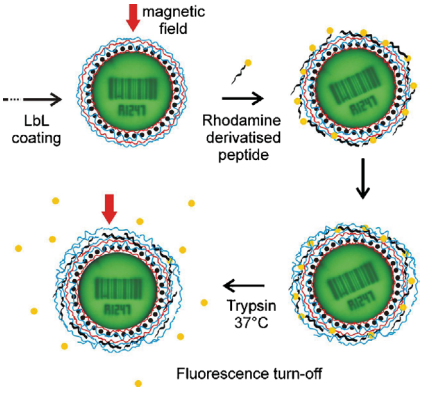
FULL PAPER

Biosensors



Layer-by-layer (LbL) polyelectrolyte coatings, containing a red-labeled trypsin substrate, are carefully designed and applied at the surface of encoded microparticles. The peptide-loaded LbL coatings lose their red fluorescence upon incubation in a trypsin solution, indicating that LbL-coated microparticles show potential to screen for the presence of active proteases in biological samples (see figure).

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Evaluation of Encoded Layer-By-Layer Coated Microparticles As Protease Sensors**

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Proteases are important pharmaceutical targets for new drugs because of their involvement in numerous disease processes. This study evaluates whether photophysically encoded microparticles carrying fluorescently labeled protease substrates (peptides) at their surface show potential for detecting proteases in a sample. Layer-by-layer (LbL) polyelectrolyte coatings, containing a red-labeled peptidic trypsin substrate, are carefully designed and applied at the surface of the encoded microparticles. The peptide-loaded LbL coatings lose their red fluorescence upon incubation in a trypsin solution, indicating that LbL-coated microparticles show potential to screen for the presence of active proteases in biological samples.

1. Introduction

Proteases, like matrix metalloproteases, secretases, and viral proteases, are important pharmaceutical targets for future drugs because of their crucial involvement in numerous human diseases.[1] Many diseases, including cancer, rheumatoid arthritis, cardiovascular and neurodegenerative diseases, are characterized by a change in the types of substrates degraded by the cellular proteases and/or by an altered protease activity. Surprisingly, in view of the high number of proteases that are potentially expressed in higher eukaryotes — the current version of MEROPS (release 7.90 at http://merops.sanger.ac.uk/) holds 612 known or putative human proteases — few proteases have thus far been characterized. Activity-based probes were introduced for monitoring active proteases in complex samples such as tissue extracts (Liu et al., 1999■ please insert full reference into list ■[Q1]). Although very promising results have been reported (amongst others for monitoring metalloproteases),[2,3] such probes often target whole protease families and do not distinguish individual members. Given recently published emerging technologies for proteome-wide characterization of protease-mediated substrate processing,[4–7] we estimate that eventually one could start using synthetic peptides carrying very specific protease-recognition motifs to monitor the activity of a specific (set of) protease(s).

Recently, our research group introduced photophysically encoded microparticles (named 'memobeads') that carry a digital code (like a number or a barcode) in their middle plane (see Fig. 1).[8,9] The code in a particular bead reads out which 'sensing molecule' ('probe') is bound at its surface. The probe can be an antibody (for screening antigens) or a single-stranded oligonucleotide (for single nucleotide polymorphism detection).[10,11] A major advantage of encoded beads is that they allow 'multiplexed' analysis of biological samples being the simultaneous analysis of numerous analytes (antigens, DNA fragments, ...) in one sample.[12] In this report we investigate whether memobeads carrying protease substrates (peptides) at their surface show potential for protease profiling in a multiplexing setup.

Figure 1 schematically shows how we consider such protease profiling studies using (green fluorescent, 40 μm sized, polystyrene) memobeads. At the surface of a memobead we first applied a layer-by-layer (LbL) coating, which is composed of polyelectrolytes (PEs) (step I in Fig. 1). The LbL coating is based on the alternate adsorption of oppositely charged PEs (or charged nanoparticles) onto a charged substrate.[13–16] We reported previously that the polystyrene core of a memobead can be successfully LbL-coated with polyanions, polycations, and magnetic CrO_2 nanoparticles. As explained in detail by Derveaux et al.,[10] the magnetic CrO_2 nanoparticles allow an optimal positioning of the memobeads in a magnetic field

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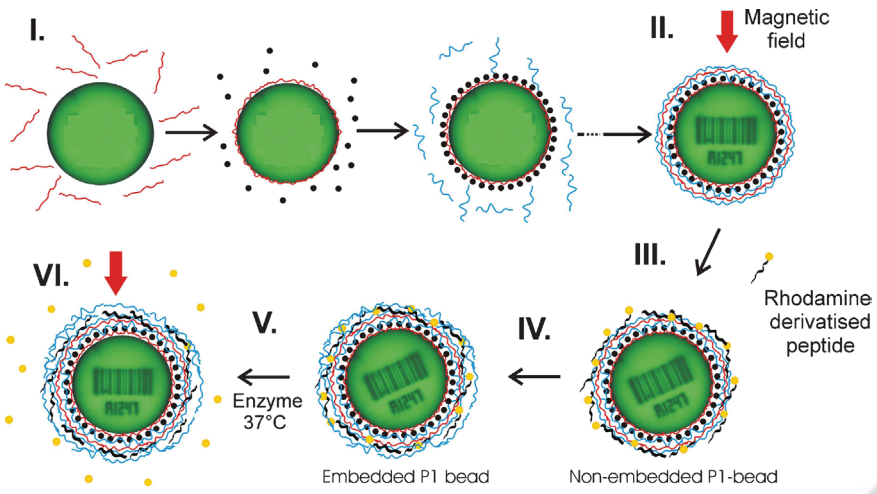

