An adsorption chromatography assay to probe bulk particle transport through hydrogels

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Abstract

Biopolymer-based hydrogels such as mucus and the basal lamina play a key role in biology where they control the exchange of material between different compartments. They also pose a barrier that needs to be overcome for successful drug delivery. Characterizing the permeability properties of such hydrogels is mandatory for the development of suitable drug delivery vectors and pharmaceutics. Here, we present an experimental method to measure bulk particle transport through hydrogels. We validate our assay by applying it to mucin hydrogels and show that the permeability properties of these mucin hydrogels can be modulated by the polymer density and pH, in agreement with previous results obtained from single particle tracking. The method we present here is easy to handle, inexpensive, and high-throughput compatible. It is also a suitable platform for the design and screening of drugs that aim at modifying the barrier properties of hydrogels. This system can also aid in the characterization and development of synthetic gels for a wide range of biomedical applications.

Keywords

adsorption; permeability; diffusion; in vitro models; mucosal delivery; hydrogels

Introduction

For successful drug delivery to the inner organs of the human body, certain barriers need to be overcome. A prominent example is given by the blood/brain barrier [1,2], a dense system of tight endothelial cell junctions supported by a basement membrane layer. A second example is given by transdermal drug applications by means of ointments or gels, where the skin layer and the underlying basal lamina need to be penetrated [3,4]. Finally, mucosal surfaces of the gastrointestinal, respiratory, and reproductive tracts are also important sites for delivering therapeutics, and thus nasal, oral, and vaginal drug delivery requires the pharmaceutics to penetrate the mucosa [5,6].

Both mucus and the basal lamina are polymer based hydrogels that establish significant diffusion barriers to nanoparticles [7-10]. Typically, drugs are loaded into such nanoscopic delivery vectors to achieve a tailored temporal release profile and spatial distribution pattern in the human body [11,12]. Yet, the rational design of suitable drug delivery vectors is

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hampered by the lack of simple methods that quantify the bulk particle transport through such hydrogel barriers.

Here we introduce a new experimental assay to quantify the bulk permeability of hydrogels. In contrast to traditional diffusion chamber setups, our method only requires relatively small sample volumes, and its multi-well format allows for simultaneous testing of different parameters and conditions. As one example, we characterize mucin hydrogels and quantify the influence of environmental parameters such as polymer density, pH and mucolytic chemicals on the gel permeability. The technique introduced here represents a potentially suitable platform for the design and screening of new drug delivery vectors, and the testing and development of novel gels and chemical modifiers for a wide range of biomedical applications.

Materials and Methods

Particles and chemicals

Fluorescent polystyrene particles (unlabeled or Biotin-labeled) were obtained from Invitrogen and from Spherotech. Coating of particles with Polyethyleneglycol (PEG) was performed using N-hydroxysuccinimide-$(PEG)_{12}$-Biotin (MW 941.09, Pierce) and methyl-$(PEG)_{12}$-N-hydroxysuccinimide (MW 685.71, Pierce) following the instructions provided by the manufacturer. The ratio of PEG-Biotin versus PEG in solution was 1 to 5 and a 5- to 20-fold molar excess of both reagents was used with respect to the molar concentration of amine groups. The size and the zeta-potential of the polystyrene particles were measured with dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Herenberg, Germany) and are compiled in Table 1. The buffer solutions used in these measurements were the same as those used for the transport experiments. Particle concentrations were determined with a hematocytometer. Dextran (MW: 2MDa), bovine serum albumin (BSA) and mucolytic chemicals (S-carboxymethyl-L-cysteine (CMC), N-acetyl-L-cysteine (NAC)) were obtained from Sigma-Aldrich and dissolved in deionized water.

