Tailoring the Surface of Magnetic Microparticles for Protein Immobilization

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ABSTRACT: Magnetic, amine-functionalized microparticles (*dynabeads*®) were surface-modified with different polyamines to suppress the nonspecific protein adsorption to a maximum extent but to allow for the enhanced covalent attachment of selected biomolecules (proteins). The stepwise chemical modification entailed the consecutive reaction of amine-functionalized dynabeads® with glutaric dialdehyde (GA) followed by the reaction with multifunctional, amine-containing compounds such as poly(allyl amine), poly(ethylene imine), and bovine serum albumin. The model proteins trypsin and concanavalin A, respectively, were finally covalently bound to the polyamine-functionalized dynabeads via GA-mediated coupling. Matrix-assisted laser-desorption/ionization time of flight/time of flight mass spectrometry confirmed the successful protein attachment. Both the nonspecific protein adsorption to the different polyamine-modified surfaces and the GA-mediated covalent binding of the target proteins to the polyamine-functionalized surfaces was quantified by standard bioassays. Compared to unmodified beads, the bioactivity of the polyamine-functionalized ones was increased by a factor of seven while keeping the nonspecific protein adsorption of a selected cationic protein at a very low level. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 3628–3634, 2011

Key words: magnetic polymers; biocompatibility; proteins; branched; functionalization of polymers; MALDI

INTRODUCTION

Currently, much effort is devoted to the development of biofunctional polymeric surfaces for specific biomedical or biological application. Because of the unique properties of polymers such as the ease of processing, their chemical inertness, conductivity, flexibility, and their low weight, they partly replaced metal or ceramic materials in biomolecule arrays. In biochemistry, standard surfaces such as poly(styrene), poly(propylene), or polyamides are most commonly used.[1] However, for particular applications, such as the immobilization of biomolecules, the properties of these inert polymeric surfaces must be tailored. There, the challenge is that the surface should allow for the specific coupling of the desired molecules while resisting nonspecific protein adsorption. Especially in the case of low-abundancy proteins, *the enhancement of specific* binding by the *reduction of nonspecific protein adsorption* is of crucial importance. So far, biomolecules have been immobilized to polymeric surfaces by physical adsorption based on hydrophobic and/or electrostatic interactions.[2] Unfortunately, some structurally unstable enzymes and proteins may undergo conformational changes during the adsorption onto hydrophobic surfaces, leading to a significant reduction of their bioactivity.[3] Furthermore, especially in fluidic systems, physically adsorbed molecules may desorb.[4] Consequently, surfaces that tolerate the preparation of stable biomolecule microarrays with remaining bioactivity need to be developed. However, the necessary grafting procedures generally require prefunctionalized surfaces. Different approaches for the initial functionalization steps have been discussed. For instance, oxidizing agents such as potassium peroxodisulfate[5] as well as chromic[6] or sulfuric[7] acid have been used. Nevertheless, these reactions generate a number of different oxygen-containing groups, that is, hydroxyl-, aldehyde, ketone, and carboxylic acid groups. A more specific approach for biomolecule attachment entails the use of photolinkers. Dankbar et al.[8] investigated nitrene- and carbene-based photolinkers for the fixation of DNA molecules onto poly(styrene) (PS) and poly(methylmethacrylate) model substrates. For the preparation

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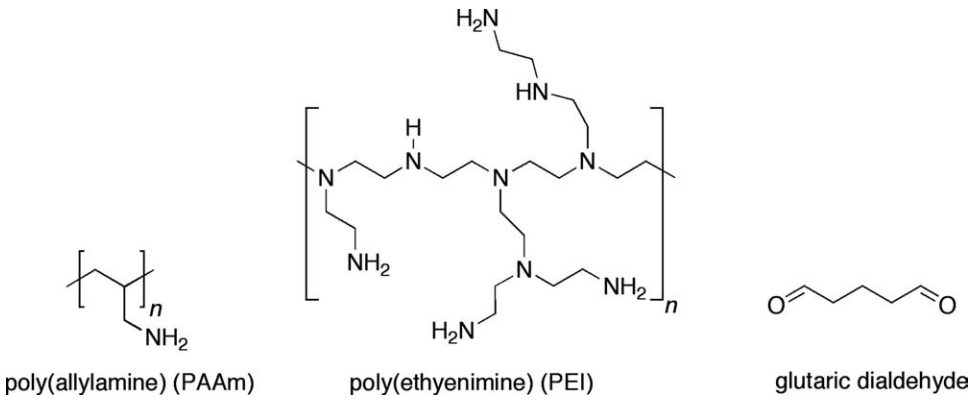


