Particle Diffusivity and Free-Energy Profiles in Inhomogeneous Hydrogel Systems from Time-Resolved Penetration Profiles

Amanuel Wolde-Kidan,†§ Anna Herrmann,‡§ Albert Prause,¶ Michael Gradzielski,¶ Rainer Haag,† Stephan Block,† and Roland R. Netz∗†

†Fachbereich Physik, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany
‡Institut für Chemie und Biochemie, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany
¶Institut für Chemie, Straße des 17. Juni 124, Technische Universität Berlin, 10623 Berlin, Germany
§These two authors contributed equally to this work.

E-mail: rnetz@physik.fu-berlin.de

Abstract

A combined experimental/theoretical method to simultaneously determine diffusivity and free-energy profiles of particles that penetrate into inhomogeneous hydrogel systems is presented. As the only input, arbitrarily normalized concentration profiles from fluorescence intensity data of labeled tracer particles for different penetration times are needed. The method is applied to dextran molecules of varying size which penetrate into hydrogels of polyethylene-glycol (PEG) chains with different lengths that are covalently cross-linked by hyperbranched polyglycerol (hPG) hubs. Extracted dextran bulk diffusivities agree well with fluorescence correlation spectroscopy data obtained separately. Scaling laws for dextran diffusivities and free energies inside the hydrogel are identified as a function of the dextran mass. An elastic free-volume model that includes dextran as well as PEG linker flexibility describes the repulsive dextran-hydrogel interaction free energy, which is of steric origin, quantitatively and furthermore suggests that the hydrogel mesh-size distribution is rather broad and particle penetration is dominated by large hydrogel pores.

Particle penetration into hydrogels is for steric particle-hydrogel interactions thus governed by an elasticity-enhanced size-filtering mechanism that involves the tail of the hydrogel pore-size distribution.
1 Introduction

The penetration of particles into hydrogels is relevant for technological applications,\textsuperscript{1,2} drug delivery\textsuperscript{3} and for biological systems such as biofilms,\textsuperscript{4} the extracellular matrix\textsuperscript{5} and mucus.\textsuperscript{6} Mucus, which is the most common biological hydrogel, lines the epithelial tissues of different organs, such as the respiratory, gastrointestinal and urogenital tracts. Mucus is mainly composed of mucins, which are glycoproteins of varying length that absorb large amounts of water and thereby lend mucus its hydrogel nature, and additional components such as enzymes and ions.\textsuperscript{7} Mucins are relevant in the cell signaling context and presumably also play a role in the development of cancer.\textsuperscript{8} But primarily, mucus is a penetration barrier against pathogens, e.g. virions or bacteria, while it allows the permeation of many non-pathogens, e.g. nutrients, that are absorbed through the mucosa of the small intestine.\textsuperscript{9} Studies have suggested that, based on the type of mucus, different mechanisms give rise to the protective barrier function.\textsuperscript{10,11} in addition to the advective transport of pathogens through mucus shedding or clearance,\textsuperscript{12,13} which is not considered here. One typically distinguishes steric size-filtering mechanisms from interaction-filtering mechanisms,\textsuperscript{6,14} the latter presumably play a major role in the defense of organisms against pathogens since they allow for precise regulation of the passage of wanted and unwanted particles and molecules.\textsuperscript{15,16} Recent studies demonstrated that attractive electrostatic interactions reduce particle diffusivity inside hydrogels substantially and much more than repulsive electrostatic interactions\textsuperscript{17,18} and that salt concentration and the distribution of charges and pore size are important parameters which influence the permeation properties of charged hydrogels.\textsuperscript{19,20}

Particle penetration into mucus and biofilms has been studied by single-particle tracking techniques\textsuperscript{21,22} as well as by methods where a diffusor ensemble is observed.\textsuperscript{15,16,23,24} In the continuum description, which is valid on length scales larger than the hydrogel pore or mesh size, particle diffusion is completely described by the free-energy and diffusivity profiles across an inhomogeneous hydrogel system, while transient particle binding to the hydrogel\textsuperscript{16} effectively reduces the diffusivity. If the free-energy and diffusivity profiles are known, particle penetration can be quantitatively predicted, provided the particle concentration is low and the particles do not modify the hydrogel properties in an irreversible manner. In this context it should be noted that both profiles depend on the interactions between particle and hydrogel and therefore are different for each distinct hydrogel-particle pair. Due to method restrictions, experiments primarily focussed on determining either the particle diffusivity inside the hydrogel\textsuperscript{6,10,21} or on the partitioning between hydrogel and the bulk solution,\textsuperscript{25} from which the free energy inside the hydrogel (relative to the bulk solution) can be determined. However, for prediction of the penetration or permeation speed of particles into the hydrogel, both the diffusivity and the free energy in the hydrogel are needed.

In this work, we study synthetic hydrogels that consist of polyethylene-glycol (PEG) linkers of different molecular masses which are permanently cross-linked by hyperbranched polyglycerol (hPG).\textsuperscript{2} Such synthetic hydrogels can be regarded as simple models for mucus, since they display size-dependent particle permeabilities,\textsuperscript{14,26} similar to mucus. As diffusing particles we employ fluorescently labelled dextran molecules of varying sizes. When using confocal laser-scanning fluorescence microscopy to investigate particle penetration into hydrogels, the sample can be oriented such that the hydrogel-bulk interface is either parallel\textsuperscript{16} or perpendicular\textsuperscript{27} to the optical axis, which makes no significant difference from a scanning perspective. However, for laterally extended samples like cell cultures that grow on a substrate, the parallel alignment causes the light path to span substantially larger distances, making this setup more prone to distortions in the imaging process. A perpendicular alignment, as employed in this work and sketched in Figure 1, is therefore preferable for biological samples\textsuperscript{27} and is also compatible with future extensions of such penetration assays to mucus-producing cell cul-
We investigate the filtering function of hydrogels by theoretical analysis of time-resolved concentration profiles of the labelled dextran molecules as they penetrate into the hydrogel. The employed numerical method allows for simultaneous extraction of free-energy and diffusivity profiles from relative concentration profiles at different times and is a significant extension of earlier methods, as it does not require absolute concentration profiles but works with relative, i.e. arbitrarily normalized, concentrations. This is a crucial advantage, as often fluorescence intensity profiles are subject to significant perturbation due to e.g. laser light intensity fluctuations or fluorescence dye bleaching over the course of the experiment, and makes the often difficult conversion of measured intensity data into absolute particle concentrations obsolete. Our method for the extraction of free-energy and diffusivity profiles from relative concentration profiles can be used for a wide range of different setups and systems. As a check on the robustness of the method, the extracted dextran bulk diffusivities are shown to agree well with fluorescence-correlation spectroscopy (FCS) data that are obtained separately. The obtained particle free energies and diffusivities inside the hydrogel are shown to obey scaling laws as a function of the dextran mass. The dextran free energy inside the hydrogel is described by a free-volume model based on repulsive steric interactions between the dextran molecules and the hydrogel linkers, which includes dextran as well as hydrogel linker flexibility. This model constitutes a modified size-filtering mechanism for repulsive particle-hydrogel interactions, according to which particle penetration into hydrogel pores is assisted by the elastic widening of pores and the elastic shrinking of dextran molecules, and matches the extracted particle free energies in the hydrogel quantitatively. The model furthermore suggests that the hydrogel mesh size distribution is rather broad and that particle penetration is dominated by the fraction of large pores in the hydrogel.
Figure 2: Exemplary time-dependent dextran concentration profiles from experimental measurements (circles) and numerical modeling (solid lines) for the hPG-G10 hydrogel. Results for the smallest dextran with $M_{\text{dex}} = 4$ kDa in A-D are compared to results for $M_{\text{dex}} = 40$ kDa in E-H.

A&E: Experimental and modeled concentration profiles agree very accurately, note that concentration profiles are shifted vertically for better visibility. B&F: Modeled concentration profiles are presented for a wide range of penetration times. The initial profile $\vec{c}_{\text{init}}$ (black line) is based on experimental data (see Methods Section 4.7). C&G: Extracted diffusivity profiles, showing that the diffusivity in the hydrogel is slightly reduced compared to the bulk solution. D&H: Extracted free-energy profiles. Significant exclusion of dextran from the hydrogel is observed, with a stronger effect for the larger dextran.
2 Results and Discussion

Fluorescence intensity profiles of fluorescein isothiocyanate (FITC)-labeled dextran molecules penetrating into PEG-based hydrogels are analyzed using the procedure explained in the Methods Section. The analysis is based on numerical solutions of the one-dimensional generalized diffusion equation\textsuperscript{31}

$$\frac{\partial c(z,t)}{\partial t} = \frac{1}{D(z)} \frac{\partial}{\partial z} \left[ D(z) e^{-\beta F(z)} \frac{\partial}{\partial z} \left( c(z,t) e^{\beta F(z)} \right) \right] \quad (1)$$

where $c(z,t)$ is the concentration at time $t$ and depth $z$ (see Figure 1), $D(z)$ and $F(z)$ are the spatially resolved diffusivity and free-energy profiles which the dextran molecules experience and $\beta = 1/k_B T$ is the inverse thermal energy. While the diffusivity $D(z)$ describes the mobility of dextran molecules at position $z$, the free energy profile $F(z)$ uniquely determines the equilibrium partitioning of dextran molecules. The numerical solution of eq. (1) provides a complete model of the penetration process into the hydrogel and at the same time allows for extraction of the diffusivity and free energy profiles by comparison with experimentally measured concentration profiles. A direct conversion of measured fluorescence intensities into absolute concentrations is often difficult due to drifts of various kinds. The method developed here circumvents this problem and allows for in-depth analysis of arbitrarily normalized concentration profiles, as explained in Methods Section 4.6. Complete profiles of free energies and diffusion constants both in the bulk and in the PEG hydrogel are obtained and the results for different hydrogels and dextran molecules of varying sizes will be analyzed in the following.