Figure 1. Schematic representation of the LbL coating (I), the encoding (II), P1 (NH_2-GRKKRRQRRRPPQC-COOH, rhodamine-labeled at its N-terminus) loading (III), embedding of P1 (IV), and degradation of P1 by trypsin (V). Step I: Carboxyl-functionalized green fluorescent polystyrene microparticles (approximately 40 μm in size) are coated by alternating adsorption of positively charged poly-allylaminehydrochloride (PAH, red strands) and negatively charged poly-styrenesulfonate (PSS, blue strands). Magnetic chromium dioxide nanoparticles (<500 nm, CrO_2 NP, black dots) are incorporated in the LbL film to allow accurate positioning of the beads in a magnetic field at the time of encoding (Step II) and decoding (Step VI) [10]. Step III: Adsorption of a (red) fluorescently labeled peptide substrate; the bar code in the bead is linked and thus reads out the peptide present on the surface of the bead. The beads obtained after step III are termed 'non-embedded P1 beads'. Step IV: An extra polyelectrolyte layer is adsorbed; The beads thus obtained are termed 'embedded P1 beads'. Step V: Dispersing these beads in a sample; the protease (if present in the biological sample) will cleave the red-labeled peptide, which lowers the red fluorescence of the microparticles. Step VI: Decoding of the microparticles occurs after proper orientation of the beads in a magnetic field.

(step II in Fig. 1), which is necessary to read the code at the time of decoding. As step III in Figure 1 illustrates, in the present study a red fluorescently labeled peptide, being the protease substrate, was applied in the LbL coating surrounding the memobeads. We hypothesized that degradation of the peptide by proteases present in the solution surrounding the polyelectrolyte multilayered (PEM) beads would lower the red fluorescence of the bead coating. Subsequently, the code in this bead would allow the substrate (and thus the identity) of the protease(s) present in the (biological) sample to be to identified. Clearly, adding tens or hundreds of differently encoded beads, each carrying a different red fluorescent peptide which can only be processed by a specific protease or family of proteases, to a protease-containing sample, may allow the simultaneous identification of the proteases present in the sample.

The immobilization of proteins like fibrinogen, trypsin, glucose oxidase, and glucoamylase in polyelectrolyte multilayers to design bioactive surfaces has been reported before.[17–21] As an example, Yudanova et al. modified the biological function of fibers by adsorption of polyethyleneimine-protease C complexes on the fibers. Antimicrobial protection in the contact zone with the material was obtained.[22] Li et al. prepared PEMs using cysteine-containing 32-mer polypeptides as PEs. These films were stabilized by disulfide bridging between cysteine residues in the different layers.[23,24] Also, Picart et al. grafted maleimide-modified polyglutamic acid with an adhesion peptide composed of 15 amino acids and a central RGD motif; thus, showing better cell adhesion and cell proliferation on these peptide-functionalised PE films.[25] To the best of our knowledge, self-assembly of (short) peptides (i.e., composed of less than 30 amino acids) in PE layers of LbL-coated beads has never been described. In particular, it is completely unknown whether peptides/proteins immobilized in PEMs are still enzymatically degradable and which factors influence this.

The specific aim of our study was to evaluate if peptides self-assembled (adsorbed) to polyelectrolyte multilayers surrounding microparticles allow the monitoring of active trypsin, chosen here as a model protease. First, we investigated whether a fluorescently labeled trypsin substrate, termed P1 (NH_2-GRKKRRQRRRPPQC-COOH, rhodamine-labeled at its N-terminus), can be adsorbed on LbL-coated microparticles. We then analyzed if the PEMs are permeable for trypsin, if the adsorbed peptides could still be cleaved by trypsin, and whether this results in a lowering of the red surface fluorescence of the microparticles. The physicochemical properties of the PEMs, like the nature and number of the polyanions and polycations used to build the layers, the conditions under which the polyelectrolytes were applied at the surface of the microparticles, and the way the peptide substrate was immobilized in the PEMs, were evaluated to find the optimal PEM for the detection of trypsin. We also investigated whether the number of PEM microparticles in the trypsin solution influences the sensitivity of the trypsin assay.

2. Results and Discussion

2.1. Loading PEM Beads with the P1 Peptide

Figure 2A shows fluorescence images of PEM beads before and after loading with P1 (NH_2-GRKKRRQRRRPPQC-COOH). Before P1 loading, green fluorescent beads are visible. As the rhodamine-derivatized peptide P1 is strongly positively charged, it should easily adsorb on the negatively charged PSS layer of the PEM beads. Indeed, red (orange) fluorescence was observed at the surface of the green beads, proving the loading of the PEM beads with the short P1 peptide.

