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Specific Detection of Gastric α-Antitrypsin by Immobilized Trypsin on PolyHEMA Films

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Abstract: Early diagnosis of gastric carcinoma is crucial for maximizing medical treatment efficacy. For the purpose of real time diagnosis ("virtual biopsy") of stomach malignancy we developed a polyHEMA platform capable of capturing human α1-antitrypsin precursor (A1AT); a model proteinaceous luminal biomarker. Its specific attachment to the polymeric platform was accomplished by immobilized trypsin, which was linked to the surface of the polyHEMA film by a series of PEG-based spacers. Recognition was enabled by adapting an ELISA-like methodology, using rabbit anti-A1AT and HRP-conjugated anti-rabbit IgG as a secondary antibody. Since this A1AT-sensing platform was designed to be detected by endoscopic means such as a video capsule, its physical stability was tested after casting on top of a polycarbonate surface. It was found that, in contrast to classical ELISA analysis performed on polystyrene plates, A1AT detection was possible only when spacer arms were used to immobilize the capturing moiety, trypsin, with a 7-fold increase in the optical signal and a saturation kinetics dependency upon the concentration of the A1AT biomarker.

Keywords: Biomarker detection, human α1-antitrypsin precursor; polyHEMA; immobilized trypsin; spacers; real time detection; ELISA

Introduction

Because early stage gastric cancer symptoms are indistinguishable from benign peptic ulcer, serological tests have been suggested as complementary diagnostic tools to endoscopy. Serum biomarkers that have been tested include pepsinogen I/II ratio, anti-*Helicobacter pylori* antibodies and gastrin 17.[1−3] Inasmuch it has been suggested to probe gastric juice given the presence of typical biomarkers for gastric malignancies. One example of such a biomarker is carcinoembryonic antigen (CEA). Its level in gastric juice was shown to be significantly higher in patients with precancerous lesions or cancer, compared to patients with normal or benign changes of the gastric mucosa.[4−6] Another biomarker for the early detection of gastric cancer may be α1-antitrypsin precursor

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(A1AT), a potent serum protein of 52 kDa belonging to the serine protease inhibitor (serpin) family. Although believed to be highly specific to neutrophil elastase, A1AT is also a broad-spectrum protease inhibitor for other serine proteases, including trypsin.[7] It has been shown that gastric juice of patients with early and advanced gastric cancer contains high levels of A1AT.[8,9]

The long-run objective of this study is to design and examine a real time diagnostic kit that will detect A1AT, *in vivo*, in gastric fluids. Consequently, the first step toward this goal is the identification and validation of a capturing entity which, after its immobilization onto a polymeric vehicle, would be able to identify A1AT. An A1AT antibody or a specific proteinaceous substrate for A1AT, such as trypsin,[10−12] may serve as the capturing component.

How to monitor the detection reaction *in vivo*? Aspiration of gastric juice is one option with the risk of pulmonary edema.[13] Probing gastric fluids for biomarkers with an endogastric capsule is another alternative that has been demonstrated for the purpose of CEA or nucleotide (e.g., E-cadherin gene) detection.[14,15] A recent development is an orally administered video pill for visual detection of GI disorders.[16] An example for such a device is the Pillcam capsule, equipped with a miniature video camera, capable of transmitting high resolution images of the epithelium of the esophagus and small intestine.[17,18] If equipped with an appropriate detection kit, the camera could be exploited for probing biomarkers *in vivo*. A detection kit could comprise a miniature sandwich ELISA-like system, in which an immobilized capturing agent would specifically bind to A1AT. At a later stage, a specific interaction with a fluorescently tagged polyclonal anti-human A1AT could result in an optical signal which would be detected by the camera inside the capsule if adjusted to the right optical band. As an appropriate polymeric platform for anchoring trypsin (the A1AT capturing moiety), 2-poly hydroxyethyl methacrylate (polyHEMA) would be an attractive choice due to its transparency, flexibility and biocompatibility.[19]

Specifically, the goals of the present study were to (a) prepare polyHEMA films and anchor trypsin to their surface with a variety of spacer arms, (b) test the capturing capability of the anchored trypsin, first toward model proteins and subsequently toward A1AT, (c) test whether the captured A1AT is recognized by a tagged anti-A1AT polyclonal antibody in a manner that could serve for future design of an A1AT detection kit and (d) test, *in vitro*, the performance of the system.

Experimental Section

Materials. *Proteins.* Trypsin from bovine pancreas (≥10,000 BAEE units/mg protein) and α1-antitrypsin (A1AT) were purchased from Sigma, St. Louis, MO. Alexa-Fluor 488 labeled ovalbumin (OVA) was purchased from Molecular Probes, Eugene, OR. Albumin bovine fraction V (BSA) was purchased from MP Biomedicals, Solon, OH.

Antibodies. Alexa Fluor 488 labeled goat anti-rabbit IgG (H+L) was purchased from Molecular Probes, Eugene, OR.

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Rabbit anti-OVA polyclonal IgG and anti-rabbit horseradish peroxidase (HRP)-conjugated IgG were purchased from Sigma, St. Louis, MO. Rabbit polyclonal anti-A1AT IgG and rabbit anti-rotavirus IgG were obtained from Novamed, Jerusalem, Israel.