Mucin preparation and hydrogel reconstitution

Porcine gastric mucin was purified from scrapings of fresh pig stomachs following a protocol outlined in Celli et al. [13], with the difference that the cesium chloride density gradient ultracentrifugation was omitted. Mass spectrometry of the purified material confirmed the presence of the major gel-forming mucins MUC5AC and MUC6. Lyophilized mucin polymers were hydrated in the presence of fluorescent polystyrene particles overnight at 4°C in 20 mM acetate buffer adjusted to pH 3, or 20 mM Hepes buffer adjusted to pH 7. NaCl was added to the hydration buffer to adjust the ionic strength to 20 mM. The same hydration protocol was used for the preparation of dextran gels. Mucolytic agents were added to the mucin gel directly before the translocation experiment was initiated. Rheological measurements were performed at 22 °C and at 37 °C and revealed comparable results. We determined the frequency dependent viscoelastic moduli $G'(f)$ and $G''(f)$ over a frequency range of two decades. Measurements were conducted on a commercial stress-controlled macrorheometer (AR-G2, TA instruments, New Castle, USA) with a 20 mm cone-plate geometry. Our reconstituted mucin gels exhibited pH-dependent viscoelastic properties as described by Celli et al [13] which served as a quality control for our mucin purification procedure; a sol-gel transition is observed at low pH. Dextran samples showed solution-like behavior and identical viscoelastic properties at both pH values.

Permeability Assay

All permeability experiments were performed in 24-well multiplex microarray reaction cassettes with streptavidin substrate glass slides (1 slide per cassette, see Fig. 1B), both of

which were obtained from Arrayit Corporation (Sunnyvale, CA). The design of the cassettes allows for temperature-controlled reactions and prevents sample evaporation. For washing and imaging the glass slides are removed from the cassettes. The substrate surface on the glass slide was blocked by incubation in blocking buffer (0.5 % BSA in 20 mM Hepes buffer, 20 mM ionic strength, pH 7) for ~30 minutes to eliminate nonspecific particle binding. Each well of the reaction cassettes was filled with 25 μL of sample including fluorescent test particles, which were allowed to diffuse through the gel for a total duration of two hours. This incubation time is motivated by the time span after which native mucus is shed from and replenished on the surface of mucosal epithelia. Also, we found that the readout of our assay was less robust at shorter translocation times. As a positive control, wells were filled with particles immersed in buffer without hydrogel polymers. All experiments were conducted at room temperature. After two hours, the glass slides were removed from the cassettes and washed twice for 15 minutes in 250 mL washing buffer (20 mM Hepes, 10 mM NaOH, 250 mM NaCl, pH 7.5). In the first washing step, 0.1 % Triton-X was added to the washing buffer to facilitate removing the hydrogel polymers from the glass surface. Typically, five images were acquired per well with a 10 × (or 20 ×) objective on a fluorescence microscope (Zeiss). Each image was processed and analyzed using ImageJ (NIH): First, the images were binarized using a threshold algorithm. Second, a counting routine implemented in the ImageJ software was employed to determined the number of bead particles per image, and the average number of surface-bound particles per well was determined. The 24 well format of our set-up allows for testing different experimental conditions (e.g. different polymer concentrations or pH values) across the same slide. For each slide, three wells were used per experimental condition. The bead counting procedure was conducted for each well, and the results were averaged. Error bars in our graphs depict the error of the mean obtained from these three data sets. For optimizing the performance of this assay, it we ensured that the Biotin-particle concentration is high enough for reliable quantification, but low enough to avoid saturation of the Streptavidin receptors. We determined particles concentration at which saturation of particle binding at the Streptavidin surface occured to be $1-6\times10^7$ particles/mL, slightly depending on particle size and buffer pH. Accordingly, the particle concentration used for our experiments was lower than this saturation limit, i.e. $\sim4-6\times10^6$ particles/mL. To quantify the gel permeability, the average number of particles that had translocated the hydrogel, N, was normalized by the average number of surface-bound particles from the positive control, N^0, where the beads diffused through buffer only. Accordingly, we used the ratio N/N^0 as a measure for the relative hydrogel permeability with respect to buffer.