Figure 2B shows the red fluorescence of non-embedded P1 and embedded P1 beads as a function of the P1 concentration of the solution in which the beads were dispersed for loading with P1. For non-embedded P1 beads, the red surface fluorescence increases linearly for P1 concentrations between

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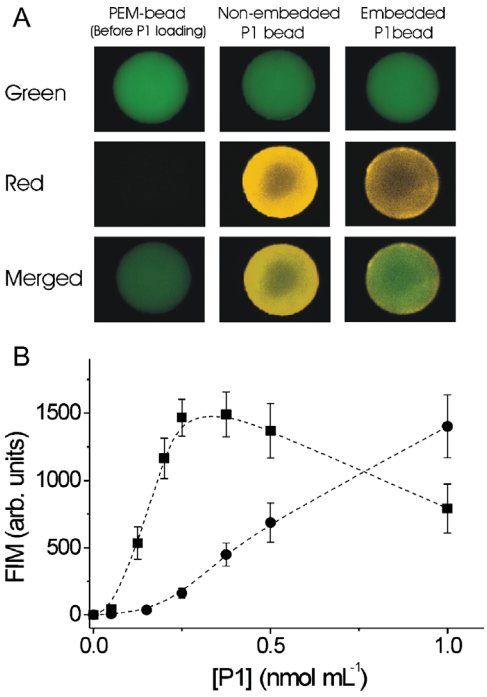

Figure 2. A) Green, red, and merged fluorecence images of beads before P1 loading, non-embedded P1beads, and embedded P1 beads. The P1 concentration used for the bead loading was 0.5 nmol mL^{-1}. To embed the P1-loaded beads, a 2 mg PSS mL^{-1} solution (in 0.5 M NaCl) was used. B) Red fluorescence (fluorescence intensity mean: FIM) of non-embedded P1 (■) and embedded P1 beads (●) as a function of the P1 concentration of the solution used for the bead loading with P1.

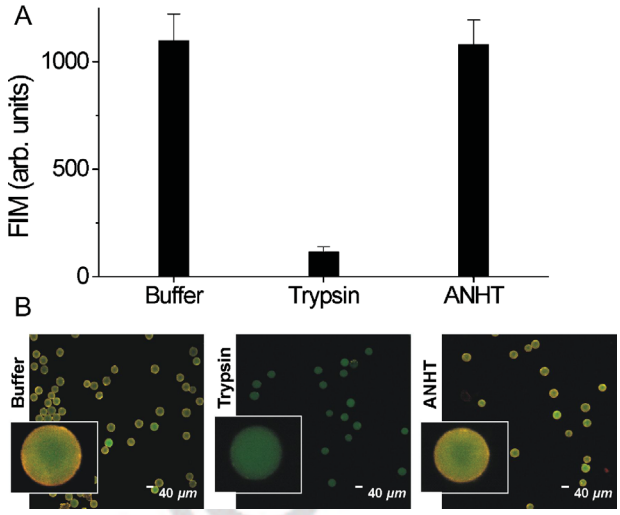

Figure 3. A) Red surface fluorescence of embedded P1 beads after incubation for 2 h in buffer, trypsin, and anhydrotrypsin (ANHT) solutions. The P1 concentration used for the loading of the beads with P1 was 0.5 nmol mL^{-1}. B) The corresponding (red and green merged) fluorescence images.

0.05 and 0.25 nmol mL^{-1}. At a higher P1 concentration, fluorescence quenching starts to occur at the surface of the beads. Clearly, at a given P1 concentration in the solution (e.g., 0.25 nmol mL^{-1}), the red fluorescence of embedded P1 beads is much lower than that of non-embedded P1 beads. Although the exact reason is unclear, possible explanations are loss of P1 during the adsorption of the extra PSS layer and/or a molecular redistribution of the fluorescent P1 in the PEM layer upon applying the extra PSS layer.

2.2. Enzymatic Cleavage of P1-loaded PEM Beads

Subsequently we investigated whether P1-loaded PEM beads could be used to detect trypsin in the solution in which the beads are dispersed. Possibly, trypsin could not access P1 anymore sufficiently, especially when embedded at the surface of the beads. Also, the PEM layer may bind and/or conformationally change the protease and thus its activity. Furthermore, to observe less red fluorescence at the surface of the beads upon adding trypsin, the fluorescent P1 fragments (i.e., the degradation products) should not remain in the PEM layer but should be released from the surface of the beads into the solution.

As Figure 3A shows, embedded P1 beads undergo a significant loss in their red surface fluorescence (a decrease of $89.5 \pm 0.3\%$) when incubated in a trypsin solution; this indicates that cleavage of P1 occurs and that fluorescent P1 degradation fragments do not remain in the PEM layer but are released into the surrounding medium. While not very likely, we cannot exclude that the change in intensity may in part be due to a shift in the fluorescence spectrum. Figure 3B shows the corresponding fluorescence images of the beads upon incubation in the buffer and trypsin solutions. As the rhodamine-labeled P1 is not covalently attached to the LbL coating, we wondered whether trypsin desorbed P1 from the beads, which would also decrease the red surface fluorescence of the beads. Therefore, we dispersed the beads in an anhydrotrypsin (ANHT) solution; ANHT is a very close structural homologue of trypsin but without proteolytic activity (see the Supporting Information). Figure 3A and B illustrates that embedded P1 beads do not significantly lose their red fluorescence upon incubation in an ANHT solution (only a minor decrease in red fluorescence of $1.7 \pm 1.2\%$ was observed), confirming that the decrease in red surface fluorescence by trypsin is due to cleavage and not desorption of P1.