Spacers. Linear polyethyleneimine (LPEI) and polyethyleneimine "MAX" (LPEI-Max, 40 kDa) were purchased from Polyscience, Warrington, PA. *O*,*O*′-Bis[2-(*N*-succinimidylsuccinylamino)ethyl]polyethylene glycol 3,000 (NHS-^{3K}PEG-NHS) and *O*-[(*N*-succinimidyl)succinyl-aminoethyl]-*O*′-methylpolyethylene glycol 2,000 (NHS-^{2K}PEG) were purchased from Sigma, St. Louis, MO.

2-Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), hexamethylenediamine, glutaraldehyde (GA) grade I, benzoyl peroxide and 3,3′,5,5′-retramethylbenzidine (TMB) reagent were all purchased from Sigma, St. Louis, MO. Alexa Fluor 647 labeled hydrazine was purchased from Molecular Probes, Eugene, OR. Tween 20 was purchased from Sigma, St. Louis, MO.

All solvents were analytical grade. Water was purified by reverse osmosis.

Methods

Preliminary A1AT Sandwich ELISA Analysis. An ELISA 96-well polystyrene plate was coated with 10 μg/mL of rabbit polyclonal anti-A1AT IgG (PBS, pH 7.4, 37 °C, 45 min) or with 10 μg/mL of trypsin (PBS pH 5, 37 °C, 45 min). After a PBS rinse, the plate was washed two times with PBS containing 0.05% w/v Tween-20. Nonspecific binding was blocked with 10% w/v BSA in PBS (37 °C, 45 min). After the addition of increasing concentrations (0−10 μg/mL) of A1AT (50 μL/well) the plate was incubated (37 °C, 45 min) and subsequently rinsed with PBS. The detecting antibody, rabbit polyclonal anti-human A1AT (30 μg/mL in blocking solution), was added to each well, followed by a similar incubation and PBS rinse. Similar concentration of polyclonal rabbit anti-rotavirus IgG served as a nonspecific control. In both cases the secondary antibody was goat anti-rabbit-HRP conjugated IgG (1:5000 in PBS). After rinsing, 100 μL of TMB reagent was added to each well. The color reaction was stopped with 1 M H_2SO_4. Color intensity was measured in a microplate reader (Synergy HT Multi Mode, Bio-Tek, Winooski, VT) at 450 nm.

HEMA Polymerization and Film Preparation. HEMA (10 mM) was polymerized in the presence of 2.5, 5, or 7.5 mol % of EGDMA (single step polymerization and cross-linking) in 50 mL of acetonitrile, by shaking at 80 °C for 24 h, under dry nitrogen, using 0.5 mol % of benzoyl peroxide as an initiator.[19] The polymerized HEMA, cross-linked with EGDMA (polyHEMA) solution, was then cooled down to room temperature. Acetonitrile was evaporated, and the monomer residues were extracted and removed with diethyl ether. The obtained soft mass was dissolved in absolute methanol and cast onto 55 mm (internal diameter) flat Teflon molds, or (after optimization) over the polycarbonate surface of Pillcam capsules (Given Imaging, Yokneam, Israel). The molds were dried at room temperature (24 h) to obtain transparent films, 0.2−0.5 mm thick (measured by Mitutoyo micrometer, Aurora, IL), depending on the concentration of EGDMA used. The higher the concentration of EGDMA, the thinner the film obtained. A decision on the optimal EGDMA concentration was taken after comparing the physical properties of the three types of films.

Table 1. The Effect of EGDMA on the Modulus of Elasticity and Adhesiveness to Polycarbonate Surfaces of PolyHEMA Films[a]

EGDMA (mol %)	modulus of elasticity (N s^{-1})	adhesion force onto polycarbonate (gF)	
		at dry state	at hydrated state
2.5	0.02 ± 0.004	0.7 ± 0.03	90 ± 20
5.0	0.29 ± 0.035	0.3 ± 0.09	46 ± 7
7.5	0.22 ± 0.02	0.2 ± 0.08	16 ± 4

[a] Shown are the mean values of 4 measurements ± SD.

Table 2. Physical Stability (Determined Gravimetrically) of the PolyHEMA Films on Polycarbonate Surface after Incubation in SGF (1 or 24 h) and in SIF (24 h)[a]

	% wt loss		
	SGF		
EGDMA (mol %)	1 h	24 h	SIF, 24 h
2.5	19 ± 6.5	21.2 ± 6.2	23 ± 1.4
5	3.1 ± 0.9	9.6 ± 0.7	9 ± 3.6
7.5	3.4 ± 1.2	12.1 ± 3.0	9.5 ± 2.5

[a] Shown are the mean values of 4 measurements ± SD.

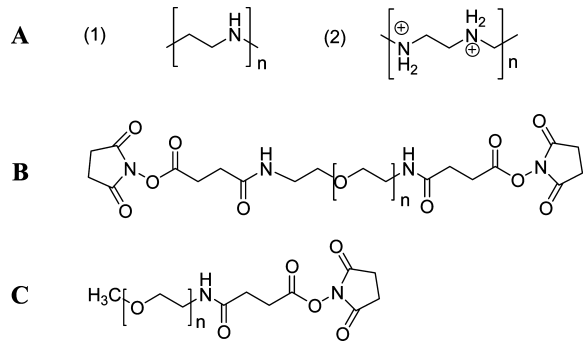
Physical Characterization of the PolyHEMA Films. Swelling properties were measured in simulated gastric fluid USP, pH 1.5, without pepsin (SGF) and in 35 mM NaCl aqueous solutions of either pH 5.5 or pH 7.5 by separately immersing preweighed dry films for 1, 4, or 24 h under gentle shaking at 25 °C. The weight gain of the hydrated (gently blotted dry) films was measured and expressed as the fraction (%) increase from the initial dry weight.