Results

Adsorption chromatography to measure the permeability of hydrogels

To reach the surface of the mucosal epithelium, a virus or drug delivery particle has to penetrate the mucus layer. Only then, the particle can bind to receptors on the epithelial surface and will become internalized. To mimic this sequence of events, we used a glass slide that is coated with a suitable receptor, in our case Streptavidin (Fig. 1A), and layer the glass slide with a mucin hydrogel that contains fluorescent particles, which are labeled with the corresponding Streptavidin ligand, Biotin. We chose the Streptavidin-Biotin pair due to its high affinity interaction[14], which is stable over a relatively wide range of pH levels and temperatures. However, in principle every other receptor/ligand system could be used, e.g. specific antibody/substrate pairs.

Particles that penetrate and exit the gel within a defined time interval will adsorb to the Streptavidin on the glass slide, while particles that fail to translocate/exit remain inside the gel. To quantify the degree of penetration, the mucin gel including the remaining particles is

removed from the slide by two washing steps, and the number of Streptavidin-bound beads is quantified via fluorescence microscopy.

One critical step for the reliable performance of this assay was to avoid unspecific (i.e. Biotin-independent) binding of the test particles. Indeed, without any further modification of the glass slide, the amount of bound Biotin-labeled and unlabeled beads was comparable (Fig. 1C), suggesting unspecific accumulation of the Biotin-free particles to the Streptavidin surface. However, this unspecific binding could be nearly eliminated by pre-treating the glass slide with Bovine Serum Albumin (BSA) (Fig. 1C). Other blocking agents might be suitable as well, however, it needs to be ensured that they do not interfere with the conjugated donor-receptor pair as e.g. milk appears to weaken the Biotin-Streptavidin bond[15].

Influence of the biopolymer concentration on the gel permeability

Having demonstrated the specific binding of our test particles to the receptor surface, we next aimed at characterizing the permeability properties of mucin hydrogels with our translocation assay. Mucin glycoproteins are the key gel-forming components of native mucus. The composition and the ensuing material properties of native mucus can vary significantly depending on the location of the gel in the human body. For instance, the concentration of mucin polymers in mucus gels on different epithelia of our body ranges from 1 % (w/v) in the lung up to 5 % (w/v) in the gastrointestinal tract[16,17]. In pathologic conditions such as in cystic fibrosis (CF), the mucin concentration can be even higher due to dehydration effects.

Earlier studies have already indicated that the permeability of mucin hydrogels depends on the biopolymer concentration: A decrease in particle transport with increasing mucin concentrations was reported for reconstituted mucin gels using a micro-Boyden chamber[18], and reduced diffusivity of test particles with increasing mucin concentration was also observed with single particle tracking techniques[19]. Here, we find a decrease of the bulk permeability of mucin gels with increasing mucin concentrations (Fig. 2A). Specifically, we report a 5fold reduction in particle transport efficiency when the mucin concentration is raised from 0.25 % (w/v) to 1 % (w/v), which agrees well with both previous studies. The same trend is observed for dextran gels when a similar concentration range is probed (Fig. 2B), which underlines the importance of the biopolymer concentration for the permeability of hydrogels.

Influence of different pH environments on the gel permeability

Another important mucus parameter that varies across human wet epithelia is pH, which ranges from very acidic conditions in the stomach[20] to neutral conditions in the lung[21], and alternating conditions in the female reproductive tract. It is possible that the body modulates the filtering properties of mucus by adjusting the pH of the hydrogel. Indeed, this is the case during ovulation when a shift from slightly acidic to more neutral pH conditions opens a window of opportunity for the sperm to penetrate the mucus barrier[22]. Also, pathogens such as *Helicobacter pylori* achieve motility in the highly acidic gastric mucus by locally altering its pH[23].