Figure 3 deals with embedded P1 beads. When non-embedded P1 beads were used, similar findings were obtained. It seems that the aqueous cavities in the PEM layers (a well-interconnected porous network[18]) allow trypsin to reach its substrate and subsequently cleave it. Earlier reports indeed show that enzymes, like glucose oxidase and glucoamylase, embedded in LbL films preserve not only their secondary structure[20,21] but also their enzymatic activity.[17–19] However, the efficiency of the reactions depends strongly on the enzyme and the nature of the multilayers.[19]

2.3. Influence of the Number of PE Layers on the Cleavage of P1 by Trypsin

In the next step, we investigated to what extent the number of PE layers adsorbed on the beads after loading them with P1

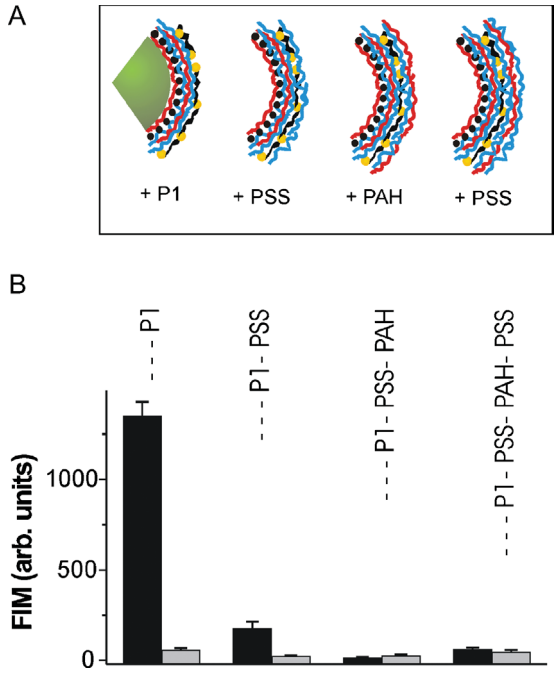

Figure 4. Red surface fluorescence of P1-loaded beads as a function of added outer layers. The beads were incubated for 2 h in ANHT (black bars) and trypsin solutions (grey bars). The drawings (A) illustrate the composition of the PE layer (red strands are PAH, blue strands are PSS). The P1 concentration used for the loading of the beads with P1 was 0.5 nmol mL^{-1}.

influences their ability to detect trypsin. As schematically illustrated in Figure 4, after P1 loading, beads were further coated with 1, 2, or 3 PE layers: PSS, PSS/PAH, and PSS/PAH/PSS, respectively (PAH: poly-allylaminehydrochloride; PSS: polystyrenesulfonate).

A first observation is that adding (even a single) PE layer(s) on top of the P1 layer strongly reduces the red surface fluorescence (compare the black bars). When only a single PSS layer is applied, trypsin can still cleave P1, lowering the red fluorescence (see grey bar). However, when two or more PE layers are applied on top of the P1 layer, adding trypsin no longer lowers the red surface fluorescence. Possibly, the extra PE layers prevent permeation of trypsin into the PE layers. Indeed, Caruso et al. showed that encapsulation of catalase in LbL-engineered polyelectrolyte capsules, comprising a large number (eight) of PE layers [$(PSS/PAH)_4$], protected catalase from protease degradation.[26] Also, Trau et al. encapsulated glucose oxidase in an LbL layer, which consisted of four PE layers [$(PSS/PAH)_2$], and showed that the enzyme became protected from the outer environment, for example, from protease or microbial activity.[27]

2.4. Influence of the PE Layer Composition on the Cleavage of P1 by Trypsin

To obtain a sensitive probe for protease screening, the composition of the PEM layer should be optimal. We wondered whether the type of PE layer that is adsorbed to the beads before P1 adsorption (which we termed the 'inner PE layer') and after P1 adsorption (the 'outer PE layer') influences the activity of trypsin. It is highly likely that the inner and outer PE layers, which are in close proximity to P1 molecules, may influence peptide loading, the fluorescent properties of P1, and the accessibility and activity of trypsin to degrade P1.[17,19,21,22] We designed PEM beads with different inner and outer PE layers: PAH was used as a positively charged inner PE layer and PSS as negatively charged inner PE layer, while PAH, PL (polylysine), PSS, or PAA (polyacrylic acid) were deposited as the outer layer.