Tensile strength and adhesion to polycarbonate surface were determined by a TA.XT Plus texture analyzer (Stable Micro Systems, Surrey, U.K.). PolyHEMA films (2 × 2 cm, $n = 4$), either in dry or in hydrated (SGF, 2 h) states, were stretched at a speed of 0.5 mm/s and a trigger force of 2 gF. Force vs time plots were drawn, and the modulus of elasticity was calculated by the apparatus data acquisition program. The same apparatus was used to assess the detachment force required to separate the films from the surface of the polycarbonate capsule (4 repeated tests for each film specimen). In this set of studies a 5 kg load cell in its compression mode was used; the compression speed was 1 mm/s, and the trigger force was 5 gF (Table 1).

Physical stability (integrity) of polyHEMA film coats on the polycarbonate capsules was measured visually and gravimetrically after incubation (37 °C) in SGF for 1 h or 24 h, or incubation (37 °C) in simulated intestinal fluid USP at pH 6.8 without pancreatin (SIF) for 24 h (Table 2).

Based on the above characterization studies, the polyHEMA polymer cross-linked with 5 mol % of EGDMA was selected to serve as the immobilization platform.

Scheme 1. The spacers: (A) LPEI (1) and LPEI-Max (2), (B) *O*,*O*′-bis[2-(*N*-succinimidyl-succinylamino)ethyl]polyethylene glycol 3,000 (NHS-^{3K}PEG-NHS, (C) *O*-[(*N*-succinimidyl)succinyl-aminoethyl]-*O*′-methylpolyethylene glycol 2,000 (NHS-^{2K}PEG)



Spacer Selection for Immobilizing Trypsin to the PolyHEMA Films. In the course of the study it became apparent that the use of a spacer arm to immobilize the capture moiety (trypsin) was inevitable. The subsequent set of studies was geared at employing trypsin[11,12] for capturing A1AT. The following spacers were tested for trypsin immobilization to the polyHEMA film: (a) linear polyethyleneimine (LPEI)[20] of increasing molecular weights (2.5, 25, and 250 kDa), (b) polyethyleneimine "MAX" hydrochloride salt (LPEI-Max; 40 kDa), (c) PEG-based spacer: the homobifunctional *O*,*O*′-bis[2-(*N*-succinimidyl-succinylamino)ethyl]polyethylene glycol 3,000 (NHS-^{3K}PEG-NHS), or a mixture of NHS-^{3K}PEG-NHS with the monofunctional *O*-[(*N*-succinimidyl)succinyl-aminoethyl]-*O*′-methylpolyethylene glycol 2,000 (NHS-^{2K}PEG) (Scheme 1).

The LPEI Spacers. The LPEI spacers (both LPEI and LPEI-Max) were attached to the polyHEMA films by separately immersing (gentle shaking, 55 °C, 16 h) the dry films with increasing concentrations (3.5, 7, or 14 mg/mL) of the various spacers, followed by a water rinse to remove unbound spacers.[20,21]

Activation of the Grafted LPEI Spacers. The terminal amine groups of the grafted LPEI or the LPEI-Max spacers were activated by incubating (room temperature, 45 min) the modified films in 1% w/v glutaraldehyde (GA) in water with a subsequent water rinse. Activation was verified by incubating (37 °C, 2 h) the films (4 mg) with 100 μL of 5 μg/mL of Alexa Fluor 647 labeled hydrazine, followed by a water rinse. The reaction with the activated spacers was verified by measuring fluorescence intensity (excitation, 648 nm; emission, 668 nm) in a microplate reader, using GA-treated polyHEMA (no spacer grafts) as controls.

Protein Binding Capacity of the Grafted Films. The capability of the activated polyHEMA-LPEI films to interact with a proteinaceous probe was initially tested with a fluorescently labeled goat anti-rabbit polyclonal (H+L) IgG.

The activated films (4 mg specimens) were incubated (4 °C, overnight) with 50 μL of 50 μg/mL of the antibody, in PBS pH 7.4. Unbound antibody was removed by washing with PBS containing 0.1% w/v Tween-20, and the presence of bound fluorescent IgG was verified spectrofluorimetrically (excitation, 485 nm; emission, 525 nm) in the microplate reader, using GA-treated polyHEMA (no spacer grafts) as controls.

To delineate the capacity of a recognizable protein to identify a target protein after immobilization, polyclonal rabbit anti-OVA was conjugated to the activated polyHEMA-LPEI film by its incubation (PBS pH 7.4, 4 °C, overnight) with 15 μg/mL of the antibody, followed by a Tween-20 (0.1% w/v in PBS) rinse to remove unbound antibody followed by an incubation in 1% w/v dry milk in PBS (room temperature, 2 h) to block nonspecific protein binding. The films were then incubated (PBS, pH 7.4, 45 min) with 2 μg/mL of Alexa 488 labeled OVA. Unbound antigen was removed by a Tween-20 (0.1% w/v in PBS) rinse.

