immobilized into SBA-15 AAPTES, SBA-15 APTES, SBA-15 (4 nm), and SBA-15 (6 nm).

against eleven fragments (55% coverage) obtained with a conventional digestion carried out with well established in solution procedure (Figure 2). Among the MPs, the lowest proteolytic activity was shown by the un-functionalized SBA-15 (6 nm) material (Figure 3 and Supporting Information), despite the fact that this material was the one that allowed the fastest diffusion (Figure 1). The increased bioactivity of trypsin confined into a mesoporous channel over the free enzyme may be due a remarkable local enzyme–substrate enrichment within the nanopores. It is foreseeable that after enzyme immobilization, myoglobin diffusion and accommodation inside the pore occurs in a more limited space, resulting in a partial substrate unfolding with increased exposition of accessible cleavage sites.[20] This is likely to occur at lower efficiency when the pore diameter is larger, thus furnishing an elegant explanation for the observed lower efficiency of the SBA-15 (6 nm) compared to the SBA-15 (4 nm).

As shown in Figure 3, compared to AAPTES and APTES derivatives, SBA-15 (4 nm), with the same pore diameter, showed a lower proteolytic efficiency over the first minute and reached its maximum in 20 min (94% sequence coverage, data not shown) as also reported in a previous study.[10] The improved proteolityc activity of trypsin immobilized into amino-functionalized SBA-15 compared to trypsin immobilized into un-functionalized SBA-15 may arise from the presence of the amine groups, which could be beneficial for the catalytic reaction. Interestingly, the increased performance correlates with the length of the functional group (Table S1 Supporting Information: APTES: 0.5 nm, AAPTES: 0.8 nm). A potential mechanism might involve a role of basic groups, in particular the longer AAPTES, in an electrostatic stabilization of the charge (which plays a crucial role in the mechanism of serine proteases activity) in the oxyanion hole, located on the external enzyme surface[21] and therefore easily accessible. It is worth noting that recent studies envisage cooperative interaction of such functional groups among organo-functionalized MPs that give rise to enhanced catalytic activity.[22] A thorough comprehension of the improved proteolysis of trypsin in our systems requires further in-depth studies.

Confinement from molecular crowding in biological cells can both stabilize and induce order-of-magnitude enhancements in catalysis for large molecules and proteins, compared to enzymes in solution reactions.[23,24] By mimicking such biological systems in this study, we have investigated the beneficial effect of organic functionalities such as AAPTES and APTES grafted on SBA-15 on in situ-proteolysis and addressed their suitability for a convenient "proteomic scale" procedure consisting of few sample-handling steps, with increased proteolytic efficiency (1000 times faster and an improved performance compared to conventional solution procedure) making it promising for high-speed and high-throughput protein identification.

Experimental Section

See the Supporting Information for detailed preparation and characterization of SBA-15 materials (Figures S1–S4, Table S1), experimental procedures for trypsin immobilization onto mesoporous silica particles (Figures S5–S7), protein digestion and MS analysis (Figures S8 and S9, Tables S2–S4).

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[1] J. R. Yates III, A. Gilchrist, K. E. Howell, J. J. Bergeron, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 702–714.
[2] C. E. Au, A. W. Bell, A. Gilchrist, J. Hinding, T. Nilsson, J. J. Bergeron, *Curr. Opin. Cell Biol.* **2007**, *19*, 376–385.
[3] S. R. Collins, P. Kemmeren, X. C. Zhao, J. F. Greenblatt, F. Spencer, F. C. Holstege, J. S. Weissman, N. J. Krogan, *Mol. Cell. Proteomics* **2007**, *6*, 439–450.
[4] D. J. Pappin, P. Hojrup, A. J. Bleasby, *Curr. Biol.* **1993**, *3*, 327–332.
[5] A. Monzo, E. Sperling, A. Guttman, *TrAC Trends Anal. Chem.* **2009**, *28*, 854–864.
[6] R. Terracciano, M. Gaspari, F. Testa, L. Pasqua, G. Cuda, P. Tagliaferri, M. C. Cheng, A. J. Nijdam, E. F. Petricoin, L. A. Liotta, M. Ferrari, S. Venuta, *Proteomics* **2006**, *6*, 3243–3250.
[7] R. Terracciano, L. Pasqua, F. Casadonte, S. Frascà, M. Preianò, D. Falcone, R. Savino, *Bioconjugate Chem.* **2009**, *20*, 913–923.
[8] R. Terracciano, F. Casadonte, L. Pasqua, P. Candeloro, E. Di Fabrizio, A. Urbani, R. Savino, *Talanta* **2010**, *80*, 1532–1538.
[9] M. Hartmann, *Chem. Mater.* **2005**, *17*, 4577–4593.
[10] L. Qiao, Y. Liu, S. P. Hudson, P. Yang, E. Magner, B. Liu, *Chem. Eur. J.* **2008**, *14*, 151–157.
[11] J. Fan, W. Shui, P. Yang, X. Wang, Y. Xu, H. Wang, X. Chen, D. Zhao, *Chem. Eur. J.* **2005**, *11*, 5391–5396.
[12] H. H. P. Yiu, P. A. Wright, *J. Mater. Chem.* **2005**, *15*, 3690–3700.
[13] H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino, S. Inagaki, *Chem. Mater.* **2000**, *12*, 3301–3305.
[14] A. Vinu, V. Murugesan, M. Hartmann, *J. Phys. Chem. B* **2004**, *108*, 7323–7330.

[15] Y. Urabe, T. Shiomi, T. Itoh, A. Kawai, T. Tsunoda, F. Mizukami, K. Sakaguchi, *ChemBioChem* **2007**, *8*, 668–674.
[16] J. F. Diáz, K. J. Balkus, Jr., *J. Mol. Catal. B* **1996**, *2*, 115–126.
[17] S. Hudson, J. Cooney, E. Magner, *Angew. Chem.* **2008**, *120*, 8710–8723; *Angew. Chem. Int. Ed.* **2008**, *47*, 8582–8594.
[18] A. K. Palm, M. V. Novotny, *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1374–1382.
[19] M. Singh-Zocchi, J. Hanne, G. Zocchi, *Biophys. J.* **2002**, *83*, 2211–2218.
[20] J. Buijs, M. Ramstrom, M. Danfelter, H. Larsericsdotter, P. Hakansson, S. Oscarsson, *J. Colloid Interface Sci.* **2003**, *263*, 441–448.
[21] T. R. Transue, J. M. Krahn, S. A. Gabel, E. F. DeRose, R. E. London, *Biochemistry* **2004**, *43*, 2829–2839.
[22] S. Shylesh, A. Wagner, A. Seifert, S. Ernst, W. R. Thiel, *Chem. Eur. J.* **2009**, *15*, 7052–7062.
[23] A. P. Minton, *J. Biol. Chem.* **2001**, *276*, 10577–10580.
[24] H. X. Zhou, K. A. Dill, *Biochemistry* **2001**, *40*, 11289–11293.

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