As shown in Figure 5, both the inner and outer PE layers influence P1 loading as well as the loss in fluorescence upon trypsin incubation. The red surface fluorescence of P1-loaded beads with PAH as the inner layer was low (see black bars), which is most likely explained by electrostatic repulsions between the positively charged P1 (pI = 12.3 ■ **define pI** ■) and the positively charged inner PAH layer. Similar observations were made when other positively charged inner PE layers were used (data not shown). In contrast, a negatively charged inner PE layer like PSS (or other negatively charged PEs; data not shown) favored loading of P1. The decrease in red surface fluorescence upon adding trypsin was generally stronger when beads with a negatively charged outer PE layer (i.e., PSS or PAA) were used, which can be explained by the higher affinity of trypsin (pI = 8.2) for the oppositely charged surface. This is in agreement with the results of Basso et al., who showed that the accessibility of penicillin G acylase (pI = 5.2–5.4) into PEGA (i.e., crosslinked acrylamide and poly(ethylene glycol)) resins could be improved by introducing positive charges into the polymer; this result was caused by the electrostatic interactions between polymer and enzyme.[28,29] When PAH

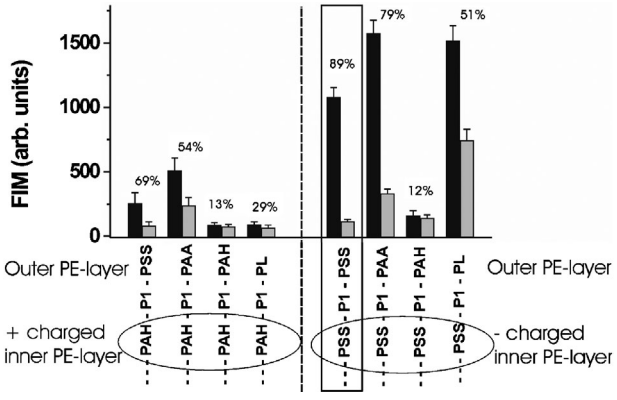

Figure 5. Red fluorescence of embedded P1 beads after being dispersed for 2 h in an ANHT solution (black bars) and a trypsin solution (grey bars). The percentages indicate the relative decrease in red fluorescence upon adding trypsin (compared to the addition of ANHT). To the left of the dashed line are beads having a positively charged inner PE layer. To the right of the dashed line are beads having a negatively charged inner PE layer. The P1 concentration used for the loading of the beads with P1 was 0.5 nmol mL^{-1}. Note that to visualize the red fluorescence of the beads having a positively charged inner (PAH) layer, a higher laser setting was necessary (gain 125; the gain was set at 103 for beads having PPS as inner PE layer).

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was used as outer PE layer, the beads were no longer able to detect trypsin activity. Figure 5 shows that the most sensitive trypsin sensor was obtained if the PE layer was chosen to be PSS, as a high initial surface fluorescence and also a strong decrease in surface fluorescence ($89.3 \pm 0.3\%$) was observed after incubation in trypsin.

2.5. Influence of Salt and Polyelectrolyte Concentration

It has been reported that the concentration of the polyelectrolyte solutions used in LbL coatings, as well as their salt concentration, affect the physicochemical properties of the resulting PE films, like their charge, elasticity, and thickness,[15,30–32] which, in turn, might influence the fluorescence of the loaded P1. As can be seen in Figure 6, embedded P1 beads are less suitable as a sensor (due to a decreased surface fluorescence) when solutions with a high PSS concentration, containing NaCl, are used to apply the outer PE layer. It has been reported that a higher PE and salt concentration results in better deposition of PEs, which results in thicker layers.[30,33] Hence, the thicker the deposited outer PSS layer, the lower the red fluorescence of the P1-loaded beads.

Figure 7A and B shows the red surface fluorescence of (non-embedded and embedded) P1-loaded beads as a function of incubation time in a trypsin and an ANHT solution. An exponential decrease in red fluorescence was observed upon incubation in trypsin. After 20 min almost all P1 molecules seemed cleaved, as can also be observed in Figure 7B. The presence of the extra PSS layer in the embedded P1 beads does not seem to influence the activity of trypsin. Also the thickness of the extra PSS layer does not seem to have an influence on the fluorescence loss (compare the beads that were prepared using $0.2\,mg\,mL^{-1}$ and $2\,mg\,mL^{-1}$ PSS solutions to apply the outer PE layer).

2.6. Redistribution of P1 Between Different Beads

To be useful in a multiplexing assay, which would imply the simultaneous presence of PEM beads loaded with different types of peptides (substrates) in the (uncharacterized) protease solution, the peptide substrates may not desorb from the surface of their beads and may not bind to the surface of other beads (carrying another code) in the same solution. To analyze whether such redistribution occurs, an equal number of (non-embedded or embedded) P1-loaded PEM beads and PEM beads (without P1) were mixed and incubated in an ANHT solution for 2 h. Figure 8 shows the outcome of the experiment. When non-embedded P1 beads were mixed with non-P1-loaded PEM beads, minute amounts of P1 were detected on the surface of the PEM beads. When embedded P1 beads (having an outer PSS layer applied in the presence of 0.5 M NaCl) were mixed with non-P1-loaded PEM beads, P1 did not redistribute (Fig. 8B–D): two populations of beads could be clearly distinguished. Note that when the outer PSS layer was loaded in water without NaCl, P1 also seemed to redistribute (data not shown). Although the redistribution of P1 was limited under all tested conditions, embedding of P1 seems necessary to avoid desorption and redistribution of the peptides over other beads.