Binding capacity of the polyHEMA film grafted, separately, with all four LPEI spacers with increasing (1, 3.5, 7, and 14 mg/mL) spacer densities, was compared. The study was conducted in a 96-well plate, and fluorescence (excitation, 485 nm; emission, 525 nm) was monitored in the microplate reader.

The PEG-Based Spacers. *Grafting.* Specimens (4 mg) of the dry polyHEMA films were immersed (gentle shaking, 3 h, 25 °C) with increasing concentrations (10−100 mg/mL) of NHS-^{3K}PEG-NHS, or a mixture of NHS-^{3K}PEG-NHS with NHS-^{2K}PEG in PBS (pH 7.4), followed by a water rinse to remove unbound linkers.

Protein Binding Capacity of the Modified Films. The ability of the PEG-based spacer-containing polyHEMA films to interact with a proteinaceous probe was tested toward fluorescently labeled goat anti-rabbit polyclonal (H+L) IgG as described above. In separate studies, films containing increasing concentrations (1, 10, 20, or 100 mg/mL) of NHS-^{3K}PEG-NHS, or films containing mixtures of NHS-^{3K}PEG-NHS and NHS-^{2K}PEG spacers (1 + 10 or 10 + 100 mg/mL, respectively), were incubated (25 °C, 2 h) with 50 μg/mL of Alexa Fluor 488 labeled goat anti-rabbit polyclonal (H+L) IgG, in PBS pH 7.4. Unbound antibody was removed by a Tween-20 (0.1% w/v in PBS) rinse. The presence of bound fluorescent IgG was verified spectrofluorimetrically (excitation, 485 nm; emission, 525 nm) in the microplate reader, using an NHS-^{2K}PEG-coated film as a control.

Trypsin Immobilization to the PolyHEMA Film. Trypsin was reacted with dry polyHEMA films pregrafted with either (a) 7 mg/mL of LPEI (25 kDa), (b) 100 mg/mL of NHS-^{3K}PEG-NHS, or (c) a mixture of NHS-^{3K}PEG-NHS and NHS-^{2K}PEG (10 + 100 mg/mL). The films were incubated (PBS pH 5, 4 °C, overnight) with 100 μg/mL trypsin followed by a Tween-20 (0.1% w/v in PBS) rinse to remove unbound trypsin. Nonspecific binding was blocked with BSA (1% w/v in PBS).

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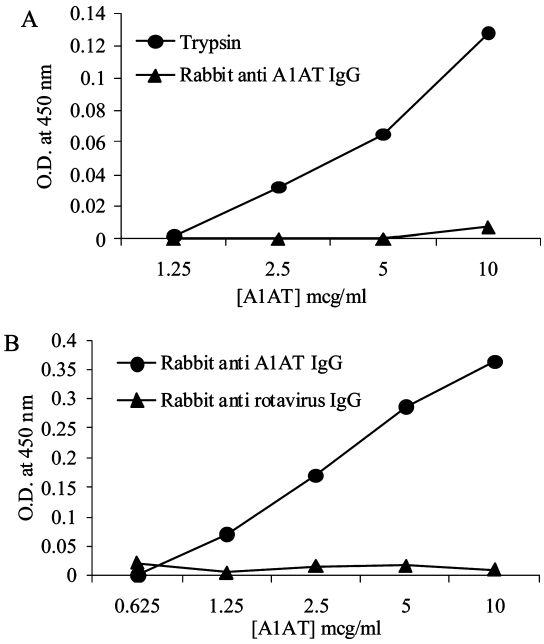


Figure 1. (A) Capturing of A1AT at increasing concentrations (1.25−10 μg/mL) by rabbit polyclonal anti-A1AT IgG (triangles) and trypsin (circles) coats on polystyrene surfaces (96-well ELISA plate). (B) Specificity assessment of the trypsin-captured A1AT coat, as analyzed by rabbit anti-A1AT IgG (circles) and polyclonal rabbit anti-rotavirus IgG (triangles, nonspecific control). HRP conjugated goat anti-rabbit IgG was used as the secondary antibody in both cases, followed by the TMB method.

A1AT Detection by the Immobilized Trypsin on the PolyHEMA Films. The study was performed in a 96-well microplate containing 100 μl/well of polyHEMA-LPEI (25 kDa), or polyHEMA-NHS-^{3K}PEG-NHS, or a mixture of polyHEMA-NHS-^{3K}PEG-NHS with NHS-^{2K}PEG films. Nonspecific binding was blocked with BSA. After the addition of increasing concentrations (0−40 μg/mL) of A1AT (50 μL/well) the plate was incubated (60 min, 37 °C) and subsequently rinsed 3 times with PBS. The first antibody, rabbit polyclonal anti-human A1AT (30 μg/mL), was added to each well, followed by a similar incubation and PBS rinse. The HRP conjugated second antibody (1:5000 in PBS) was then added to the wells and incubated (45 min, 37 °C), after which the plate was rinsed and reacted with a TMB reagent (in citrate buffer, pH 5 to a final volume of 100 μL/well). The reaction was stopped with 1 M H_2SO_4 (100 μL/well). Color intensity was measured in a microplate reader at 450 nm.

Results

Using sandwich ELISA, we first compared the capacity of trypsin to that of rabbit polyclonal anti-A1AT IgG, to capture A1AT in a 96-well polystyrene plate. Figure 1 shows that in the concentrations range used, the trypsin layer was able to capture A1AT as opposed to the polyclonal antibody layer, highlighting the higher specificity of the former toward its serpin.