Figure 1 Reagents and polymers used for the surface functionalization of dynabeads.

of dense-albuminated layers, Magoshi et al.[9] grafted acrylic acid via visible light-induced photopolymerization onto segmented polyurethane films. Another versatile strategy for the surface modification is the nonthermal low-pressure plasma treatment.[10,11] Depending on the process gas (Ar, N_2, O_2, H_2, and NH_3) and process parameters (pressure, power, and time), the type of generated functionalities can be controlled to a large extent. However, after such initial activation, the functionalization density is quite low. Wang et al.[12] developed a versatile method for the surface functionalization of LDPE films. Poly(ethylene glycol) (PEG) chains were grafted onto plasma-pretreated polymeric surfaces. The remaining surface-hydroxyl groups were then modified with different reagents for the particular application, while at the same time the nonspecific adsorption was suppressed by the hydrophilic PEG. To overcome the problem of low-functional group density, bi- or multifunctional spacer molecules can be immobilized to the surface, for example, by the fabrication of polymer brushes[12–15] or of hyperbranched polymers or dendrimers.[16]

We recently reported on the plasma-based surface functionalization of PS-microtiter plates in combination with a suitable linker chemistry to produce highly branched, amine-terminated systems for bioassay applications.[17] Here, we report on the functionalization of polyamine-terminated microparticles by the stepwise, *in situ* construction of amine-rich structures. The focus was to develop a system that allows for a highly specific functionalization while being virtually resistant to nonspecific protein adsorption. Nonporous magnetic *dynabeads*® were selected as model supports and functionalized with different reagents. Focusing on the development of cost-effective functionalization methods, this system was chosen as a model system also for large-scale applications. The advantage of such microparticles is that they already exhibit a defined number of amine functionalities. In addition, they can be easily removed from a reaction mixture applying a magnetic field, a simple process that both reduces the loss of functional particles and speeds up the screening process. For functionalization, linear poly(allyl amine) (PAAm)[18] and a branched poly(ethylene imine) (PEI) served as reagents. Glutaric dialdehyde (GA) was chosen as a bifunctional coupling agent.[1] The differently functionalized microparticles were used for the covalent attachment of trypsin for the preparation of bioreactors and of the protein concanavalin A, respectively. The quality and quantity of specific and nonspecific protein attachment were monitored by a common bioassay (digestion of *N*-benzoyl-L-arginine-*p*-nitroanilide) and by MALDI–TOF analysis.

EXPERIMENTAL

Materials

A branched PEI [50 wt % in H_2O, M_w (LS) 750.000 g/mol; Figure 1] and iodoacetamide were purchased from Sigma-Aldrich (Germany). GA (25% aqueous solution, Fig. 1) and pentafluorobenzaldehyde (PBFA, 98%) were obtained from ABCR (Germany). PAAm (15 wt % solution in water, Fig. 1) was obtained from Polysciences (Eppelheim, Germany). *N*-Benzoyl-L-arginine-*p*-nitroanilide (L-BAPA) was purchased from Peptide Institute (Japan). Ethanol and trifluoroacetic acid (TFA) were from Merck (Germany), dithiothreitol from Fluka (Germany), and acetonitrile was purchased from Carl Roth GmbH (Germany, LC–MS grade, $\geq$99.95%). All chemicals were used as received.