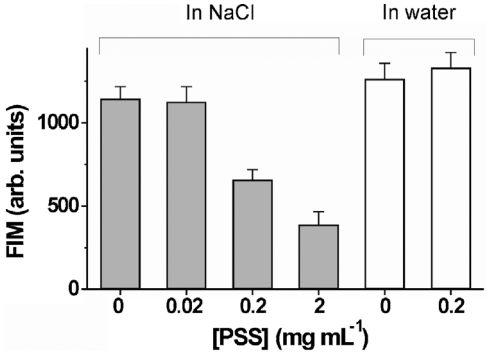

Figure 6. Red fluorescence of embedded P1 beads. The *x*-axis indicates the PSS concentration of the solution used to apply the outer PSS layer. The PSS solution was prepared in water (white bars) or in 0.5 M NaCl (grey bars). The P1 concentration used for the loading of the beads with P1 was $0.5\,mg\,mL^{-1}$.

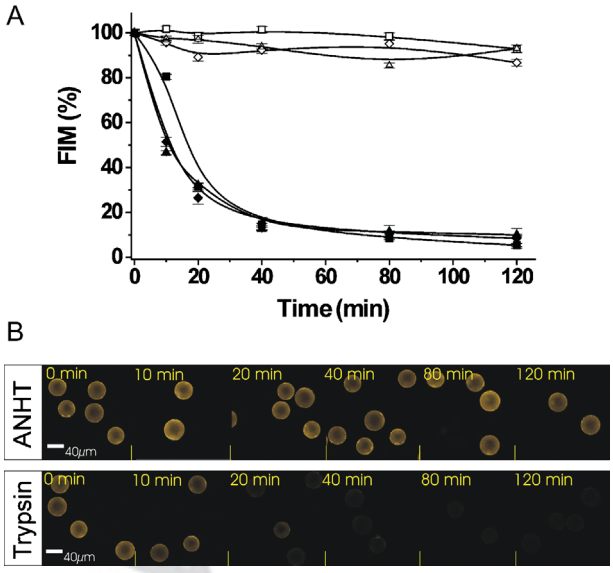

Figure 7. A) Red fluorescence of non-embedded P1 (squares) and embedded P1 beads (diamonds, triangles) upon incubation in an ANHT (open symbols) and trypsin solution (closed symbols) for different time periods ■ **ok?** ■. The concentration of PSS in the solutions used to apply the outer PSS layer was $0.2\,mg\,mL^{-1}$ (diamonds) or $2\,mg\,mL^{-1}$ (triangles). The solvent of the PSS solutions was 0.5 M NaCl. The P1 concentration used for loading of the beads with P1 was $0.5\,mg\,mL^{-1}$. B) Red fluorescence images of the embedded P1 beads incubated in an ANHT and a trypsin solution.

2.7. Influence of the Number of P1 Beads on the Sensitivity of the Assay to Detect Trypsin

We anticipated that the number of beads in the assay may affect its sensitivity towards the detection of proteases. Indeed,

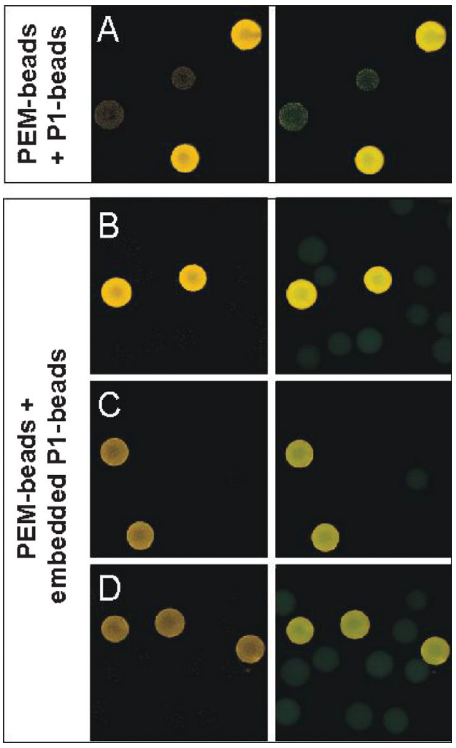

Figure 8. An equal number of embedded P1 beads and beads without P1 were mixed. The PSS concentration in the solution used to apply the outer PSS layer was A) 0 (i.e., nonembedded P1 beads), B) 0.02, C) 0.2, and D) 2 mg mL^{-1} (solvent was 0.5 M NaCl). The left column shows red fluorescence images while the right column shows red and green merged fluorescence images of the mixtures after 2 h incubation in an ANHT solution.