Comparison Between Experimental and Modeled Concentration Profiles. Figure 2A&E shows exemplary concentration profiles for dextran molecules with molecular masses of $M_{\text{dex}} = 4$ kDa and $M_{\text{dex}} = 40$ kDa penetrating into the hPG-G10 hydrogel (see Methods Section 4.1). Measurements are performed over a total time span of about 30 minutes and concentration profiles are recorded every 10 seconds, leading to a total of about 180 concentration profiles as input for the numerical extraction of the diffusivity and free-energy profiles. The first measured concentration profile at $t = 0$ min represents the start of the experiment, approximately 10 seconds after the dextran solution was applied onto the gel (see Methods Section 4.4). The numerically determined concentration profiles (lines) reproduce the experimental data (data points) very accurately, as seen in Figure 2A&E. The deviation is estimated from the normalized sum of residuals, $\sigma$ (according to eq. (13)) which is below 2 mg/L for both measurements. A stationary concentration profile is obtained in the theoretical model only after 4 hours of penetration for the smaller 4 kDa dextran, see Figure 2B, for the larger dextran molecule the stationary profile is reached only after an entire day, see Figure 2F. These times significantly exceed the duration of the experiments.

The extracted diffusivity and free-energy profiles in Figure 2C, D, G, H, reveal the selective hydrogel permeability for dextran molecules of varying size. The free energy difference in the hydrogel is positive $\Delta F_{\text{gel}} > 0$ for both dextran sizes, indicating that dextran is repelled from the hydrogel. The dextran partition coefficient $K_{\text{gel}}$ between the hydrogel and the bulk solution is related to the change in the free energy $\Delta F_{\text{gel}}$ as

$$K_{\text{gel}} = e^{-\beta \Delta F_{\text{gel}}} \quad (2)$$

According to eq. (2), the obtained free energy differences $\Delta F_{\text{gel}} = 0.6 k_B T$ and $\Delta F_{\text{gel}} = 1.9 k_B T$, correspond to partition coefficients of about $K_{\text{gel}} \approx 1/2$ and $K_{\text{gel}} \approx 1/7$ for the smaller and the larger dextran molecules, respectively, which illustrates a significant exclusion in particular for the larger dextran. Compared to the partition coefficients, the diffusion constants in the hydrogel decrease only slightly as a function of the dextran mass. This suggests that the dextran molecules are only modestly hin-
dered in their motion, a conclusion that will be rationalized by our elastic free-volume model further below.

Figure 3: Comparison of experimental results (circles) and modeling results based on the extracted diffusivity and free-energy profiles (lines) for the mean dextran concentration \( \bar{c} \) over time in three different regions, the far solution \((-z_{\text{top}} < z < 0)\), the near solution \((0 < z < z_{\text{int}})\) and the gel \((z_{\text{int}} < z < z_{\text{bot}})\), see Figure 1. The systems are the same as shown in Figure 2. A non-monotonic dextran concentration is measured over time in the near and far solution regions. The fact that \( \bar{c} \) in the gel does not vanish for \( t \to 0 \) reflects that the first measurement at \( t = 0 \) is done approximately 10 seconds after the application of the dextran solution onto the gel.

Figure 3 shows the temporal evolution of the average dextran concentration \( \bar{c} \) in three different regions, namely inside the gel for \( z_{\text{int}} < z < z_{\text{bot}} \), in the near solution for \( 0 < z < z_{\text{int}} \), and in the far solution for \(-z_{\text{top}} < z < 0\) for the same data shown in Figure 2. The lines show the predictions based on the extracted diffusivity and free-energy profiles, the circles the experimental data, which are not available in the far solution range. The average concentration in the gel (black) increases monotonically and saturates after about one hour for both dextran sizes. Note that the stationary final concentration in the hydrogel is considerably less for the larger dextran with \( M_{\text{dex}} = 40 \) kDa. In contrast, the average concentration in the far solution saturates more slowly and shows a slight non-monotonicity for both dextran masses (blue). This non-monotonicity is more pronounced in the near solution (red) and is caused by the fact that dextran molecules diffuse quickly into the hydrogel from the near solution in the beginning of the experiment, while the replenishment from the bulk solution takes a certain time, as also seen in the concentration profiles in Figure 2B&F. Very good agreement between experiments and modeling results is observed.

Influence of Dextran Size on Hydrogel Penetration. The same analysis is performed for dextran molecules of molecular masses ranging from \( M_{\text{dex}} = 4 \) kDa to \( M_{\text{dex}} = 70 \) kDa that penetrate into PEG hydrogels with two different linker lengths, namely \( hPG-G6 \) with a PEG linker size of \( M_{\text{PEG}} = 6 \) kDa and \( hPG-G10 \) with \( M_{\text{PEG}} = 10 \) kDa. Figure 4 shows the extracted diffusivities and free energies, which result from averages over at least three experiments for each system, except for \( M_{\text{dex}} = 70 \) kDa dextran, where only one experiment was performed.

Figure 4A shows the bulk diffusivities \( D_{\text{sol}} \) extracted from measured concentration profiles as colored symbols, in principle there should be no difference between results for \( hPG-G6 \) and \( hPG-G10 \). A power law relation between the dextran mass and the diffusivity according to \( D_{\text{sol}} \propto M_{\text{dex}}^{-\nu} \) is shown as straight lines for \( \nu = 1 \) (broken line) and for \( \nu = 1/2 \) (dotted line). An exponent of \( \nu = 1/2 \) agrees nicely with our FCS data (solid black triangles; see Methods Section 4.5) as well as with literature FRAP measurements\(^{32}\) (open black triangles). The value \( \nu = 1/2 \) follows from combining the Stokes-Einstein relation \( D_{\text{sol}} = k_B T/(6\pi \eta w r_0) \)}
Figure 4: Results for the diffusivity and free energy obtained from the experimental measurements as a function of dextran mass. A: Fitted diffusivities in the bulk solution (squares and circles) agree within the error with FCS data measured in the current work (solid black triangles) and with FRAP measurements from literature\(^{32}\) (open black triangles). B: Fitted diffusivities in the hydrogel are reduced relative to the bulk values and are compared to different power laws. C: Dextran molecules are excluded from the hydrogel and \(\Delta F_{\text{gel}} > 0\) for all dextran masses. For larger dextran molecules, \(\Delta F_{\text{gel}}\) increases as a square root with the dextran mass. The results from the free-volume model of eq. (7) (continuous lines) agree nicely with the measurements. Error bars have been estimated as explained in Section ?? of the Supporting Information. The inset in B presents a schematic depiction of the two different gels. Even though the \(hPG-G10\) gel is composed of larger linkers, the mass density is larger than in the \(hPG-G6\) gel, which results in an effectively smaller pore size.

with the scaling of the dextran hydrodynamic radius according to \(r_0 \propto M_{\text{dex}}^{\nu}\)\(^{33,34}\) by assuming that the bulk solution is a theta solvent for dextran polymers\(^{35,36}\) (see Section ?? in the Supporting Information for details). The exponent \(\nu = 1/2\) is only expected for linear polymers, while dextran is in fact a branched polymer. The good agreement of FCS and FRAP data with the power law for \(\nu = 1/2\) suggests that the degree of branching is low\(^{37}\) or that branching effectively compensates self-avoidance effects. The dextran hydrodynamic radii estimated from the FCS measurements compare well with the values reported by the supplier, see Table 1. The data for \(D_{\text{gel}}\) obtained from the time-dependent dextran concentration profiles show rather large uncertainties, which is due to the fact that the concentration profiles are rather insensitive to the bulk diffusivities; they are within error bars consistent with our FCS results but do not allow extraction of the power-law scaling with any reasonable confidence.