Buffer

Sodium chloride, phosphate-buffered saline (PBS, pH 7.4), and HEPES were ordered from Carl Roth GmbH (Karlsruhe, Germany); anhydrous sodium carbonate and ammonium hydrogen carbonate from Fluka. Tween®20 was from Sigma-Aldrich. Anhydrous $CaCl_2$ was from Lancaster. 0.1*M* Na_2CO_3 pH 9.2 and 50 m*M* HEPES (100 m*M* NaCl and 10 m*M*

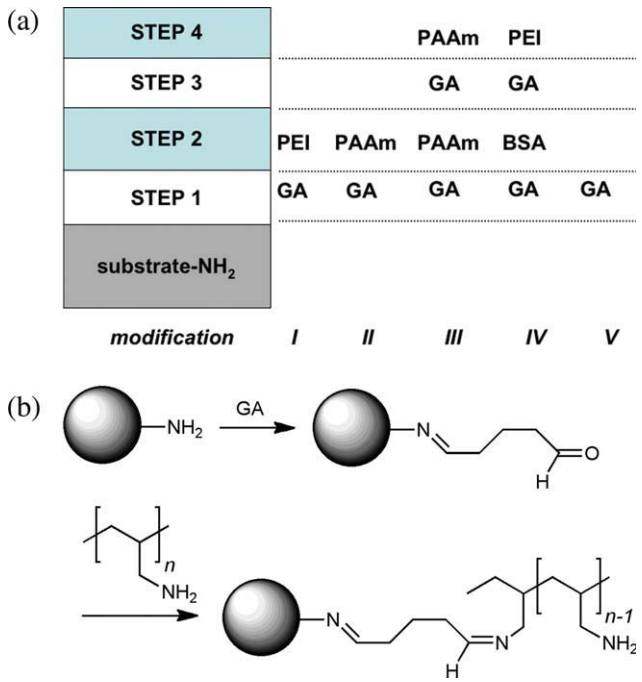


Figure 2 (a) Description of the layer composition, and (b) functionalization of aminofunctionalized dynabeads with PEI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$CaCl_2$) pH 8 buffer solutions were prepared with Milli-Q grade water. The pH was adjusted with hydrochloric acid and sodium hydroxide, respectively. PBS was prepared by diluting a stock solution to a pH of 7.4. For rinsing the protein-modified surface, the detergent Tween20 (0.01 wt %) was added to the PBS buffer.

Proteins

Two globular proteins were chosen. Trypsin (Tn) from bovine pancreas was obtained from Sigma-Aldrich (quality grades: essentially salt free and suitable for protein sequencing) and bovine serum albumin (BSA) from Fluka. Both were used as proteins for the study of the bioactivity of the model surfaces. BSA also served as a reagent for the functionalization of the dynabeads. For MALDI–TOF experiments, concanavalin A from the jack bean *Canavalia ensiformis* was purchased from Sigma-Aldrich. The physical properties of the proteins are summarized in the Supporting Information.

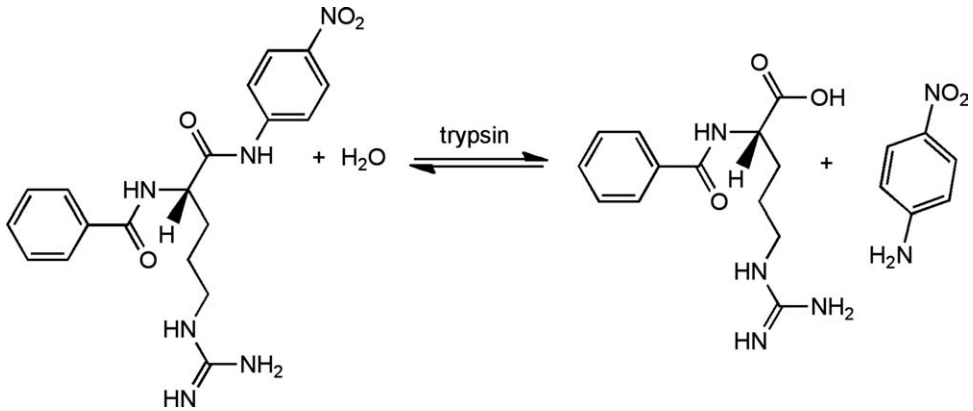
Bioassay supports

Amine-functionalized, magnetic, nonporous PS microparticles (dynabeads M270 amine) were obtained from Invritogen GmbH (Germany) as aqueous suspension with a concentration of 2×10^9 beads/mL. The beads consisted of highly crosslinked PS-containing magnetic iron oxide particles. Before use, they were washed three times thoroughly with deionized water. The beads were collected with a magnetic separator; this way, both the water and the buffer could be removed effectively without any loss of beads.