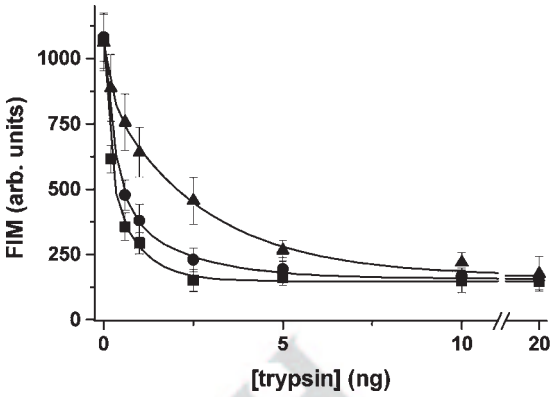

Figure 9. Influence of the number of embedded P1 beads (2000 (▲), 1000 (●), and 200 (■)) on the decrease in red fluorescence 2 h after incubation in different amounts of trypsin. The P1 concentration used for the loading of the beads with P1 was 0.5 mg mL^{-1}, while the concentration of the PSS solution to apply the outer PE layer was 2 mg mL^{-1} in 0.5 M NaCl.

one can expect that the higher the number of P1-loaded beads in the assay, the more trypsin has to be present to significantly decrease the red surface fluorescence of the beads. Indeed, by increasing the number of beads, protease molecules are distributed along a greater total surface area, and the amount of substrate (peptide on the bead) present in the assay increases. Figure 9 shows the decrease in red fluorescence of the beads after 2 h incubation in a trypsin solution. The number of beads and the amount of trypsin were varied. Clearly, using fewer beads allowed detecting a lower amount of enzyme; 0.2 ng of trypsin could still be detected when only 200 beads were used but could no longer be detected when 2000 beads were used in the assay. At higher amounts of trypsin (10 and 20 ng), the red fluorescence of the beads remaining after 2 h of incubation seemed independent of the number of beads, indicating maximal cleavage was reached. Note that a complete loss of the red fluorescence was never observed, indicating that some (intact) P1 remained in the PEM layers.

3. Conclusions and Future Outlook

This work shows that LbL coatings, containing the short (red-labeled) peptide P1, can be designed at the surface of photophysically encoded microparticles. Importantly, we succeeded in engineering P1-loaded LbL coatings in which the peptide becomes degraded by trypsin if present in the solution in which the microparticles are dispersed. Indeed, trypsin degraded P1 into lower molecular weight fragments, which did not remain attached at the surface of the beads but were released into the surrounding medium. The presence of trypsin could thus be recorded as the beads significantly lost their red fluorescence. To our knowledge this is the first report that shows LbL coatings loaded with an enzyme substrate can detect the presence of the enzyme.

We showed that numerous parameters, like the nature of the inner and outer PE layers, the number of outer PE layers adsorbed after P1 loading, and the conditions under which the layers were applied at the surface of the beads (e.g., polyelectrolyte solutions with or without NaCl), all influence the sensitivity of the bead assay for trypsin. While P1-loaded beads with PSS as the inner and outer PE layers were found to be the most sensitive to trypsin (a decrease in red surface fluorescence of 89% was observed upon adding trypsin), other P1-loaded LbL coatings were obtained that were not at all sensitive for trypsin.

As outlined in the Introduction, another objective of our research is to investigate whether encoded microparticles carrying protease substrates at their surface can be used for simultaneous identification of different proteases in a biological sample. This would imply the simultaneous use of PEM beads loaded with different types of peptides (substrates). A peptide substrate may thus not desorb from the surface of a bead and may not bind to the surface of another bead. We showed that embedding the P1 molecules through the application of an outer PSS layer successfully prevented desorption and redistribution of P1.

An important lesson from this study is that a universal LbL layer in which all kinds of peptide/protein substrates, independent of their physicochemical nature, can be (sufficiently) loaded in such a way that they remain degradable by

their corresponding enzyme, is highly challenging to construct. In other words, to proceed with multiplex protease profiling by LbL-coated encoded microparticles, for each substrate–enzyme pair, an LbL coating with an optimal composition should be designed, which would be a huge effort. However, one could think about the design of a universal tail, for example, a low-molecular-weight polyelectrolyte, that binds to the substrates and immobilises them into the LbL coating so that loading of the beads becomes less dependent on the physicochemical nature of the protein or peptide.

Another critical issue to realize protease profiling by multiplexing with encoded carriers is the 'substrate specificity' of the enzymes. Clearly, to detect protease *x* in a biological sample by multiplexing, we should make sure that the substrate loaded on the beads only becomes degraded by protease *x* and not by (an)other protease(s) in the solution. It currently appears that this would first need biologists to build comprehensive substrate catalogues of the proteases of interest as such information is not yet readily available. Here, a further challenge is the fact that, instead of just identifying potential protease substrates, the exact cleavage sites need to be characterized. However, only rarely have such systemic substrate cataloguing approaches been published (see an example in the literature[7]), but the advent of novel technologies[34] could make such data available at a faster paste.

Finally, we showed that the number of beads used in the assay influences the sensitivity of the assay: using fewer beads detects a lower amount of trypsin. This is an interesting observation that would allow the use of low volumes of biological samples when one wants to use such microcarriers for assaying in microchips.