Values for the diffusion constant in the hydrogel \(D_{\text{gel}}\) are compared to power laws with exponents \(\nu = 1/2\) and \(\nu = 1\) in Figure 4B. The

| \(M_{\text{dex}}\) (kDa) | \(r_0\) (nm) | \(r_{\text{FCS}}\) (nm) |
|-----------------|-------------|----------------|
| 4               | 1.4         | 1.5            |
| 10              | 2.3         | 2.7            |
| 20              | 3.3         | 3.2            |
| 40              | 4.5         | 4.3            |
| 70              | 6.0         | 6.4            |

\(r_0\) and \(r_{\text{FCS}}\) as reported by the supplier, in comparison to estimated hydrodynamic radius \(r_{\text{FCS}}\) based on our FCS measurements using the Stokes-Einstein relation and the viscosity of water as \(\eta_{\text{w}} = 0.8 \cdot 10^{-3}\) Pas.
difference of the diffusion constants between the two different hydrogels is within the error bars, in agreement with the fact that the estimated mean hydrogel mesh-sizes are $l_0^{hPG-G6} = 7.1$ nm and $l_0^{hPG-G10} = 7.5$ nm (see Methods Section 4.2) and thus quite similar to each other. It is to be noted that for $M_{dex} \leq 20$ kDa, the estimated mesh sizes are larger than twice the dextran hydrodynamic radii from Table 1, which would not suggest any dramatic confinement effect on the diffusion constant. Interestingly, for the data where $M_{dex} \gtrsim 20$ kDa, the hydrogel with the larger linker length ($hPG-G10$), which has a slightly higher mesh size, is seen to reduce the diffusion constant slightly more, which at first sight is counterintuitive. This finding is rationalized by the fact that the $hPG-G10$ gel has a higher mass density compared to the $hPG-G6$ gel (see Methods Section 4.1), and thus the effective pore size is in fact substantially smaller. This is schematically illustrated in the inset in Figure 4B. A diffusivity scaling with an exponent $\nu = 1$, which describes the data for $hPG-G10$ slightly better, could be rationalized by screened hydrodynamic interactions or by reptation-like diffusion.\(^{38}\) In fact, a cross-over in the scaling of the diffusivity with increasing hydrogel density from $\nu = 1/2$ to $\nu = 1$ has been described before for dextran penetrating into hydroxypropylcellulose.\(^{34}\) However, because of the large error bars, extraction of the diffusivity scaling with respect to dextran mass in the two gels is not uniquely possible. This is mostly due to the fact that the diffusivities change rather mildly with varying dextran mass.

Figure 4C shows the extracted values of $\Delta F_{gel}$ for the two hydrogels as a function of the dextran mass. In all measurements we find $\Delta F_{gel} > 0$, which suggests exclusion of the dextran molecules from the hydrogel. Also the value of $\Delta F_{gel}$ increases with the dextran mass. Since dextran as well as the PEG-hPG based hydrogels are uncharged,\(^{39}\) this exclusion must be due to steric repulsion, possibly enhanced by hydration repulsion.

Elastic Free-Volume Model for Dextran Penetration in Hydrogels. For the larger dextran molecules, the hydrogel with the smaller PEG linkers, $hPG-G6$, displays a slightly stronger exclusion. The power law relation between the hydrogel free energy and dextran mass according to $\Delta F_{gel} \propto M_{dex}^\alpha$ with an exponent of $\alpha = 1/2$ describes the data well for larger dextran masses $M_{dex} \gtrsim 20$ kDa, as shown by the dotted black line in Figure 4C. This power law behavior is in fact compatible with an elastic free-volume model for the penetration of dextran molecules into hydrogels, which yields the solid lines and will be derived in the following.

The model geometry is sketched in Figure 5A and consists of a single dextran molecule of radius $r$ (green sphere) inside a cubic unit cell of the PEG based hydrogel (grey cylinders). The presence of the hPG hubs connecting the PEG linkers is neglected in the following. The dextran experiences a reduction of its free volume compared to the bulk solution, due to steric interactions with the PEG linkers. In the simple model geometry, the PEG linkers are located at the edges of the cubic unit cell and are modeled as impenetrable cylinders of radius $a$ and length $l$. The excluded volume $V_{ex}$ for dextran in the cubic unit cell consists of a quarter of each of the twelve cylinders at the edges. The accessible or free volume in the hydrogel $V_{free}$ depends on the sum of sphere radius $r$ and cylinder radius $a$ and is given by

$$V_{free} = V_{unit} - V_{ex} = l^3 - \frac{12}{4} \pi (r + a)^2 l + 2V_{cyl} \tag{3}$$

Here, $V_{unit} = l^3$ is the volume of the unit cell and $V_{cyl} = \frac{16}{3} \pi (r + a)^3$ is the volume of two intersecting cylinders,\(^{40}\) which is subtracted from the excluded volume to avoid over counting of the unit-cell corners. The entropic contribution to the total free energy is given by

$$\Delta F_{vol} = -k_B T \ln \left( \frac{V_{free}}{V_{unit}} \right) \tag{4}$$

$$= -k_B T \ln \left( 1 - 3\pi \left[ \frac{r + a}{l} \right]^2 + \frac{32}{3} \left[ \frac{r + a}{l} \right]^3 \right)$$
Figure 5: Elastic free-volume model for the partitioning of a particle in a hydrogel. A: Schematic sketch of the cubic unit-cell model for the hydrogel, made up of connected linkers of length $l$ and a finite radius of $a$. The diffusing particle is modeled as a sphere of radius $r$. Both the particle and the linkers are elastic and can stretch or contract. B: Partition coefficient $K_{gel}$ extracted from the experimentally measured dextran concentration profiles (symbols) in comparison to the elastic free-volume model predictions according to eq. (7) (solid lines). The results of the non-elastic model according to eq. (4) are shown as dashed lines. The inset shows the equilibrium values of $l^*$ and $r^*$ obtained for the $hPG-G6$ gel. C: Illustration of a disordered pore in the hydrogel which has a mesh size $l_0$ and consists of more than four linkers.

Since dextran and the PEG linkers are elastic polymers, they are both flexible and can deform. For small deformations, the polymers behave like Gaussian chains. The elastic deformation free energy for a cubic unit cell consisting of 12 equally deformed PEG linkers can be written as (for a detailed derivation see Section ?? in the Supporting Information)

$$\Delta F_{PEG} = \frac{12}{2} k_B T \left( \left[ \frac{l}{l_0} \right]^2 + \frac{1}{2} \left[ \frac{l}{l_0} \right]^2 \right)$$  \hspace{1cm} (5)

Here $l/l_0$ is the relative stretching of the PEG linkers, where $l_0$ denotes the edge length of the unit cell in the absence of dextran molecules. The elastic deformation energy of dextran is obtained in the same fashion and reads

$$\Delta F_{dex} = \frac{3}{2} k_B T \left( \left[ \frac{r}{r_0} \right]^2 + \left[ \frac{r_0}{r} \right]^2 - 2 \right)$$  \hspace{1cm} (6)

where $r$ denotes the deformed dextran radius and the equilibrium dextran radius is denoted by $r_0$ and is taken from Table 1. The complete free energy follows as

$$\Delta F_{gel}(r, l) = \Delta F_{vol}(r, l) + \Delta F_{PEG}(l) + \Delta F_{dex}(r)$$  \hspace{1cm} (7)

The equilibrium free energy is given by its minimal value, obtained for the optimal stretched unit cell length $l^*$ and the optimal dextran radius $r^*$, which are determined numerically. The values of the unit cell length $l_0$ and the PEG linker thickness $a$ are adjusted by fits. The model results are shown in Figure 5B in terms of the partition coefficient as solid lines and compared to the experiments (circles and squares) as a function of the length ratio $r_0/l_0$. The inset shows the obtained equilibrium values for $l^*$ and $r^*$ for the $hPG-G6$ gel. A considerable stretching of PEG-linkers and compression of dextran is observed, which shows that elasticity effects of both PEG linkers and dextran molecules are important.

The fit to the experimental data yields $l_0^{hPG-G6} = 16.7$ nm, $l_0^{hPG-G10} = 23.7$ nm, $a_{hPG-G6} = 3.4$ nm and $a_{hPG-G10} = 5.4$ nm. The values of $a$ certainly represent an effective PEG linker radius, including the effects of poly-
mer crumpling and a layer of tightly bound hydration water. In fact, the free-volume model yields estimates of the number of hydration waters per PEG monomer that scatter around 8 and slightly depend on dextran mass and PEG length, in rough agreement with literature values (see Figure ?? and Section ?? in the Supporting Information). The fit values for the unit cell length \( l_0 \) are significantly larger than the mean mesh size estimated based on eq. (12), which yields \( l_{\text{PEG-G6}}^0 = 7.1 \text{ nm} \) and \( l_{\text{PEG-G10}}^0 = 7.5 \text{ nm} \), but still considerably shorter than the PEG contour length \( L = b_0^\text{PEG}N_{\text{PEG}} \), which is \( L_{\text{PEG-G6}} = 48.5 \text{ nm} \) and \( L_{\text{PEG-G10}} = 80.9 \text{ nm} \), where \( b_0^\text{PEG} = 0.356 \text{ nm} \) is the PEG monomer length\(^4^1\) and \( N_{\text{PEG}} \) is the respective number of PEG monomers. A priori there is no reason why the linkers should be stretched to such a considerable fraction of their contour length. We therefore rationalize this surprising result in terms of a broad distribution of pore sizes that exhibit different topologies. To illustrate this, a random pore is schematically shown in Figure 5C. Based on the 3:1 number ratio of linkers and cross linkers in the hydrogel formulation (cf. Methods Section 4.1), a perfectly cubic lattice could form, where each hub is connected to 6 different linkers. Such an ideal cubic connectivity is of course entropically highly unfavorable and the connectivity distribution of hubs, i.e. the distribution of the number of linkers that connect to one hub, will be rather broad and the network topology will be disordered. While in a cubic lattice each cubic facet consists of four hubs and four linkers, the pores present in the actual hydrogel will show a broad distribution of the number of participating linkers. For illustration, the pore shown in Figure 5C consists of eight linkers. Clearly, dextran molecules will tend to be located in larger pores in order to maximize their free volume, and therefore the fit parameters of our model will be dominated by the tail of the pore size distribution, which explains the large fit values for \( l_0 \). This finding also explains why the dextran diffusivities in the hydrogel differ only mildly from the bulk diffusivities, since large pores restrict the diffusion of particles only slightly. Clearly, the precise topology and compositional distribution of pores cannot be predicted by our analysis, our results should thus be merely interpreted as an indication of the presence of large pores and a disordered network topology.