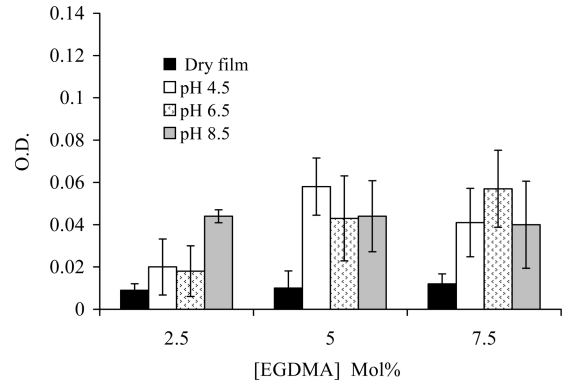


Figure 2. The effect of cross-linking density (expressed in mol % of EGDMA used for polyHEMA cross-linking) on the polyHEMA film transparency (expressed in optical density) as measured at 600 nm in both dry and hydrated states, at 3 different pH values. Shown are the mean values of 3 experiments ± SD.

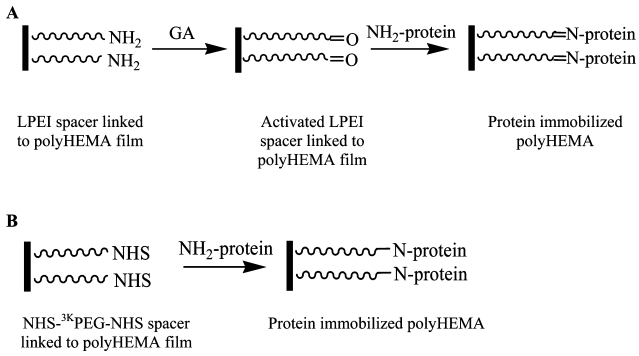
Once we verified that trypsin bears improved capturing properties toward A1AT, we focused our efforts to fabricate a polymeric platform into which trypsin could be immobilized while maintaining its capturing capabilities toward A1AT.

A series of polyHEMA films were prepared, differing from each other by the amount of cross-linker (EGDMA) used (2.5, 5, and 7.5 mol %). The swelling properties of all films were similar (33 ± 2% within 1 h). However, using 7.5 mol % EGDMA resulted in films with reduced adhesiveness to the polycarbonate surface by 3.5- and 5.6-fold in dry and hydrated states, respectively (Table 1). The stability of films to physical erosion upon long incubation periods in simulated GI fluids was superior when prepared with 5 and 7.5 mol % of EGDMA (Table 2). Thus it was decided to employ the more stable film with the lesser amount of cross-linker (5 mol %). Figure 2, which summarizes the effect of EGDMA on the transparency of the polyHEMA films in dry and hydrated states at three pH values, demonstrates that cross-linking had negligible effect on light transmittance through the films. Although wetting caused a 3-fold increase (pH-independent in nature) in the optical density of the films, the overall OD values did not exceed 0.06.

Spacer arms were then grafted on the surface of the selected polyHEMA film in order to immobilize trypsin in a controllable manner. The various spacers tested are shown in Scheme 1. The first set of studies made use of LPEI spacers which required activation by GA for the subsequent conjugation with the protein (whether antibody or trypsin) through the formation of an imine bond between the aldehyde (on the spacer) and the protein's primary amine (Scheme 2).

Activation of the spacer's end was verified by reacting the modified film with Alexa Fluor 647 labeled hydrazine. Figure 3 shows that while the reaction between the fluorescent hydrazine and the polyHEMA was negligible without

Scheme 2. The Methods Used To Attach Proteins (Whether Antibody or Trypsin) to Grafted LPEI (A) or NHS-^{3K}PEG-NHS (B) to the PolyHEMA Backbone



a spacer (similarly treated with GA), the presence of activated LPEI on the polyHEMA film profoundly increased the amount of fluorescent hydrazine on the film's surface in a spacer-density dependent manner (5.5- and 8-fold binding to the films grafted with 3.5 and 7 mg/mL of LPEI, respectively, compared with the film without a spacer).

After confirming the necessity for a spacer in order to improve the overall capturing capacity on the polymer surface, a comparison study was conducted with different types of spacers, using Alexa Fluor 488 labeled goat anti-rabbit polyclonal IgG as a proteinaceous target molecule. In one study LPEI spacers with increasing molecular weights (2.5, 25, and 250 kDa) were compared to LPEI-Max (M_W of 40 kDa). In a second study, increasing concentrations of NHS-^{3K}PEG-NHS, NHS-^{2K}PEG and a mixture of the two were tested. The findings are summarized in Figure 4 and indicate that, in general, LPEI was more effective than the NHS-^{3K}PEG-NHS or its mixture with NHS-^{2K}PEG in binding the goat anti-rabbit polyclonal IgG to the polyHEMA films. Figure 4A demonstrates that, out of all LPEI activated spacers, the 25 kDa was superior in its ability to bind the goat anti-rabbit polyclonal IgG (13-, 11- or 5-fold in comparison to spacer-free films for 25 kDa, 2.5 kDa and