The effect of pH on particle diffusion in mucin hydrogels has been studied with single particle tracking, and a reduced particle mobility was observed in acidic mucin gels compared to neutral pH conditions[19]. Similarly, HIV particles are reported to be trapped in acidic but not in neutral cervical mucus[24]. As depicted in Fig. 2A, our assay confirms this pH-dependence of the mucin hydrogel permeability. However, it is important to note that a pH dependent permeability is not a generic hydrogel property. Dextran gels, in contrast,

exhibit comparable permeability properties both at acidic and neutral pH (Fig. 2B). These findings also correlate with the viscoelastic behavior of the two hydrogels: Whereas mucin solutions undergo a gelation transition when shifted from neutral to acidic pH[13], the viscoelastic properties of dextran solutions are independent from the pH level of the microenvironment (data not shown). In conclusion, our findings demonstrate that our assay can be used both in acidic and neutral buffer conditions due to the high stability of the chosen Biotin-Streptavidin ligand-receptor pair.

Influence of hydrogel-altering chemicals on the gel permeability

We next aimed at testing whether our assay can also be used to quantify the impact of putative hydrogel-altering chemicals. As an example, we chose N-acetyl-L-cysteine (NAC) and S-carboxymethyl-L-cysteine (CMC), chemicals that are able to break disulfide bonds. Such disulfide bonds contribute to the viscoelastic properties of mucus by establishing covalent cross-links between distinct mucin polymers [16]. Accordingly, NAC and CMC are key components in various mucolytic drug formulations where their mucus-dissolving abilities are harnessed as an adjuvant in respiratory conditions with excessive mucus production. Moreover, NAC can also modulate the inflammation response in cystic fibrosis[25].

Given their ability to liquefy native mucus, it seems reasonable to assume that those mucolytic chemicals might also have an impact on the microscopic permeability of mucin hydrogels. We tested this hypothesis by adding increasing amounts of either mucolytic agent to a 0.5 % (w/v) mucin hydrogel at pH 3, i.e. to a hydrogel that constitutes a strong barrier towards our test particles (see Fig. 1A). Indeed, with an increasing dose of either NAC or CMC we observed a continuous increase in the mucin hydrogel permeability, demonstrating the weakening effect of those chemicals on the barrier function of mucus. This example shows that our translocation assay can also successfully quantify the dose-dependent efficiency of drugs and pharmaceuticals that target the microarchitecture of hydrogel barriers.

Selected particle surface properties facilitate particle translocation through mucin solutions

In a last step, we tested whether our assay can report differences in particle translocation efficiencies that arise from distinct particle surface properties. Studies on native and reconstituted mucus have demonstrated that PEGylation of polystyrene particles increases their mobility in mucus hydrogels[19,26,27]. Indeed, here we also find that PEGylation increases the transport efficiency of polystyrene particles through mucin solutions compared to unPEGylated particles (Fig. 4). PEGylation of particles was suggested to reduce interactions between diffusion particles and mucus biopolymers[26,27], and in part these interactions appear to be based on electrostatic forces[19]. This picture agrees with the observation that the zeta-potential of the PEGylated particles used here is less negative than that of the unmodified carboxyl-particles (Table 1).

Discussion and further applications

The method presented here has several critical advantages over existing methods that measure hydrogel permeability. For example, in contrast to single particle tracking techniques[28], which estimate bulk transport by ensemble-averaging local diffusion coefficients of individual particles, our method directly measures the fraction of particles which permeates through a hydrogel. In addition, compared to diffusion chambers, the sample volume required for the analysis of the hydrogel permeability is relatively small in our assay. This is important as it is often very difficult to obtain large amounts of biological

specimen, especially if the samples are acquired from individuals with pathological conditions.

In general, the passage of nanoparticles through a hydrogel will require a series of events: partitioning into the gel, translocation through the gel, and exit from the gel. In our assay we circumvent the effect of partitioning by pre-loading the particles into the gel, which simplifies the data interpretation. However, our assay can still not distinguish between the actual translocation through the gel, and exit of the particles from the gel.