An approximate non-elastic version of the free-volume model is obtained by neglecting the polymer deformation term and just keeping the excluded volume term, eq. (4), which becomes accurate in the limit of \( l_0 \gg r_0 \), where \( r^* \approx r_0 \) and \( l^* \approx l_0 \). These approximate results are shown as broken lines in Figure 5B and describe the experimental data only for small values of \( r_0/l_0 \). When additionally approximating the logarithm in eq. (4), the obtained expression for the free energy is similar to results derived for a random-fiber network.\(^4^2\)

**Derivation of Particle Permeabilities through Hydrogel Barriers.** Permeation through biological barriers is quantified by the permeability coefficient \( P \), which is defined as

\[
P(z_1, z_2) = \frac{J}{c(z_1) - c(z_2)} \quad (8)
\]

where \( c(z_1) \) and \( c(z_2) \) are the particle concentrations at the two sides \( z_1 \) and \( z_2 \) of the barrier, and \( J \) denotes the particle flux through the barrier. Based on the diffusion eq. (1), the permeability can be written as (for a detailed derivation see Section ?? of the Supporting Information)

\[
\frac{1}{P(z_1, z_2)} = \int_{z_1}^{z_2} \frac{e^{\beta F(z)}}{D(z)} \, dz \quad (9)
\]

For a step-like barrier one obtains

\[
\frac{1}{P} = \frac{e^{\beta \Delta F_{\text{gel}}}}{D_{\text{gel}} L} \quad (10)
\]

Here \( \Delta F_{\text{gel}} \) and \( D_{\text{gel}} \) are the particle free energy relative to the solution and the diffusivity inside the hydrogel and \( L \) denotes the width of the hydrogel barrier.

Figure 6A shows normalized permeability coefficients \( PL \) for a single step-like barrier according to eq. (10), which are independent of the thickness of the barrier \( L \), as a function of the gel free energy and the gel diffusivity. The values extracted from the experimental data
Figure 6: A: Normalized permeability coefficient $PL$ through a step-like single hydrogel barrier of width $L$ as a function of the hydrogel free energy $\Delta F_{\text{gel}}$ and the hydrogel diffusivity $D_{\text{gel}}$ from eq. (10). High permeability is observed for low free-energy barriers and high diffusivities in the hydrogel. The symbols denote the experimental data from Figure 4. Due to opposing trends in the free-energy barrier and the diffusivity, both hydrogels display comparable permeability coefficients. B: Schematic layered structure of a mucous membrane, as found in the stomach. Examples for different diffusers are shown, including nutrients such as glucose and pathogens such as virions or bacteria. The diffusers have to penetrate different layers of varying permeabilities to enter the tissue below the mucous membranes, the total permeability of a layered structure follows from eq. (11).

3 Conclusion

The method introduced in this paper allows for the simultaneous extraction of diffusivity and free-energy profiles of particles that permeate into spatially inhomogeneous hydrogel systems. The advantage over alternative methods is that both quantities are obtained from a single experimental setup. This is important, as only the combination of diffusivity and free-energy profiles completely determines the diffusion of particles.

From concentration profile measurements of fluorescently labeled dextran molecules permeating into PEG-hPG-based hydrogels, diffusivities and free energies in the hydrogel are extracted and analyzed in terms of scaling laws as a function of dextran mass. Dextran and PEG linkers repel each other via steric interactions. A modified free volume model that includes the elasticity of PEG linkers and of the diffusing dextran molecules quantitatively accounts for the extracted free energy values and demonstrates that elastic deformations of both the diffusor and the hydrogel network are important, in line with previous computational \cite{44-46} and experimental studies. \cite{47} Our model additionally suggests that the effective hydrogel mesh size is substantially larger than expected for a regular cubic hydrogel topology. This means that
the hydrogel presumably is topologically disordered and that dextran molecules preferentially move into larger hydrogel pores that are locally even more enlarged due to the osmotic pressure exerted by the dextran molecule on the local hydrogel network.

Diffusional barriers in biological systems often show a layered structure, as previously demonstrated for skin$^{28–30}$ and is also the case for mucous membranes, as found for instance in the gastrointestinal tract, schematically indicated in Figure 6B. For a layered system, eq. (9) shows that the individual piecewise constant permeability coefficients $P_i$ add up inversely as

$$\frac{1}{P_{\text{tot}}} = \sum_i \frac{1}{P_i} = \sum_i \frac{e^{\beta \Delta F_i}}{D_i L_i} = \sum_i \frac{L_i}{D_i K_i}$$

Equation (11)

where the sum goes over all layers, represented by their respective diffusion constants $D_i$, free energy values $\Delta F_i$ or partition coefficients $K_i$ and thicknesses $L_i$. Here, $P_{\text{tot}}$ denotes the total permeability, which is dominated by the smallest permeability in the inverse sum.

Figure 6B schematically illustrates permeation through a layered system which represents the mammalian stomach.$^{48}$ The outermost layer of mucus is only loosely bound and characterized by the permeability $P_1$, it is followed by a layer of more tightly bound mucus, characterized by $P_2$, and adheres onto the first layer of epithelial cells, characterized by $P_3$. The total thickness of this diffusional barrier is about a millimeter, with the two mucus layers spanning a few hundred micrometers only.$^{49}$ Measurements in rat gastrointestinal mucosa suggest typical values of $L_1 = 109$ $\mu$m, $L_2 = 80$ $\mu$m and $L_3 \approx L_2$, which are close to the range of gel thicknesses studied in this work.

The total permeability is determined by the free energies and the mobilities inside all layers. Nutrients for instance can easily penetrate through the epithelia of the gastrointestinal tract, displaying large permeabilities in the different layers. Pathogens on the other hand are in healthy environments kept from reaching the epithelium, due to low permeability in the tightly bound mucus layer ($P_2 \ll P_1$).$^{48}$ From eq. (11), it is apparent that the lowest permeability in such a layered system dominates the total permeability, leading to an effective barrier function that for different particles can be effected by different parts of the layered barrier structure.

The method introduced in this work can be used to determine free-energy and diffusivity profiles, and thereby effective permeabilities, of different kinds of fluorescently labeled molecules, particles or even organisms that penetrate into various layered systems, including systems that contain hydrogels and mucus. This will help to shed light on the underlying mechanisms of the function of biological barriers including mucous membranes.

## 4 Methods

### 4.1 Hydrogel Preparation.

The hydrogel is formed by cross-linking end-functionalized polyethylene glycol-bicyclo[6.1.0]non-4-yn (PEG-BCN) linkers with hyperbranched polyglycerol azide (hPG-N$_3$) hubs via strain-promoted azide-alkyne cycloaddition (SPAAC). The two macro-monomers PEG-BCN and hPG-N$_3$ are synthesized as previously described.$^{2,51}$ The click reaction of binding the PEG-BCN linkers to the hPG-N$_3$ hubs works in water, at room temperature, without the addition of a catalyst or external activation like heat or UV radiation and without the formation of byproducts. Two different sizes of PEG-BCN linkers are employed, having a molecular weight of either $M_{\text{PEG}} = 6$ or $M_{\text{PEG}} = 10$ kDa (for details about the mass distributions see Section ?? of the Supporting Information), the hydrogels are denoted as hPG-G6 and hPG-G10, respectively. The number ratio of the PEG-BCN linkers to the hPG-N$_3$ hubs ($M_{\text{hPG}} = 3$ kDa, 20% azide) is kept constant at 3:1 for both hPG-G6 and hPG-G10. This ratio can ideally lead to a cubic lattice structure if each hPG-hub
Table 2: Composition of the hydrogels used in this study. Here, \( V_{\text{PEG}}^{\text{sol}} \) and \( V_{\text{hPG}}^{\text{sol}} \) denote the volumes of the stock solutions, \( V_{\text{H}_2\text{O}} \) is the volume of purified water added to the resulting gel solutions and \( n_{\text{PEG}} \) and \( n_{\text{hPG}} \) denote the amount of PEG linkers and hPG-hubs in the gel solutions. From the total resulting volume of the gel solutions \( V_{\text{gel}}^{\text{sol}} \) only \( V_{\text{app}} = 1 \mu\text{L} \) was placed as a gel spot on the glass substrate, leading to the applied amount \( n_{\text{app}} \) and applied mass \( m_{\text{app}} \). Solution is of 8.5 wt% for 6 kDa PEG and 8.4 wt% for 10 kDa PEG. hPG solution is of 5 wt%.