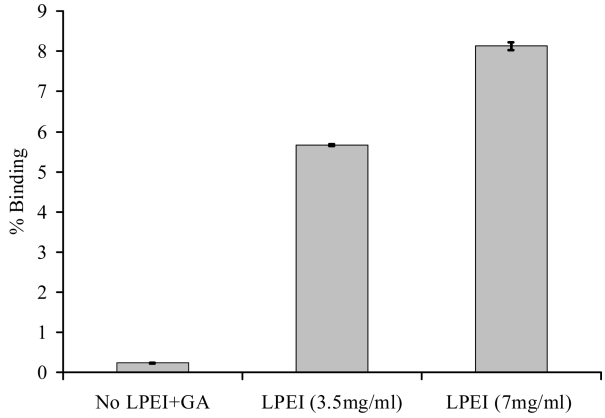


Figure 3. Binding of Alexa Fluor 647 labeled hydrazine to polyHEMA films grafted with three concentrations (0, 3.5, and 7 mg/mL) of activated (GA) LPEI spacers. Binding is expressed as the fraction (in %) bound from the initial amount of the antibody.

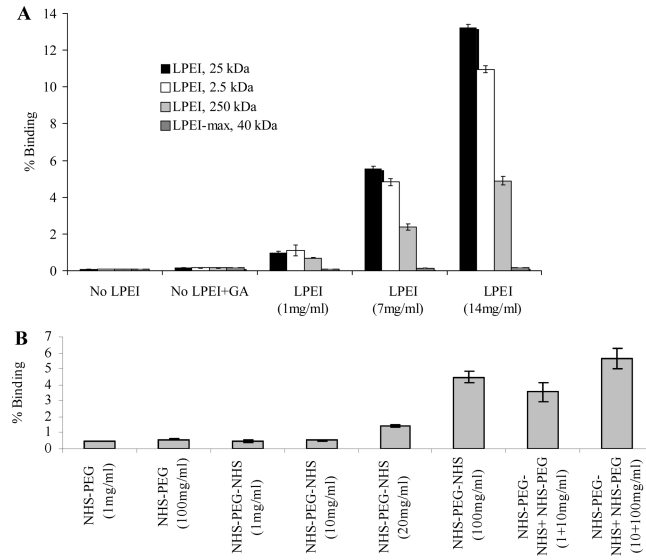


Figure 4. Binding of Alexa Fluor 488 labeled goat anti-rabbit polyclonal IgG to polyHEMA films grafted with: (A) LPEI spacers of increasing molecular weights (2.5 kDa, white columns; 25 kDa, black columns; and 250 kDa, light gray columns) and LPEI-Max, 40 kDa, dark gray columns. The background fluorescence is an intrinsic fluorescence of the polymer normalized to background (polymer) fluorescence. Note the negligible antibody adsorption to the polyHEMA films without spacers. (B) Two concentrations of NHS-^{2K}PEG, four concentrations of NHS-^{3K}PEG-NHS and two concentrations of a mixture of the two PEG spacers. Binding is expressed as the fraction (in %) bound from the initial amount of the antibody.

250 kDa, respectively). Negligible amounts of the goat anti-rabbit polyclonal IgG were adsorbed, nonspecifically, to naked (no spacers) polyHEMA films with or without pretreatment with GA. Negligible also were the amounts of goat anti-rabbit polyclonal IgG bound to those polymer films grafted with the LPEI-Max spacer.

To assess how surface density of the immobilized capturing protein affects the overall capability of grafted polyHEMA film to identify a biomarker, polyclonal rabbit anti-OVA IgG was added to polyHEMA films pregrafted with increasing concentrations (elevated surface density) of the LPEI 25 kDa spacer. The ability of the polyclonal rabbit anti-OVA IgG-bound polymer to identify, in turn, a model antigen, Alexa 488 labeled OVA, is shown in Figure 5. The data illustrates surface density dependence (saturation kinetics, typical to the limited recognition sites of the monolayer coat) of the grafted antibody on the overall recognition capacity of the polymer toward the Alexa 488 labeled antigen.

Nonspecific binding of Alexa 488 labeled OVA to the nongrafted (no LPEI spacer) polyHEMA films preincubated with polyclonal rabbit anti-OVA IgG was also measured and was found to be negligible (data not shown).

Lastly, in a set of recognition studies, an *in vitro* competence ELISA analysis was performed to examine the

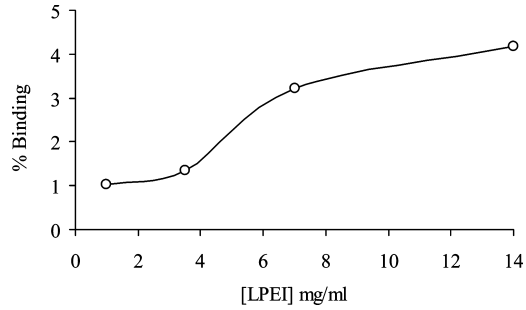


Figure 5. Capturing Alexa 488 labeled OVA by immobilized (via LPEI, 25 kDa of increasing surface densities) polyclonal rabbit anti-OVA IgG on polyHEMA films. Shown are the mean values of 2 experiments.