Sample preparation

GA (2 wt %), PAAm (1.5 wt %), and PEI (1 wt %) reagent solutions were prepared in a pH 9.2 Na_2CO_3 buffer, which was reported to provide the optimum reaction conditions for the irreversible reaction of GA with an amine.[19] The beads (10 μL per Eppendorf tube) were modified with 200 μL of each reagent for 1 h in Eppendorf tubes. In course of this study, four different surface functionalizations, all based on imine-formation via reaction with GA, were carried out. Using branched PEI and PAAm, modifications **I–III** were realized. In modification **IV**, BSA, a protein, was used for the GA-mediated immobilization of PEI. For the estimation of the bioactivity of the pure dynabeads, the unmodified beads were only reacted with GA (modification **V**). Figure 2(a) summarizes the different modification steps. After each step, the samples were rinsed three times with Millipore water. Figure 2(b) exemplifies the GA-based immobilization of a polyamine as used for modification **II**. Before protein attachment, the modified surfaces were activated with GA in a 0.1*M*, pH 9.2 carbonate buffer followed by 2-h treatment with trypsin (HEPES, pH 8, and 3 mg/mL) or ConA solution (PBS, pH 7.4, and 3 mg/mL).

The trypsin activity was determined by quantifying the extent of the digestion of L-BAPA. Trypsin (a common bioactive protein) is capable of cleaving peptides or proteins at the lysine and arginine sequence. The mechanism of the reaction is presented in Scheme 1. The optimum for the activity of trypsin lies in the pH range of 7–9[20] and a temperature of 37°C.[21] Noncovalently bound protein was removed by rinsing the beads twice for 5 min with both PBS and PBS/0.01% Tween20 solutions, respectively. A solution of L-BAPA (2 mg in 10 mL of DMSO and 10 mL of HEPES, pH 8) was added to the Eppendorf tubes containing a suspension of modified magnetic particles, and, for every 30 min, 50 μL of the L-BAPA solution was withdrawn from the reaction tube (particle free) and added to a clean PS microtiter plate. The time-dependent release of the redundant *p*-nitroaniline was measured at 37°C by following the extinction at 405 nm using an Infinite® 200 microplate reader (TECAN Trading AG, Switzerland). After the measurement, the solution was returned to the tubes. From the slope of the kinetic experiment, the dE/min values were calculated. The covalently attached amount Γ_{cov} (mg/mL) of trypsin was calculated with the aid of a calibration curve.



Scheme 1 Release of *p*-nitroaniline by trypsin-catalyzed hydrolysis of L-BAPA.

Complementary to these experiments, ConA was covalently attached onto PAAm-modified beads (modification **II**). The immobilized ConA was then cleaved from the microparticles by digestion of the protein with trypsin. The following protocol was applied: first, the ConA-modified beads were incubated for 30 min at 88°C in 15 μL of a water/acetonitrile mixture (60/40 vol %) containing 0.1% TFA. Acetonitrile reduces the intermolecular hydrophobic interactions, and TFA is responsible for the unfolding of the protein. Then, the disulfide bonds were reduced by dithiothreitol (10 μL of a 10 m*M* solution in 100 m*M* NH_4HCO_3, 30 min at 56°C). The thiol groups were further reacted to stabile *S*-carboxyamidomethyl groups by adding 10 μL of a 55 m*M* iodacetamide solution in 100 m*M* NH_4HCO_3 (30 min at room temperature, exclusion of light) followed by addition of 5 μL of a 0.2 mg/mL trypsin solution in 50 m*M* NH_4HCO_3 (overnight incubation at 37°C). The eluate was spotted onto a PAC-target after ZipTip® (Millipore™) cleaning for further MALDI–TOF/TOF analysis.