| Gel       | \( n_{\text{PEG}} \) | \( V_{\text{PEG}}^{\text{sol}} \) | \( n_{\text{hPG}} \) | \( V_{\text{hPG}}^{\text{sol}} \) | \( V_{\text{H}_2\text{O}} \) | \( V_{\text{gel}}^{\text{sol}} \) | \( V_{\text{app}} \) | \( m_{\text{app}} \) | \( n_{\text{app}} \) |
|-----------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| hPG-G6    | 142 nmol             | 10 \( \mu\text{L} \) | 47 nmol              | 2.8 \( \mu\text{L} \) | 13.0 \( \mu\text{L} \) | 25.8 \( \mu\text{L} \) | 1 \( \mu\text{L} \) | 38 \( \mu\text{g} \) | 7.3 nmol             |
| hPG-G10   | 84 nmol              | 10 \( \mu\text{L} \) | 28 nmol              | 1.7 \( \mu\text{L} \) | 12.7 \( \mu\text{L} \) | 24.4 \( \mu\text{L} \) | 1 \( \mu\text{L} \) | 38 \( \mu\text{g} \) | 4.6 nmol             |

exactly binds to six PEG linkers. The chemical structure of the hPG-N\(_3\) hubs, however, allows on average for eight binding sites, making the hydrogel presumably quite disordered.

The two components of the hydrogel are stored as aqueous stock solutions at concentrations of 8.5 wt% (6 kDa PEG-BCN), 8.4 wt% (10 kDa PEG-BCN) and 5 wt% (hPG-N\(_3\)). To initiate hydrogel formation, they are mixed according to Table 2. The resulting gel solution is thoroughly vortexed before being placed as 1 \( \mu\text{L} \) drops on the glass substrate. Both hydrogel solutions are adjusted to have the same mass concentration. However, after drying and re-swelling on the glass substrate, volumes of the formed hydrogels are different and measured as \( V_{\text{tot}}^{\text{hPG-G6}} = 0.42 \pm 0.03 \mu\text{L} \) and \( V_{\text{tot}}^{\text{hPG-G10}} = 0.31 \pm 0.04 \mu\text{L} \) for hPG-G6 and hPG-G10, respectively (see Figure ?? in Section ?? of the Supporting Information). This results in a final hydrogel concentration of 9 wt% (\( \approx 90 \text{ mg/mL} \)) for hPG-G6 and 12 wt% (\( \approx 120 \text{ mg/mL} \)) for hPG-G10.

4.2 Estimate of Mean Hydrogel Mesh Size.

Assuming a cubic hydrogel network structure, the mean mesh size can be easily estimated. The length of a cubic unit cell \( l_0 \) follows from the total gel volume \( V_{\text{tot}} \) and the total number of hPG hubs \( n_{\text{hPG}}^{\text{tot}} \) in mol as

\[
l_0 = \frac{\sqrt[3]{V_{\text{tot}}}}{n_{\text{hPG}}^{\text{tot}}N_A} \tag{12}
\]

where \( N_A \) is the Avogadro constant. The total volumes for the re-hydrated gels are \( V_{\text{tot}}^{\text{hPG-G6}} = 0.42 \mu\text{L} \) and \( V_{\text{tot}}^{\text{hPG-G10}} = 0.31 \mu\text{L} \) as mentioned above. The total number of hPG hubs is given as \( n_{\text{hPG}}^{\text{tot}} = n_{\text{hPG}} \cdot V_{\text{app}} / V_{\text{gel}}^{\text{sol}} \), with the values from Table 2 for the respective gel and where we account for the fact that only \( V_{\text{app}} = 1 \mu\text{L} \) of the total gel solution \( V_{\text{gel}}^{\text{sol}} \) is applied onto the gel substrate.

This results in a rough estimates for the mesh size of \( l_0^{\text{hPG-G6}} = 7.1 \text{ nm} \) and \( l_0^{\text{hPG-G10}} = 7.5 \text{ nm} \), which shows that even though PEG linkers of significantly different masses were used, the mesh sizes of the two gels differ only slightly.

4.3 Dextran Preparation.

Dextrans conjugated with the dye fluorescein isothiocyanate (FITC) are obtained from Sigma-Aldrich as d4-FITC, d10-FITC, d20-FITC, d40-FITC and d70-FITC, the number stating the molecular weight in kDa of the commercial product. To remove unbound FITC from the dextran solutions, all batches are subjected to a desalting PD-10 column, which eliminates low-molecular weight compounds such as free FITC dye. This step is done according to the manufacturers recommendations and the column is equilibrated using phosphate buffer saline (PBS). Afterwards, the molecular weight distribution of all dextrans is determined by gel permeation chromatography (GPC) (see Section ?? of the Supporting Information).

4.4 Penetration Assay of FITC-labeled Dextrans.

After preparation of the hydrogel solutions and purification of the dextrans (see above),
penetration assays are performed with five different dextran solutions and two different gels. For these assays, coverslips (Menzel #1; VWR, Darmstadt, Germany) with a diameter of 25 mm and a thickness of 0.13-0.16 mm are thoroughly washed with water and absolute ethanol and subsequently dried under a stream of nitrogen. For every experiment, 1 µL of the respective hydrogel solution is placed in the center of the coverslip. The substrates with the applied gel spots are kept in a humid environment overnight, allowing hydrogel formation to be completed before the hydrogel spots are left to dry for 30 min at ambient conditions. Permeation experiments are performed within one day after hydrogel formation. To start a permeation experiment, a home-made polydimethylsiloxane (PDMS) stamp (1 x 1 cm) prepared with a cylindrical cavity in the middle (5 mm diameter) is placed on the coverslip, so that the dried hydrogel is located in the middle of the stamp’s cavity. The PDMS surrounding the dried hydrogel allows for the addition of solutions such as buffer or dextran. Prior to the measurement, 30 µL of PBS buffer are added to re-swell the hydrogel for 30 min, which typically creates hydrogel volumes of semi-spheroid shape with a base radius of 1050 µm and heights of about 150 µm for hPG-G10 and about 210 µm for hPG-G6 (see Section ?? of the Supporting Information). Afterwards, the coverslip is mounted on a Leica SP8 confocal laser scanning microscope (CLSM; Leica, Wetzlar, Germany) and imaged using a 20x objective (0.75 HC PL APO water immersion objective with correction ring). In a first step, the hydrogel is visually identified by imaging the sample like this, the PBS buffer is removed from the cavity and replaced by 35 µL of the FITC-dextran solution (0.07 mg/mL for all dextrans). This fixes the total length from the bottom of the glass dish at \( z = z_{\text{bot}} \) to the air-water interface at \( z = -z_{\text{top}} \), where \( z = 0 \) corresponds to the end of the measurement region (see Figure 1A). The total length of the solution is thus \( z_{\text{tot}} = z_{\text{top}} + z_{\text{bot}} = 1780 \mu m \). The individual contributions to \( z_{\text{tot}} \) vary, due to different gel thicknesses, changing the extent of the measured region, ranging from \( z = 0 \) to \( z = z_{\text{bot}} \) (cf. also Figure 1A).

About 10 seconds after the application of the dextran solution, the spatial distribution of the FITC-based fluorescence intensity is measured using a z-stack that starts 30 µm below and ends 410 µm above the glass-hydrogel interface (with 10 µm increments). The recorded intensities are afterwards truncated to probe the spatial FITC distribution within the hydrogel starting from the glass bottom (located at \( z_{\text{bot}} \)) and extending about 100 µm into the bulk solution, away from the gel-water interface located at \( z = z_{\text{int}} \) (cf. Figure 1A). In these measurements, the sample is excited at \( \lambda = 488 \text{nm} \) and the emission is recorded between 500 nm and 550 nm using a PMT. For the \( M_{\text{dex}} = 4 \text{ kDa} \) to the \( M_{\text{dex}} = 40 \text{ kDa} \) dextrans, one z-stack is recorded every \( \Delta t = 10 \text{ s} \), yielding time-resolved FITC distributions following the penetration of the dextran molecules into the hydrogel network over time. For the \( M_{\text{dex}} = 70 \text{ kDa} \) dextrans a period of \( \Delta t = 30 \text{ s} \) is used instead, in order to account for the much smaller diffusion coefficient of the larger dextran molecules. For all dextran types, measurements are performed at least three times with total measurement times of about 30 minutes, with the exception of the \( M_{\text{dex}} = 70 \text{ kDa} \) dextrans. Here only one measurement is performed for each gel but with a longer recording time of about 1 hour.