With our assay, multiple experiments can be run in parallel allowing for a comparative analysis of particles with different properties or gels of different composition. Particles adsorbed to the glass slide can be quantified by automated microscopy, which is convenient if systematic larger scale screens are performed, e.g. comparing various particle types or hydrogel modifications.

Here, we have used the biotin/streptavidin pair to allow for the specific binding of translocating particles to the glass surface. The high stability of the biotin/streptatvidin bond is convenient as it prevents the removal of adsorbed particles during the washing steps of our assay. However, the system is not limited to this particular pair, and could be adapted to the use of other receptor/substrate complexes such as antibodies/target peptides.

In contrast to other fluorescence techniques such as single particle tracking or fluorescence recovery after photobleaching, our method is not limited by high background fluorescence from the gel, which is a common problem with protein-based hydrogels. However, we note that the imaging of small particles in the size range of 50-100 nm is more challenging and would require more advanced optics than used for the relatively large beads used in this study.

Finally, our method can be used to optimize the fabrication of synthetic gels. As the mucus barrier consists of linear polymers which entangle cross-link to form a hydrogel, then it may be possible to mimic the gel barrier by synthetic polymers[29]. Our assay easily allows for testing different synthetic polymers and probing the permeability properties of the resulting hydrogels. If successful, these results would have broad medical applications and could lead to the development of synthetic gels that support the natural barrier function of mucus against viral or parasitic infections, act as contraceptives, or recover conditions of oral or postmenopausal dryness.