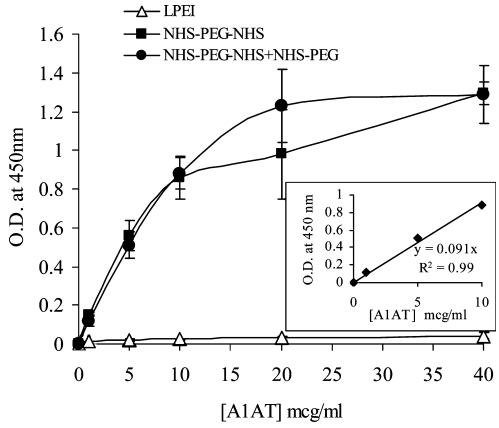


Figure 6. A1AT capturing (expressed in OD arbitrary units) by trypsin (100 μg/mL), immobilized by 7 mg/mL of LPEI 25 kDa (open triangles), or 100 mg/mL NHS-^{3K}PEG-NHS (filled squares), or a mixture of 10 mg/mL of NHS-^{3K}PEG-NHS + 100 mg/mL of NHS-^{2K}PEG (filled circles) to the surface of the polyHEMA film, as analyzed by ELISA. The recognition antibody was rabbit anti-A1AT. The secondary antibody was HRP-conjugated anti-rabbit IgG. Inset: The linear section of the A1AT recognition in the range of 1 to 10 μg/mL. Shown are the mean values of 3 independent experiments ± SD.

ability of a polyHEMA immobilized-trypsin to capture A1AT. In these studies, trypsin was attached to the surface of polyHEMA films with different spacers chosen according to the data obtained for the model systems examined thus far. Thus, LPEI, NHS-^{3K}PEG-NHS and a mixture of NHS-^{3K}PEG-NHS with NHS-^{2K}PEG were tested for their effect on the capturing capacity of grafted trypsin. ELISA analysis of the captured A1AT was conducted using rabbit anti-A1AT (the detecting antibody), followed by quantification with HRP-conjugated anti-rabbit IgG (the secondary antibody). The results, summarized in Figure 6, demonstrate that using NHS-^{3K}PEG-NHS as a linker of trypsin to the polyHEMA surface leads to a significantly improved capturing capacity of A1AT in comparison to LPEI. Overall, the use of a spacer for trypsin immobilization was crucial for A1AT capturing. The inhibitor was hardly recognized when trypsin was adsorbed, nonspecifically, directly to the surface of the polyHEMA film (data not shown).

Discussion

The present *in vitro* study, the first in a series of experiments aimed at exploring the likelihood to detect, in real time, typical gastric cancer biomarkers for diagnostic purpose, examined the capability of immobilized trypsin to capture A1AT. The design of the detecting polymeric platform presented in this study can outfit a remote device (e.g., an ingestible video pill) in a tailor-made manner. PolyHEMA, which was selected for this purpose, is commonly used in the drug delivery and contact lenses industries.[19,22] Trypsin was selected as the capturing moiety due to (1) its reported high specificity toward A1AT,[7,10] (2) failure to identify A1AT by rabbit polyclonal anti-A1AT IgG immune sandwich reaction and (3) verification that immobilized trypsin could identify A1AT in a specific manner (Figure 1). Since the *in vivo* diagnosis is planned to be conducted on top of an optic device, a series of studies were performed in order to identify an optimal film with satisfactory mechanical as well as biorecognition properties. Control over the mechanical properties of polyHEMA was accomplished by identifying the right amount of EGDMA used to cross-link the polymer during its synthesis. After attaining a stable film with satisfactory transparency (Figure 2, Tables 1, 2), our efforts concentrated on functional attachment of trypsin to the film.

Commonly, proteins are immobilized onto solid surfaces by adsorption techniques or covalent binding, with or without the employment of spacer arms.[23] Direct adsorption is routinely used in conventional ELISA assays or in microfluidic chip devices.[24] Examples include the human secretory immunoglobulin, directly adsorbed onto polystyrene beads[25] and protein A, directly adsorbed onto a glass surface.[24] However, passive surface adsorption of proteins (e.g., antibodies), for immunoaffinity chromatography, lacks an orientated array and an even distribution.[26,27] For these

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reasons, as well as for attaining sufficient spatial flexibility and preserving the activity of the attached proteins, it was decided to employ a spacer arm for the immobilization of trypsin to the polyHEMA surfaces. Two types of spacer groups were compared in terms of specific protein binding capacity and concomitant reduction of nonspecific adsorption: the amine-bearing poly(ethyleneimine)[20,21] and the PEG-based spacers[28,29] bearing NHS single protein-binding motifs.

After grafting to the surface of the polyHEMA films, LPEI spacers were activated with GA. Functionality of the spacer's active groups was tested with Alexa Fluor 647 labeled hydrazine and was found to be surface density-dependent (Figure 3), allowing continuation of the embedding process with the LPEI spacers up to 7 mg/mL. The broad activity of the activated, embedded, LPEI spacers toward proteins was then tested with Alexa Fluor 488 labeled goat anti-rabbit polyclonal IgG. The other types of spacers used in this study, the PEG spacers, were tested similarly without activation. Here, bifunctional PEG-based spacers or their mixtures with monofunctional PEG-based spacers were tested for their activity toward the fluorescently tagged antibody. Figure 4 shows that protein binding was highest with immobilized 25 kDa LPEI and immobilized NHS-^{3K}PEG-NHS in a surface density-dependent manner. The immobilized LPEI-Max, in its HCl salt form, did not show any specific binding capability (Figure 4A) most likely due to the absence of an active nucleophile to interact with the polyHEMA and thus was excluded from the screening test. As seen in Figure 4B, increasing the surface density of the monofunctional NHS-^{2K}PEG spacer did not affect the binding of the Alexa Fluor 488 labeled goat anti-rabbit polyclonal IgG, indicating that a nonspecific adsorption was not involved in the process. However, binding of the model protein to the bifunctional NHS-^{3K}PEG-NHS spacer or to a mixture of the two spacers increased protein binding in a concentration (surface density) dependent manner. It is assumed that the addition of the monofunctional NHS-^{2K}PEG led to the formation of a brushlike structure with a hindrance effect,[30] which prevented a cross reaction of the bifunctional NHS-^{3K}PEG-NHS spacer with the surface of the anchoring film at both ends, leading to an improvement in the binding capacity of the brushed polymeric surface.