### 4.5 Fluorescence Correlation Spectroscopy of FITC-labeled Dextrans.

Reference diffusion coefficients for the FITC-labeled dextran molecules in the bulk solution are obtained using fluorescence correlation spectroscopy (FCS). The measurements are performed on a Leica TCS SP5 II CLSM with a FCS set-up from PicoQuant. The CLSM is equipped with an HCX PL APO 63x/1.20 W
CORR CS water immersion objective. Samples are put on high precision cover glasses (18 x 18 mm, 170 ± 5 µm thick) and excited with the 488 nm Argon laser line. The fluorescent light is passed through a 50/50 beam splitter with a lower wavelength cut-off of λ = 515 nm. Both channels are detected separately with a single photon avalanche diode (SPAD). Afterwards a pseudo-cross correlation is performed between both channels to eliminate the influence of detector after-pulsing. Prior to a measurement, the optical setup is calibrated with the water soluble Alexa-Fluor 488 dye. The correlated signal is fitted with two components and accounting for triplet states. The first component is fixed to a freely diffusing FITC-dye molecule where only the fraction is a fit parameter. The second component is set to a log-normal distributed species. The component fractions and means of distribution are fitted and the width of distribution is taken from previously performed gel permeation chromatography (GPC) measurements (see details about the fitting procedure see Section ?? of the Supporting Information). The fitted diffusion times are used to calculate the diffusion coefficients and hydrodynamic radii using the Stokes-Einstein relation.

4.6 Numerical Model and Discretization.

Extending a previously introduced method, spatially resolved diffusivity and free energy profiles are estimated from experimentally measured concentration profiles. Numerical profiles are computed by discretizing the entire experimental setup from the glass bottom through the gel and at least 100 µm into the bulk solution, away from the hydrogel-bulk interface, which leads to values of zbot ≈ 300 µm, depending on the exact thickness of the hydrogel in the respective measurement.

The numerical optimization problem is given by the cost function, which is defined as

$$\sigma^2(D, F, \vec{f}) := \frac{1}{N \cdot M} \sum_{j=1}^{N} \sum_{i=1}^{M} [c_i^{\text{num}}(t_j) - f_j \cdot c_i^{\text{exp}}(t_j)]^2$$

with N the total number of experimental profiles, M the total number of experimental data points per concentration profile and \(\sigma^2(D, F, \vec{f})\) being the mean squared deviation between the experimental and numerical profiles. The diffusivity profile \(D = D(z)\), the free energy landscape \(F = F(z)\) and the vector containing all scaling factors (see below for details) \(\vec{f} = (f_1, ..., f_j, ..., f_N)\) are all optimized to find the minimal value of \(\sigma^2\). This non-linear regression is performed using the trust region method implemented in python’s scipy package.

The numerical profiles

$$\vec{c}_{\text{num}}(t_j) = (c_1^{\text{num}}(t_j), ..., c_i^{\text{num}}(t_j), ..., c_M^{\text{num}}(t_j))^T$$

are computed from the diffusivity and free energy profiles as

$$\vec{c}_{\text{num}}(t_j) = e^{W_{i,k}} \cdot \vec{c}_{\text{init}}$$

where the rate matrix \(W(D, F)\) is defined as

$$W_{i,k} = \frac{D_i + D_k}{2\Delta z^2} e^{-\frac{E_i - E_k}{RT}}, \text{ with } k = i \pm 1$$

as explained previously. Numerical profiles at time \(t_j\) depend on the initial profile \(\vec{c}_{\text{init}}\) at \(t = 0\), which is determined as explained below.

The numerically computed profiles are fitted to the re-scaled experimental profiles \(\vec{c}_{\text{exp}}(t_j)\) at time \(t_j > 0\). The scaling factors \(\vec{f}\) are obtained
simultaneously from the fitting procedure and correct drifts in the experimentally measured fluorescence intensity profiles (see Section ?? of the Supporting Information). As a check, the numerical model is compared to the analytical solution for a model with piece-wise constant values of the diffusivity and free energy in the respective regions. Results from the numerical model agree perfectly with those from the analytical solution (see Section ?? of the Supporting Information).

4.7 Construction of the Initial Concentration Profile.

The initial profile \( \tilde{c}_{\text{init}} \), used for the computation of all later profiles according to eq. (14), needs to cover the entire computational domain and is generated by extending the first experimentally measured profile \( \tilde{c}_{\text{exp}}(t = 0) \) into the bulk regime (from \( z = 0 \) to \( z = -z_{\text{top}} \), cf. Figure 1A). We define \( t = 0 \) as the time of the first measurement, which is performed approximately 10 seconds after application of the dextran solution onto the gel-loaded substrate. For the extension, a constant initial concentration is assumed in the bulk, the value of which is taken as the experimentally measured value furthest into the bulk \( c_0 := c_1^{\text{exp}}(t = 0) \) at \( z = 0 \). This leads to the following expression used for the initial profile

\[
\tilde{c}_{\text{init}}^i := \begin{cases} 
    c_0, & \text{if } -z_{\text{top}} \leq z_i \leq 0 \\
    c_1^{\text{exp}}(t = 0), & \text{if } 0 < z_i \leq z_{\text{bot}} 
\end{cases} \tag{15}
\]

which by construction is continuous at \( z = 0 \). The initial profiles used for the fit procedure are shown in Figure 2B and F as black lines. In order to obtain concentration profiles in physical units, we set the first measured value furthest into the bulk equal to the applied dextran concentration \( c_0 = 0.07 \text{ mg/mL} \).

4.8 Free Energy and Diffusivity Profiles.

The diffusivity \( D(z) \) and free energy \( F(z) \) profiles are assumed to change in a sigmoidal shape from their values in the bulk solution to their values in the hydrogel. This sigmoidal shape is modeled using the following expressions

\[
D(z) = \frac{D_{\text{sol}} + D_{\text{gel}}}{2} + \frac{D_{\text{sol}} - D_{\text{gel}}}{2} \text{erf} \left( \frac{z - z_{\text{int}}}{\sqrt{2}d_{\text{int}}} \right) \\
F(z) = \frac{\Delta F_{\text{gel}}}{2} + \frac{\Delta F_{\text{gel}}}{2} \text{erf} \left( \frac{z - z_{\text{int}}}{\sqrt{2}d_{\text{int}}} \right) \tag{16}
\]

where \( \text{erf}(z) := 1/\sqrt{\pi} \int_{-z}^{z} e^{-z'^2} \, dz' \) is the error function. The fit parameters \( z_{\text{int}} \) and \( d_{\text{int}} \) determine the transition position and width, respectively, and are the same for the free energy and diffusivity profiles. Since only free energy differences carry physical meaning, the free energy in the bulk solution is set to zero, so that \( F_{\text{sol}} = 0 \). The values of the diffusivity and free energy in the hydrogel and in the bulk solution are thus determined by fitting the five parameters of eqs. (16), namely \( D_{\text{gel}}, \Delta F_{\text{gel}}, D_{\text{sol}}, z_{\text{int}} \) and \( d_{\text{int}} \), to the experimentally measured concentration profiles.

Confidence intervals for the obtained parameters of \( D_{\text{sol}}, D_{\text{gel}} \) and \( \Delta F_{\text{gel}} \) are estimated by determining the parameter values that change \( \sigma \) by not more than 50% (for details see Section ?? of the Supporting Information). The error bars shown in Figure 4 are then obtained by averaging the confidence intervals over all measurements.

Supporting Information Available

The following files are available free of charge.

??: Scaling of Diffusion Constant with Dextran Size; ??: Expression for the Elastic Deformation Free Energy; ??: Estimating PEG-Monomer Hydration Number; ??: Permeability Coefficient; ??: Molecular Mass Distributions of PEG linkers and Dextran Molecules; ??: Hydrogel Volume Reconstruction; ??: Fitting Procedure for FCS Measurements; ??: Drifts in the Measured Fluorescence Intensity Data; ??: Analytical Solution for Two-Segment System; ??: Error Estimate for Numerical Analysis.
Acknowledgement The authors acknowledge funding by the Deutsche Forschungsgemeinschaft (DFG) via grant SFB 1449.

References

(1) Wirthl, D.; Pichler, R.; Drack, M.; Kettlguber, G.; Moser, R.; Gerstmayr, R.; Hartmann, F.; Bradt, E.; Kaltseis, R.; Siket, C. M.; Schausberger, S. E.; Hild, S.; Bauer, S.; Kaltenbrunner, M. Science Advances 2017, 3, e1700053, DOI: 10.1126/sciadv.1700053.

(2) Herrmann, A.; Kaufmann, L.; Dey, P.; Haag, R.; Schedler, U. ACS Applied Materials & Interfaces 2018, 10, 11382–11390, DOI: 10.1021/acsami.8b01860.

(3) Li, J.; Mooney, D. J. Nature Reviews Materials 2016, 1, 16071, DOI: 10.1038/natrevmats.2016.71.

(4) Billings, N.; Birjiniuk, A.; Samad, T. S.; Doyle, P. S.; Ribbeck, K. Reports on Progress in Physics 2015, 78, 036601, DOI: 10.1088/0034-4885/78/3/036601.

(5) Rosales, A. M.; Anseth, K. S. Nature Reviews Materials 2016, 1, 15012, DOI: 10.1038/natrevmats.2015.12.

(6) Lieleg, O.; Vladescu, I.; Ribbeck, K. Biophysical Journal 2010, 98, 1782–1789, DOI: 10.1016/j.bpj.2010.01.012.