The amount of free NH_2 groups of each two modification **II** and **V** beads was determined using a labeling reaction with a 5 (w/v) % solution (0.05 g/mL) of PBFA in absolute ethanol. The amine-containing beads were equilibrated three times with 200 μL of ethanol, reacted for 1 h with 200 μL of PBFA, and finally washed three times with ethanol and Millipore water. The beads were then collected in 10 μL of Millipore water and spotted on cleaned silicon wafers. The atomic concentration and ratios of N, F, C, and O were determined by XPS using an AXIS Ultra system (Kratos Analytical, UK) equipped with a monochromatic-Al Kα 1,2-X-ray source of 150 W at 15 kV. The kinetic energy of the electrons was analyzed with a pass energy of 160 eV.

Maldi–TOF analysis

An autoflex III smartbeam (Bruker Daltonics, Bremen, Germany) matrix-assisted laser-desorption/ionization time of flight/time of flight (MALDI–TOF/TOF) mass spectrometer equipped with the LIFT™ technology was used to acquire the mass spectra. The samples were prepared on an AnchorChip™ (PAC) target prespotted with an α-cyano-4-hydroxycinnamic acid matrix and peptide calibration standards, which covered the mass range between 700 and 4000 g/mol. The software packages flexControl and flexAnalysis (Bruker Daltonics, Germany) were used. Spectra from 500 laser shots were summed to obtain the final spectrum. The spectrometer was operated both in reflection mode for qualitative analysis and in the TOF/TOF (LIFT) mode for detailed characterization. The latter mode provides peptide mass fingerprints with high sensitivity. The generated mass list was used for the screening of the protein databases with the help of the Mascot search engine (Matrix Science). The matches between the mass fingerprint and the sequence database were scored.

RESULTS AND DISCUSSION

Surface modification of magnetic microparticles

The dynabeads were stepwise modified as illustrated in Figure 2. For that purpose, the surface-located amino groups of the beads were first reacted with an excess of GA. Then, the aldehyde-functionalized beads were reacted with either PEI or PAAm (modifications **I** and **II**). In modification **III**, the top PAAm layer as realized in modification **II** was again reacted with GA followed by reaction with PAAm. In modification **IV**, the GA-derivatized beads were first reacted with BSA, then again with GA, and finally with PEI. BSA was chosen for the saturation of the unspecific binding sites. All these modifications resulted in the formation of polyamino-funtionalized surfaces containing different amounts of surface-bound amino groups. Then, trypsin was *covalently* attached to the thus amino-functionalized beads using the same GA-based coupling sequence. Thus, the modified dynabeads were first reacted with GA and then with trypsin aiming for the preparation of a