4. Experimental

LbL Coating of the Microparticles: The LbL coating of the green fluorescent (carboxyl-functionalized) polystyrene microparticles (Spherotech, Libertyville, IL, USA, diameter = 39 μm) occurred as follows according to the literature [10]: PAH (70 kDa, Sigma–Aldrich) and PSS (70 kDa, Sigma–Aldrich) stock solutions were prepared in 0.5 M NaCl (2 mg mL^{-1}). As illustrated in Figure 1 (step I), the microspheres were LbL-coated by suspending approximately 400 000 microspheres in 1 mL of PAH solution; the suspension was continuously vortexed (1000 rpm, 25 °C) for 15 min. Non-adsorbed PAH was removed by repeated centrifugation (4000 rpm; 30 s) and washing (3 times in 1 mL deionized water containing 0.05% Tween). Subsequently, the microspheres were dispersed in deionized water containing $CrO_{2\ NP}$ nanoparticles less than 500 nm in size. This dispersion was continuously shaken for 15 min, and the excess of $CrO_{2\ NP}$ was removed by repeated centrifugation/washing steps. Subsequent polyelectrolyte layers were applied in a similar way as the first one. The LbL-coated microspheres (called PEM beads) thus obtained were resuspended in 1 mL of Hepes buffer (100 mM, pH 7.4). Unless otherwise specified, the LbL coating surrounding the beads was as follows (starting from the surface of the microparticles): PAH/<500 nm $CrO_{2\,NP}$/PAH/PSS/PAH/PSS.

Loading LbL-Coated Microparticles with Red Fluorescent Peptides: A 15 amino acid peptide (P1; NH_2-GRKKRRQRRRPPQC-COOH; pI ~ 12.3) was synthesized on an Applied Biosystems 433A peptide synthesizer (Framingham, MA, USA) using Fmoc chemistry [35]. Carboxytetramethylrhodamine (CTMR) was attached to the peptide at its amino terminus and used as fluorescent marker (λ_{ex}: 543 nm, λ_{em}: 578 nm). As described below, P1 was immobilized on the PEMs in two different ways.

In the first way, the positively charged peptide P1 was adsorbed to the PSS layer of the PEM beads (step III in Fig. 1). Thus, the coating surrounding the microparticles was as follows (starting from the surface of the microparticles): PAH/<500 nm $CrO_{2\ NP}$/PAH/PSS/PAH/**PSS**/**P1**. These P1-loaded beads are termed "non-embedded P1 beads", as P1 is 'freely' present at the surface of the PEM beads. Non-embedded P1 beads were obtained by incubating 10^4 LbL-coated beads in 50 μL P1 solution (the P1 concentration varied between 0 and 10 nmol mL^{-1}; solvent was 100 mM Hepes at pH 7.4) for 15 min on a rocker platform (250 rpm). Excess of peptide was removed by repeated centrifugation/washing steps.

In the second way, an extra PSS layer was applied to the non-embedded P1 beads described above (see step IV in Fig. 1). The coating surrounding the microparticles was thus as follows (starting from the surface of the microparticles): PAH/$CrO_{2\,NP}$/PAH/PSS/PAH/**PSS/P1/PSS**. These beads were termed "embedded P1 beads". The PSS solution used for assembling the extra PSS layer ranged between 0–2 mg mL^{-1} (solvent 0.5 M NaCl).

Fluorescence Microscopy on Beads: The (green) LbL-coated beads containing the CTMR (red)-labeled peptides were imaged with a Nikon EZC1 confocal microscope (PL APO 10 × 0.45 objective) using the 488 nm laser line (for imaging the green fluorescence) and the 561 nm laser line (for imaging the red fluorescence). The lasers were used at, respectively, 3% and 30% of their maximal power in a sequential line-scanning mode to avoid crosstalk. The beads were visualized on the 'green image' and subsequently the contours of the beads were identified with home-built software. These contours were plotted on the 'red image'. The fluorescence intensity mean (FIM) values were determined by quantifying the red fluorescence inside the contours of at least 20 beads. The FIM data are given as mean ± standard deviation (SD).

Measuring the Cleavage of P1 by Trypsin: The cleavage of P1 immobilized at the surface of the microparticles was evaluated by incubating the P1-loaded beads in solutions of trypsin (Promochem) and anhydrotrypsin (ANHT, Innovative Research, Southfield, ■ **state?** ■ USA), respectively. Trypsin preferably catalyzes the hydrolysis of peptide bound at the carboxyl-terminal side of lysine or arginine. Anhydrotrypsin (ANHT) is a chemically modified variant of trypsin in which the active site serine residue has been chemically converted to dehydroalanine. In our study, ANHT is used as inactive trypsin. The activity was verified spectrophotometrically (see the Supporting Information).

The cleavage of P1 on the beads by trypsin and ANHT was measured as follows. Approximately 1000 P1-loaded beads were incubated for 2 h in 50 μL of a trypsin or an ANHT solution (0.4 μg mL^{-1} in 50 mM TrisHCl (pH 7.4; 1 mM $CaCl_2$)) on a rocker platform (250 rpm, at 37 °C, in the dark). The cleaved fluorescent peptide fragments were removed by repeated centrifugation and washing. Subsequently, the microspheres were dispersed in 50 μL of 100 mM Hepes buffer (pH 7.4), and the red surface fluorescence was measured as described above.

When cleavage of P1 was followed as a function of time, 2000 P1-loaded beads were incubated in 50 μL of trypsin or ANHT solution. After different incubation times, 10 μL of the suspension was removed from the assay and added to 200 μL of ice-cold deionized water containing 0.05% Tween. The supernatant (containing the enzyme and cleaved fluorescent peptide fragments) was immediately removed by centrifugation and washing. Subsequently the microspheres were dispersed in 25 μL of 100 mM Hepes buffer (pH 7.4) and their red surface fluorescence was measured.

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