(7) Wagner, C.; Wheeler, K.; Ribbeck, K. Annual Review of Cell and Developmental Biology 2018, 34, 189–215, DOI: 10.1146/annurev-cellbio-100617-062818.

(8) Hollingsworth, M. A.; Swanson, B. J. Nature Reviews Cancer 2004, 4, 45–60, DOI: 10.1038/nrc1251.

(9) McGuckin, M. A.; Lindén, S. K.; Sutton, P.; Florin, T. H. Nature Reviews Microbiology 2011, 9, 265–278, DOI: 10.1038/nrmicro2538.

(10) Dawson, M.; Wirtz, D.; Hanes, J. Journal of Biological Chemistry 2003, 278, 50393–50401, DOI: 10.1074/jbc.M309026200.

(11) Lai, S. K.; O’Hanlon, D. E.; Harrold, S.; Man, S. T.; Wang, Y.-Y.; Cone, R.; Hanes, J. Proceedings of the National Academy of Sciences 2007, 104, 1482–1487, DOI: 10.1073/pnas.06086110104.

(12) Johansson, M. E. V. PLoS ONE 2012, 7, e41009, DOI: 10.1371/journal.pone.0041009.

(13) Button, B.; Cai, L.-H.; Ehre, C.; Kesimer, M.; Hill, D. B.; Sheehan, J. K.; Boucher, R. C.; Rubinstein, M. Science 2012, 337, 937–941, DOI: 10.1126/science.1223012.

(14) Witten, J.; Ribbeck, K. Nanoscale 2017, 9, 8080–8095, DOI: 10.1039/C6NR09736G.

(15) Li, L. D.; Crouzier, T.; Sarkar, A.; Dunphy, L.; Han, J.; Ribbeck, K. Biophysical Journal 2013, 105, 1357–1365, DOI: 10.1016/j.bpj.2013.07.050.

(16) Marczynski, M.; Käsdorf, B. T.; Altaner, B.; Wenzler, A.; Gerland, U.; Lieleg, O. Biomaterials Science 2018, 6, 3373–3387, DOI: 10.1039/C8BM00664D.

(17) Zhang, X.; Hansing, J.; Netz, R. R.; DeRouchey, J. E. Biophysical Journal 2015, 108, 530–539, DOI: 10.1016/j.bpj.2014.12.009.

(18) Hansing, J.; Ciemer, C.; Kim, W. K.; Zhang, X.; DeRouchey, J. E.; Netz, R. R. The European Physical Journal E 2016, 39, 53, DOI: 10.1140/epje/i2016-16053-2.

(19) Hansing, J.; Netz, R. R. Biophysical Journal 2018, 114, 2653–2664, DOI: 10.1016/j.bpj.2018.04.041.

(20) Hansing, J.; Duke, J. R.; Fryman, E. B.; DeRouchey, J. E.; Netz, R. R. Nano Letters 2018, 18, 5248–5256, DOI: 10.1021/acs.nanolett.8b02218.
(21) Dawson, M.; Krauland, E.; Wirtz, D.; Hanes, J. Biotechnology Progress 2004, 20, 851–857, DOI: 10.1021/bp0342553.

(22) Wagner, C. E.; Turner, B. S.; Rubinstein, M.; McKinley, G. H.; Ribbeck, K. Biomacromolecules 2017, 18, 3654–3664, DOI: 10.1021/acs.biomac.7b00809.

(23) Wilking, J. N.; Zaburdaev, V.; De Volder, M.; Losick, R.; Brenner, M. P.; Weitz, D. A. Proceedings of the National Academy of Sciences 2013, 110, 848–852, DOI: 10.1073/pnas.1216376110.

(24) Lawrence, J. R.; Wolfaardt, G. M.; Korber, D. R. Applied and Environmental Microbiology 1994, 60, 1166–1173, DOI: 10.1128/AEM.60.4.1166-1173.1994.

(25) Sassi, A. P.; Shaw, A. J.; Sang Min Han; Blanch, H. W.; Prausnitz, J. M. Polymer 1996, 37, 2151–2164, DOI: 10.1016/0032-3861(96)85860-5.

(26) Herrmann, A.; Rödiger, S.; Schmidt, C.; Schierack, P.; Schedler, U. Analytical Chemistry 2019, 91, 8484–8491, DOI: 10.1021/acs.analchem.9b01586.

(27) Furter, M.; Sellin, M. E.; Hansson, G. C.; Hardt, W.-D. Cell Reports 2019, 27, 2665–2678.e3, DOI: 10.1016/j.celrep.2019.04.106.

(28) Schulz, R.; Yamamoto, K.; Klossek, A.; Flesch, R.; Hönzke, S.; Rancan, F.; Vogt, A.; Blume-Peytavi, U.; Hedtrich, S.; Schäfer-Korting, M.; Rührl, E.; Netz, R. R. Proceedings of the National Academy of Sciences 2017, 114, 3631–3636, DOI: 10.1073/pnas.1620636114.

(29) Schulz, R.; Yamamoto, K.; Klossek, A.; Rancan, F.; Vogt, A.; Schütte, C.; Rührl, E.; Netz, R. R. Biophysical Journal 2019, 117, 998–1008, DOI: 10.1016/j.bpj.2019.07.027.

(30) Lohan, S. B.; Saeidpour, S.; Colombo, M.; Staufenbiel, S.; Unbehauen, M.; Wolde-Kidan, A.; Netz, R. R.; Bodmeier, R.; Haag, R.; Teutloff, C.; Bittl, R.; Meinke, M. C. Pharmaceutics 2020, 12, 400, DOI: 10.3390/pharmaceutics12050400.

(31) Risken, H.; Frank, T. The Fokker-Planck Equation: Methods of Solution and Applications; Springer Series in Synergetics; Springer Berlin Heidelberg, 2012.

(32) Gribbon, P.; Hardingham, T. E. Biophysical Journal 1998, 75, 1032–1039, DOI: 10.1016/S0006-3495(98)77592-7.

(33) Cheng, Y.; Prud’homme, R. K.; Thomas, J. L. Macromolecules 2002, 35, 8111–8121, DOI: 10.1021/ma0107758.

(34) Bu, Z.; Russo, P. S. Macromolecules 1994, 27, 1187–1194, DOI: 10.1021/ma00083a017.

(35) Rubinstein, M.; Colby, R. H. Polymer Physics; OUP Oxford, 2003.

(36) Netz, R. R.; Andelman, D. Physics Reports 2003, 380, 1–95, DOI: 10.1016/S0370-1573(03)00118-2.

(37) Granath, K. A. Journal of Colloid Science 1958, 13, 308–328, DOI: 10.1016/0095-8522(58)90041-2.

(38) de Gennes, P. G. The Journal of Chemical Physics 1971, 55, 572–579, DOI: 10.1063/1.1675789.

(39) Wang, Y.-Y.; Lai, S. K.; Suk, J. S.; Pace, A.; Cone, R.; Hanes, J. Angewandte Chemie International Edition 2008, 47, 9726–9729, DOI: 10.1002/anie.200803526.

(40) Moore, M. The Mathematical Gazette 1974, 58, 181, DOI: 10.2307/3615957.

(41) Liese, S.; Gensler, M.; Krysiak, S.; Schwarzl, R.; Achazi, A.; Paulus, B.; Hugel, T.; Rabe, J. P.; Netz, R. R. ACS Nano 2017, 11, 702–712, DOI: 10.1021/acsnano.6b07071.
(42) Giddings, J. C.; Kucera, E.; Russell, C. P.; Myers, M. N. *The Journal of Physical Chemistry* **1968**, *72*, 4397–4408, DOI: [10.1021/j100859a008](https://doi.org/10.1021/j100859a008).

(43) Diamond, J. M.; Katz, Y. *The Journal of Membrane Biology* **1974**, *17*, 121–154, DOI: [10.1007/BF01870176](https://doi.org/10.1007/BF01870176).

(44) Zhou, H.; Chen, S. B. *Physical Review E* **2009**, *79*, 021801, DOI: [10.1103/PhysRevE.79.021801](https://doi.org/10.1103/PhysRevE.79.021801).

(45) Godec, A.; Bauer, M.; Metzler, R. *New Journal of Physics* **2014**, *16*, 092002, DOI: [10.1088/1367-2630/16/9/092002](https://doi.org/10.1088/1367-2630/16/9/092002).

(46) Kumar, P.; Theeyancheri, L.; Chaki, S.; Chakrabarti, R. *Soft Matter* **2019**, *15*, 8992–9002, DOI: [10.1039/C9SM01822K](https://doi.org/10.1039/C9SM01822K).

(47) Mohamed, H. F. M.; Kobayashi, Y.; Kuroda, C. S.; Ohira, A. *The Journal of Physical Chemistry B* **2009**, *113*, 2247–2252, DOI: [10.1021/jp808960w](https://doi.org/10.1021/jp808960w).

(48) Johansson, M. E. V.; Sjövall, H.; Hansson, G. C. *Nature Reviews Gastroenterology & Hepatology* **2013**, *10*, 352–361, DOI: [10.1038/nrgastro.2013.35](https://doi.org/10.1038/nrgastro.2013.35).