The idea of capturing one protein by another after immobilization of the latter to a polyHEMA film was also verified in this study using polyclonal rabbit anti-OVA IgG immobilized (via LPEI, 25 kDa spacer) to the polymer. The recognition reaction toward Alexa 488 labeled OVA was found to be protein density-dependent (Figure 5).

Finally, the entire diagnostic analysis of A1AT by immobilized trypsin was performed, using an ELISA-like analysis with rabbit anti-A1AT as the recognition antibody and a HRP-conjugated anti-rabbit IgG as the secondary antibody. The efficiency of the detection process was compared between two polymeric products employing several different spacers, LPEI (25 kDa), NHS-^{3K}PEG-NHS and a mixture of NHS-^{3K}PEG-NHS with NHS-^{2K}PEG (Figure 6). According to Figure 4 it would have been expected that films containing LPEI spacers would possess better recognition properties than films with PEG-based spacers. Thus it is apparent that the choice of a specific spacer must be optimized for a given capturing system in order to provide a real estimate on its diagnostic capacity.

It is interesting to compare the ELISA-like detection of A1AT by trypsin, as performed on the surface of a conventional polystyrene 96-well microplate (Figure 1), to that on the surface of polyHEMA film grafted with PEG-based spacers used to immobilize the trypsin (Figure 6). In both systems, similar amounts of recognition and secondary antibodies were employed, leading to a linear relationship between A1AT concentration and the optical signal at a concentration range of $1-10\ \mu$g/mL with a minimal detection concentration (above the background noise) of $1\ \mu$g/mL. This linearity indicates the "working" range of the biomarker concentrations (see inset of Figure 6) for further optimization. Importantly, the optical signal of the polyHEMA detecting system was 7-fold higher than that obtained when detection of A1AT was performed in the 96-well system without spacers (Figure 1).

The strong affinity between immobilized trypsin and its inhibitor, A1AT, can be used for detection purposes, enabling the latter to serve as a biomarker and the former as a capturing moiety. The recognition reaction requires the involvement of a spacer arm to increases the trypsin mobility, affinity and hence, sensing capabilities toward A1AT. If indeed A1AT is presented in detectable amounts in the lumen of the stomach at early stages of malignancy, the system presented here could be exploited for a real time early detection of gastric cancer, by endoscopic means or a video capsule. However, to translate the competence of the above immunopolymer into a viable diagnostic kit, additional engineering acts should be conducted. For example, the design should include a two-compartment construct in which one chamber, having a free access to gastric juice, will contain the polyHEMA film with the immobilized trypsin. The other compartment, adjacent to the sensing polymer, should include fluorescent (preferably in the near IR range, due to background interference) particulate carrier. The antibodies, aimed at recognition of the captured A1AT, would be conjugated to the surface of the particles, thus allowing revelation by the optical machinery of the video capsule. This compartment would also allow a controlled input rate of the recognition particles. Miniaturization of this

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construct will allow its inclusion inside the capsule, ascertaining that most of the captured A1AT molecules are identified and illuminated by the components of the recognition portion of the kit.

Since the immunorecognition reaction in our system initiated 10 min after incubation with the various test media, augmenting over a period of 60 min, it is reasonable to assume that this illumination kinetics fits with gastric emptying rates of a large, nondigestible object from a liquid-filled stomach.[31] For optimal performance, pH and proteolysis should also be taken into consideration. Thus, protease inhibitor(s) and buffering agents should be incorporated into the system. Functioning of the immobilized A1AT polymeric system was tested after its exposure to aqueous solutions with a pH range of 1−3 for up to 30 min (after which the pH was elevated to 6), and found to be unaffected (data not shown). Results from a set of studies performed in a biorelevant gastric buffer, as well as in aspirated gastric juice, are a subject of a succeeding study which will be reported in due course.

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Abbreviations Used

A1AT, human α1-antitrypsin precursor; EGDMA, ethylene glycol dimethacrylate; GA, glutaraldehyde; HEMA, 2-hydroxyethyl methacrylate; IgG, immunoglobulin G; LPEI, linear polyethyleneimine; LPEI-Max, linear polyethyleneimine "MAX"; NHS-PEG-NHS, *O*,*O*′-bis[2-(*N*-succinimidyl-succinylamino)ethyl]polyethylene glycol; PEG-NHS, *O*-[(*N*-succinimidyl)succinyl-aminoethyl]-*O*′-methylpolyethylene glycol; OVA, ovalbumin; polyHEMA, poly 2-hydroxyethyl methacrylate cross-linked with EGDMA; SGF, simulated gastric fluid USP without pepsin; SIF, simulated intestinal fluid USP without pancreatin.

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