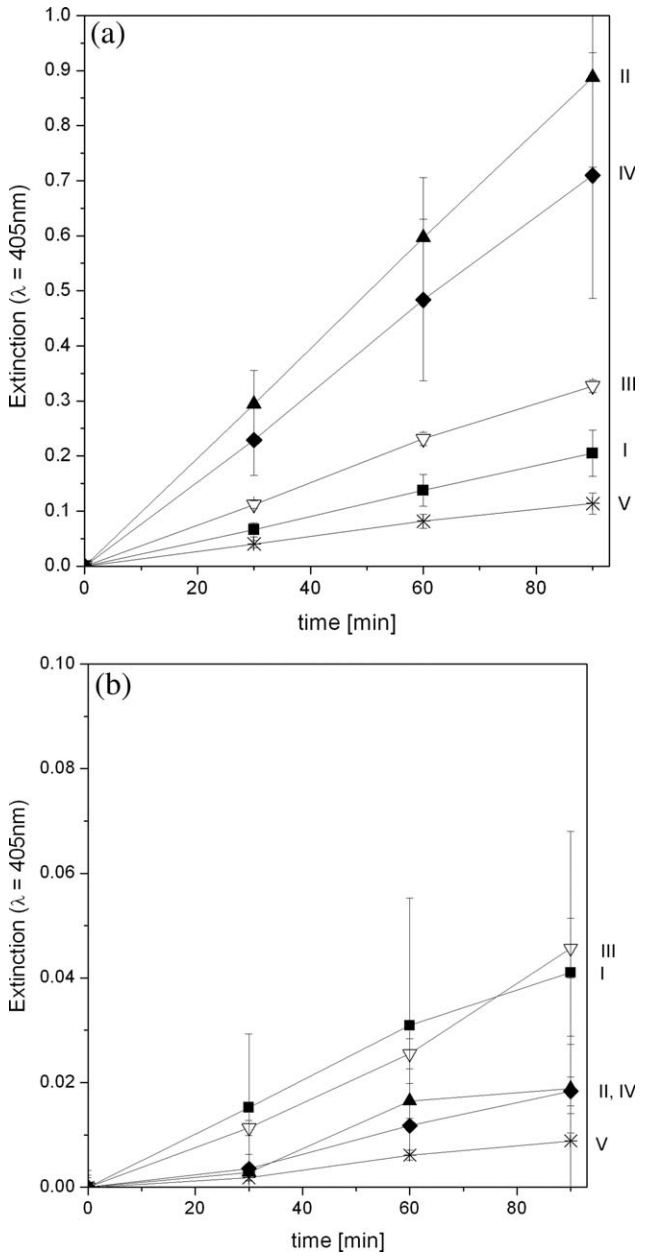


Figure 3 Extinction values of the L-BAPA digestion of trypsin on differently modified dynabeads® (modifications I–IV) and GA-modified beads (modification V). Left (a): covalent attachment of trypsin; right (b): nonspecific adsorption onto the beads.

magnetic particle-based bioreactor. For the purposes of comparison, GA-modified beads (modification **V**) were also treated with trypsin. To determine the *nonspecific protein adsorption*, the modified beads were also treated with trypsin *without* any preceeding reaction with GA. The different amount of trypsin present at the surface of the bead, whether covalently or nonspecifically bound, was quantified using the trypsin-mediated hydrolysis of L-BAPA (Fig. 3). The results shown are the average and median values of three independent measurements. The self-hydrolysis of L-BAPA during the digestion reaction was negligible (dE/min $\sim$ 0). As can be seen from Figure 3(b), the nonspecific adsorption caused by physical interaction between the protein and the functionalized beads was very low (dE/min $\sim$ 0). Consequently, the extinction values in Figure 3(a) result virtually exclusively from the successful *covalent* attachment of the protein onto the modified beads. Figure 3(a) also clearly indicates significant variations in the bioactivity of the different modifications. Consequently, we compared the average *dE/min* values of the specific and nonspecific protein adsorption experiments (Fig. 4). The best results were obtained with dynabeads modified with PEI (modification **I**), PAAm (modifications **II** and **III**), and the PEI/BSA system (modification **IV**). In comparison with the results obtained with microparticles only activated with glutaric dialdhehyde (modification **V**), we found an increase in the *dE/min* values up to factor of seven.

As demonstrated by XPS measurements on the PBFA-labeled modifications **II** and **V**, the bioactivity of amine-modified magnetic beads directly corresponds to the increased number of amine groups introduced by the grafting reaction (Table I).

As can be deduced from the F/C ratio in Table I, the amount of fluorine introduced via the reaction of the amino groups with pentfluorobenzaldehyde (corresponding to the accessable NH_2-groups) on PAAm-modified beads was about four times higher than on pristine beads.

Calculation of the amount of trypsin covalently bound to the surface

From the *dE/min* values, the amount of trypsin covalently attached to the beads, Γ_{cov} (mg/m^2), can be calculated as follows:

$$\Gamma_{cov} = \frac{m_{cov}}{A_{cov} \cdot 1000} \quad (1)$$

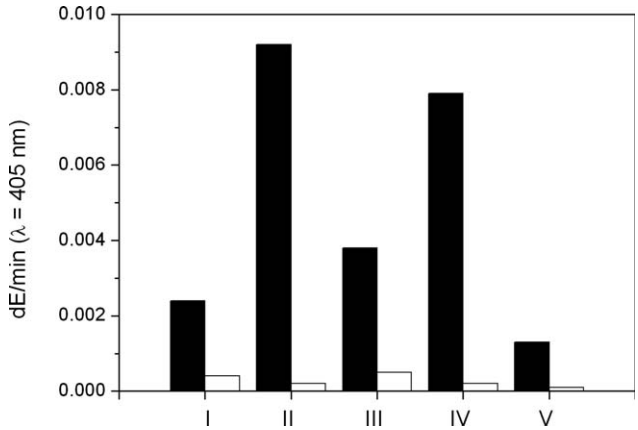


Figure 4 Average dE/min values of the L-BAPA digestion with differently modified dynabeads (modifications I–V, black bar = covalently bound trypsin, white bar = nonspecifically bound trypsin).

TABLE I
Atomic Concentrations at the Surface of GA-Modified (V) and Multiply Modified (II) Beads

	C (atm %)	O (atm %)	N (atm %)	F (atm %)	N/C	F/C
II	59.8 ± 2.4	31.9 ± 3.3	5 ± 0.6	3.4 ± 0.2	8.3 ± 0.7	5.6 ± 0.2
V	63 ± 0.1	32.4 ± 0.0	3.7 ± 0.0	0.8 ± 0.0	5.9 ± 0.0	1.2 ± 0.0

with m_{cov} (μg) representing the mass of the covalently bound protein and A_{cov} (m^2) representing the surface area of the substrate. A 10-μL volume of dynabeads (2×10^7 particles) corresponds to a surface area of 4.9×10^{-4} m^2. The amount of immobilized protein can be evaluated by using a calibration curve. The calibration curve (Supporting Information) was created by measuring the L-BAPA hydrolysis in microtiter plate wells (with a known surface area) using known trypsin concentrations. The results are summarized in Table II. For modifications **II** and **III**, values for $\Gamma_{cov.}$ of 1.1 and 1.0 mg/m^2, respectively, were calculated. Malmsten et al.[22] reported on the covalent attachment of trypsin onto porous unmodified and hydrophilized glycidyl methacrylate beads. There, the highest surface concentration of trypsin was determined to be 1.8 mg/m^2 for the unmodified particles. The saturation value for the nonspecific trypsin adsorption on hydrophobic surfaces was reported to be between 2.5 and 4.5 mg/m^2.[23,24] However, it is worth notifying that in the above-mentioned reports, the surface area of the particles was denoted with 47 m^2/g (58-nm average pore size, 125–315-μm particle diameter). In our case, the specific surface area of the unmodified nonporous dynabeads was very low, for example, around 1.6 m^2/g. Thus, a comparably large amount of trypsin was covalently attached on a quite low-specific surface area by the different approaches used here and underlines the efficiency of the modification approach used here.

In terms of unspecific protein adsorption, the isoelectric point (IEP) of a protein (IEP_{BSA} = 4.7, $IEP_{trypsin}$ = 10.1–10.5) plays a crucial role. Because PEI and PAAm are highly basic reagents with IEPs around 11, they are protonated at the chosen pH. Especially PEI is well known for its antifouling properties.[25–27] Under the used conditions, trypsin carries the same charge as the cationic polymers, which easily explains for the highly trypsin-repelling character of surfaces modified by such polymers resulting in low unspecific adsorption of this protein. It is, however, worth underlining that the nonspecific adsorption of other proteins, for example, ones with *acidic* IEPs, such as BSA, may be different from the one of trypsin. Our interface is well suited for proteins with a *basic* isolelectric point, for *acidic* proteins like BSA other surfaces should be developed.