Acknowledgments

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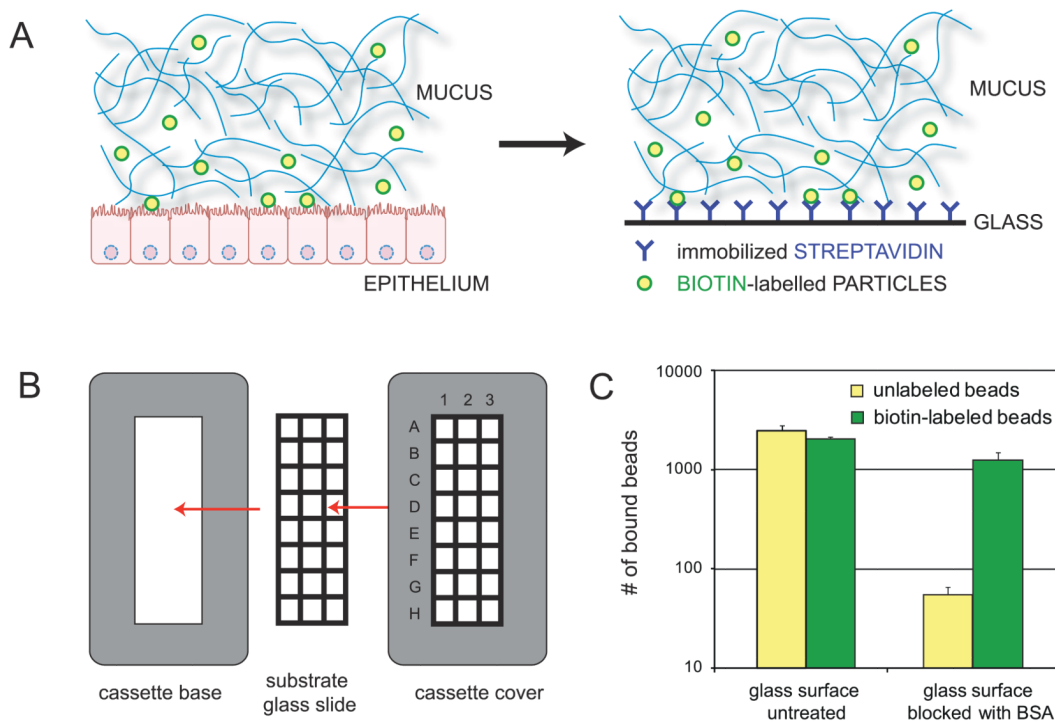
Figure 1.
Schematic representation of our bead translocation assay. (A) Our setup mimics the conditions a drug delivery particle encounters on its way to the mucosal epithelium. The epithelial surface is represented by a glass slide that is coated with Streptavidin receptor molecules and lined with a mucin hydrogel. Particles coated with the corresponding Streptavidin ligand, Biotin, are embedded in the gel and allowed to diffuse through the gel layer. Successful gel penetration entails binding of particles to the surface of the functionalized glass slide. (B) The translocation cassette consists of a base and cover unit between which the functionalized glass slide is sandwiched. By placing the metal enclosure on a heating plate, this setup allows for conducting the translocation experiment in an environment where the temperature can be controlled whereas sample evaporation is prevented. (C) Unspecific binding of unlabeled carboxyl-terminated polystyrene beads (1 μm in size) is prevented by blocking the surface of the functionalized glass slide with BSA.
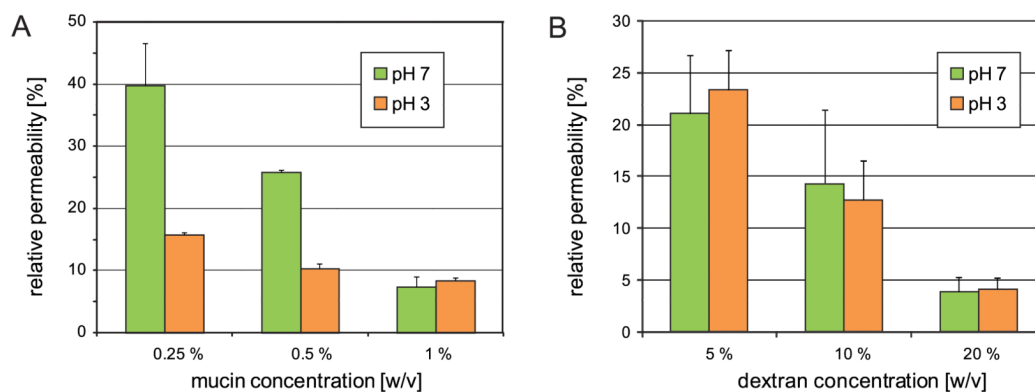
Figure 2.
The permeability of hydrogels depends on internal parameters. (A) The bulk permeability of mucin hydrogels decreases with increasing mucin concentrations and is reduced at acidic pH compared to neutral pH conditions. (B) Dextran hydrogels show a similar