TABLE II
Calculation of the Masses of Trypsin Covalently Attached to the Beads and Γ_{cov} Values Obtained from the Calibration Curve

	m_{well} (μg)	Γ_{cov} (mg/m^2)
I	0.1	0.3
II	0.6	1.1
III	0.2	0.5
IV	0.5	1.0
V	0.1	0.2

Detection and identification of covalently attached concanavalin a via Maldi–TOF

In biochemistry, mass spectrometry is a versatile tool for the characterization and identification of proteins, enzymes, and peptide sequences.[28]

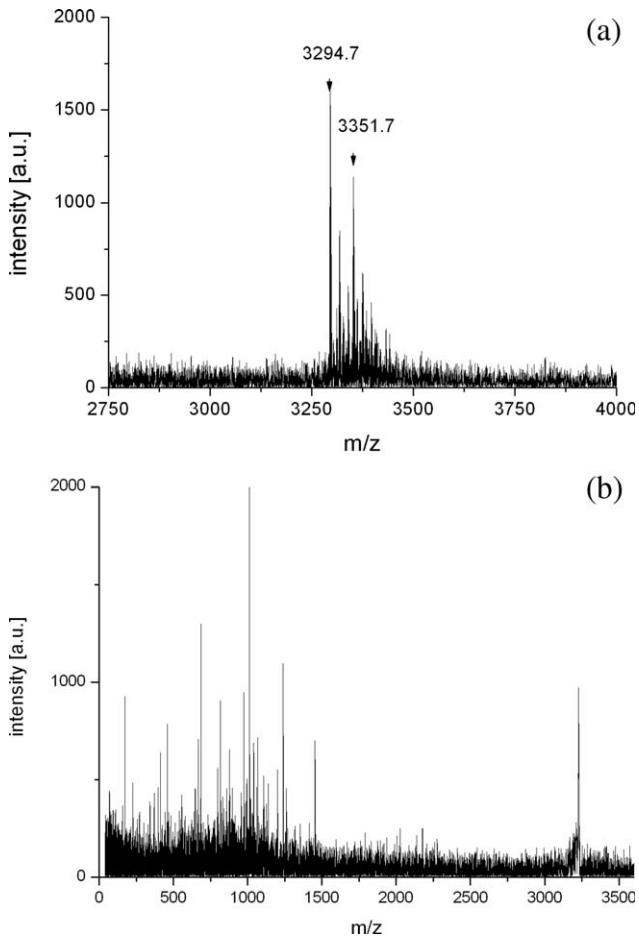


Figure 5 (a) Positive reflection ion mode spectra and (b) LIFT™ mode spectra of the mass at m/z = 3294.7 g/mol.

Recently, Sparbier et al.[28] reported on the purification of glycoproteins in human serum by means of lectine- or boronic acid-modified magnetic particles. The isolated glycoproteins were identified via LC–MALDI–TOF/TOF analysis. Our goal was to attach concanavalin A to PAAm-modified magnetic microparticles and then, by means of tryptic digestion, to identify the protein by MS/MS analysis. The aim of the MALDI–TOF and MALDI–TOF/TOF experiment was to detect the peptide mass fingerprint of ConA obtained by enzymatic digestion. Figure 5 represents the MS and MS/MS spectra of the trypsin-triggered digestion of ConA. For the MS/MS fragment ion analysis, the peptide mass of 3294.6 g/mol was selected. Using the sequence number 224–253 (sequence R.LSAVVSYPNADSATVSYDVDLDNVL-PEWVR.V), the fragments could be unambiguously identified by Mascot as a peptide sequence of ConA with good agreement of the observed [3294.647 g/mol $(M-H)^+$] with the expected mass (3293.640 g/mol).

This result provides further evidence for the excellent suitability of polyamine-modified magnetic microparticles for covalent protein attachment. Even more important, it opens the door to the selective extraction of biomolecules. Thus, using the sugar-binding properties of Con-A, ConA-modified microparticles are of interest for the selective attachment of functional sugar-containing molecules (glycosidic peptides).[29] Work related to that particular aspect will be reported in due course.

CONCLUSIONS

In summary, we investigated the bioactivity of differently modified microparticles and thereby developed a trypsin bioreactor and established a rapid screening process based on the trypsin-triggered hydrolysis of L-BAPA for the covalent binding capacity as well as protein repelling properties of differently modified beads. PAAm- and PEI-modified beads turned out to be the best-suited systems. Applying a PAAm modification to the dynabeads, the bioactivity and the surface concentration of immobilized trypsin could be increased by the factor of seven. MALDI–TOF/TOF mass spectrometry was successfully applied for the detection of covalently bound concanavalin A.

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