dependence of their bulk permeability on the polymer concentration as mucin gels, but are insensitive towards pH changes. For both experiments, Biotin-labeled carboxyl-terminated polystyrene particles (840 nm in size) have been used. Experiments have been conducted in triplicates, error bars denote the error of the mean.
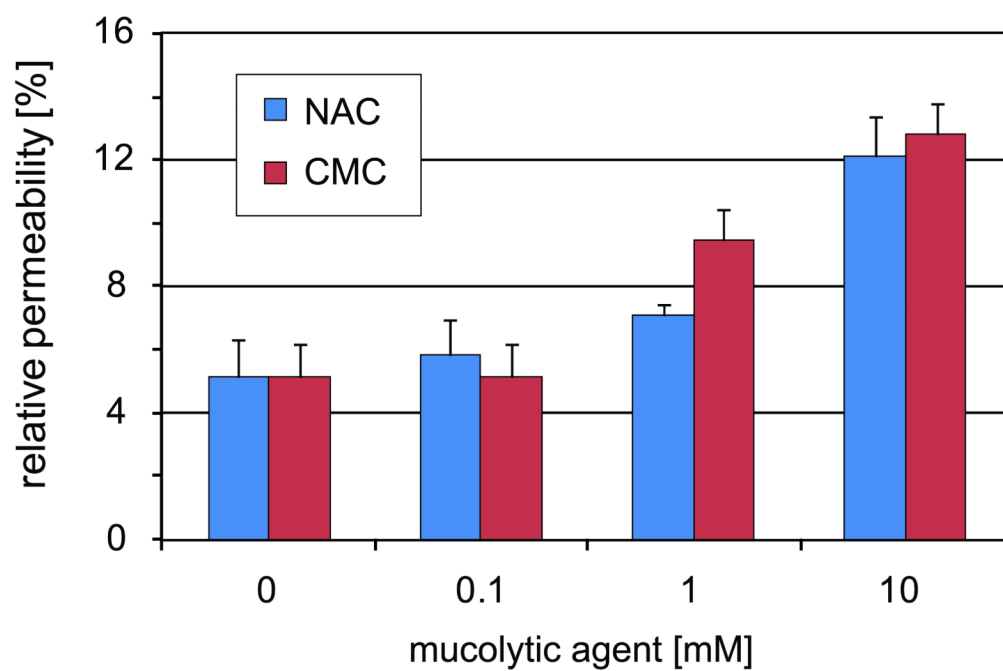
Figure 3.
The barrier properties of mucin hydrogels can be weakened by mucolytic chemicals. Increasing amounts of either S-carboxymethyl-L-cysteine (CMC) or N-acetyl-L-cysteine (NAC) increase the bulk permeability of a 0.5 % mucin hydrogel at pH 3. As test particles, biotin-labeled carboxyl-terminated polystyrene particles (840 nm) were used. Experiments have been conducted in triplicates, error bars denote the error of the mean.
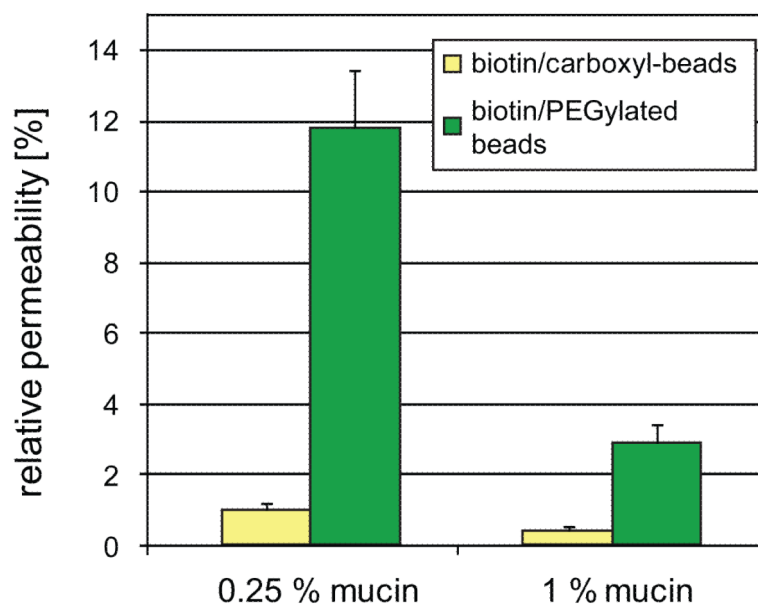
Figure 4.
The permeability of mucin solutions towards polystyrene particles depends on the particle surface properties. PEGylated carboxyl-terminated polystyrene particles (1 µm) exhibit higher transport efficiencies than the non-PEGylated beads.

Table 1

Size, ζ-potential, polydispersity index (PDI), and surface modifications of the test particles used in this study

Nominal particle size [nm]	Surface modification	Measured particle size [nm]	PDI	ζ-potential at pH 7 [mV]	ζ-potential at pH 3 [mV]
1000	Biotin-carboxyl	1135	0.39	−42.2 ± 0.8	N.D.
1000	PEG/biotin-carboxyl	1320	0.26	−29.1 ± 1.1	N.D.
840	Biotin-carboxyl	1020	0.16	−47.7 ± 1.9	12.8 ± 2.8