(49) Jordan, N.; Newton, J.; Pearson, J.; Allen, A. *Clinical Science* **1998**, *95*, 97–106, DOI: [10.1042/cs0950097](https://doi.org/10.1042/cs0950097).

(50) Atuma, C.; Strugala, V.; Allen, A.; Holm, L. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **2001**, *280*, G922–G929, DOI: [10.1152/ajpgi.2001.280.5.G922](https://doi.org/10.1152/ajpgi.2001.280.5.G922).

(51) Dey, P.; Schneider, T.; Chiappisi, L.; Gradzielski, M.; Schulze-Tanzil, G.; Haag, R. *Macromolecular Bioscience* **2016**, *16*, 580–590, DOI: [10.1002/mabi.201500377](https://doi.org/10.1002/mabi.201500377).

(52) Branch, M. A.; Coleman, T. F.; Li, Y. *SIAM Journal on Scientific Computing* **1999**, *21*, 1–23, DOI: [10.1137/S1064827595289108](https://doi.org/10.1137/S1064827595289108).
Graphical TOC Entry

\[ \Delta F \propto M \]
\[ D \propto M \]
Supporting Information:

Particle Diffusivity and Free-Energy Profiles in Inhomogeneous Hydrogel Systems from Time-Resolved Penetration Profiles

Amanuel Wolde-Kidan,†,§ Anna Herrmann,‡,§ Albert Prause,¶ Michael Gradzielski,¶ Rainer Haag,‡ Stephan Block,‡ and Roland R. Netz*,†

†Fachbereich Physik, Freie Universität Berlin, Arnimallee 14, 14195 Berlin, Germany
‡Institut für Chemie und Biochemie, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany
¶Institut für Chemie, Straße des 17. Juni 124, Technische Universität Berlin, 10623 Berlin, Germany
§These two authors contributed equally to this work.

E-mail: rnetz@physik.fu-berlin.de

arXiv:2006.10676v2  [cond-mat.soft]  1 Jul 2020
Scaling of Diffusion Constant with Dextran Size

According to the Stokes-Einstein relation, the diffusion constant of dextran molecules in the bulk solution $D_{\text{sol}}$ is expected to scale with the dextran radius $r_0$ as

$$D_{\text{sol}} \propto r_0^{-1} \quad (S1)$$

Assuming the dextran polymer behaves like a freely jointed chain, its radius is related to the number of monomers $N_{\text{dex}}$ as

$$r_0 = b_0^{\text{dex}} \sqrt{N_{\text{dex}}} \quad (S2)$$

where $b_0^{\text{dex}}$ is the dextran monomer length and $N_{\text{dex}}$ can be estimated from the total molecular mass of a dextran molecule $M_{\text{dex}}$, when the monomer mass $M_{\text{mono}}^{\text{dex}}$ is known

$$N_{\text{dex}} = \frac{M_{\text{dex}}}{M_{\text{mono}}^{\text{dex}}} \quad (S3)$$

This leads to the following equality

$$D_{\text{sol}} = \frac{k_B T}{6 \pi \eta \left( b_0^{\text{dex}} \sqrt{M_{\text{mono}}^{\text{dex}}} \sqrt{M_{\text{dex}}^{\text{dex}}} \right)} \quad (S4)$$

which gives rise to a scaling of $D_{\text{sol}} \propto M_{\text{dex}}^{-1/2}$. 
S2 Expression for the Elastic Deformation Free Energy

The free energy cost for stretching a polymer chain from an initial equilibrium mean square end-to-end distance $\langle \vec{R}_0^2 \rangle$ to a larger end-to-end distance $\langle \vec{R}^2 \rangle$ can be written as\textsuperscript{1,2}

$$\Delta F_{\text{stretch}} = \frac{3}{2} k_B T \frac{\langle \vec{R}^2 \rangle - \langle \vec{R}_0^2 \rangle}{\langle \vec{R}_0^2 \rangle}$$  \hspace{1cm} (S5)

where $k_B T$ denotes the thermal energy. In the case of the PEG linkers we define the z-component of the end-to-end distance as $l := \langle R_z \rangle$, so that $l_0 := \langle R_{0,z} \rangle$. The PEG polymer chain is now only stretched in the z-direction, thus eq. (S5) reduces to

$$\Delta F_{\text{stretch}} = \frac{3}{2} k_B T \left[ l^2 - \langle R_{0,x}^2 \rangle - \langle R_{0,y}^2 \rangle - l_0^2 \right] = \frac{3}{2} k_B T \left[ l^2 - l_0^2 \right]$$  \hspace{1cm} (S6)

since $\langle R_{x}^2 \rangle = \langle R_{0,x}^2 \rangle$ and $\langle R_{y}^2 \rangle = \langle R_{0,y}^2 \rangle$. As the PEG polymer chain performs a random walk in all three spatial dimensions, all components of the mean squared end-to-end distance contribute equally and so

$$\frac{\langle \vec{R}_0^2 \rangle}{3} = \langle R_{0,x}^2 \rangle = \langle R_{0,y}^2 \rangle = \langle R_{0,z}^2 \rangle = l_0^2$$  \hspace{1cm} (S7)

Together with eq. (S6), eq. (S7) leads to the stretching free energy for a single PEG linker polymer chain

$$\Delta F_{\text{stretch}} = \frac{1}{2} k_B T \left[ \frac{l^2}{l_0^2} - 1 \right]$$  \hspace{1cm} (S8)

For the compression of a polymer chain from an initially larger mean square end-to-end distance $\langle \vec{R}_0^2 \rangle$ to a smaller one $\langle \vec{R}^2 \rangle$ we write\textsuperscript{1,2}

$$\Delta F_{\text{compress}} = \frac{3}{2} k_B T \frac{\langle \vec{R}_0^2 \rangle - \langle \vec{R}^2 \rangle}{\langle \vec{R}^2 \rangle}$$  \hspace{1cm} (S9)

In the same way as above, we only allow compression along the z-axis, which leads to
\[\Delta F_{\text{compress}} = \frac{3}{2} k_B T \frac{\langle R_{0,x}^2 \rangle + \langle R_{0,y}^2 \rangle + l_0^2 - \langle R_x^2 \rangle - \langle R_y^2 \rangle - l^2}{\langle R_x^2 \rangle + \langle R_y^2 \rangle + l^2} = \frac{3}{2} k_B T \frac{l_0^2 - l^2}{\langle R_{0,x}^2 \rangle + \langle R_{0,y}^2 \rangle + l^2}\]  

(S10)

Using eq. (S7) to substitute the x- and y-components of the equilibrium end-to-end distance gives the expression for the compression free energy of a single PEG linker

\[\Delta F_{\text{compress}} = \frac{1}{2} k_B T \frac{3l_0^2 - 3l^2}{2l_0^2 + l^2}\]  

(S11)

The total elastic deformation free energy per PEG linker is the sum of eq. (S8) and eq. (S11)

\[\Delta F_{\text{PEG}} = \frac{1}{2} k_B T \left( \left[ \frac{l}{l_0} \right]^2 + \frac{l_0^2 - 4l^2}{2l_0^2 + l^2} \right)\]  

(S12)

For the twelve PEG linkers of the hydrogel unit cell this leads to eq. (??) of the main text.
S3 Estimating PEG-Monomer Hydration Number

Based on eq. (??) and eq. (??) from the main text, we obtain the following relation between the partition coefficient $K_{\text{gel}}$ and the volume accessible to the dextran diffusors $V_{\text{free}}$

$$K_{\text{gel}} = \frac{V_{\text{free}}}{V_{\text{unit}}} e^{-\beta(\Delta F_{\text{dex}} + \Delta F_{\text{PEG}})}$$  \hspace{1cm} (S13)

The volume inaccessible to the dextran molecules $V_{\text{ex}}$ (see eq. (??) of the main text) is composed of a part occupied by the gel or dextran directly and a part due to tightly bound hydration water, so that

$$V_{\text{ex}} = V_{\text{unit}} - V_{\text{free}} = V_{\text{gel}} + V_{\text{hyd}} + V_{\text{dex}}$$  \hspace{1cm} (S14)

where $V_{\text{gel}} = V_{\text{PEG}} + V_{\text{hPG}}$ denotes the excluded volume due to both gel components, $V_{\text{dex}} = 3\pi r^2 l - \frac{32}{3} \pi r^3$ (with $r$ and $l$ as explained in Figure ??A in the main text) denotes the excluded volume due to dextran, $V_{\text{unit}} = l^3$ is the unit cell volume and $V_{\text{hyd}}$ is the volume occupied by hydration water. Since $r$ denotes the hydrodynamic radius of the spherical dextran, we assume $V_{\text{hyd}}$ to be the volume of hydration water only binding to the gel components.

The mass fraction $\Phi_{\text{gel}}$ of the gel components inside the hydrogel is defined as the ratio of the mass of the gel components $m_{\text{gel}}$ to the total mass $m_{\text{tot}}$, but since the mass density of the gel components is comparable to that of water, it also represents the fraction of inaccessible volume due to the gel components

$$\Phi_{\text{gel}} := \frac{m_{\text{gel}}}{m_{\text{tot}}} \approx \frac{V_{\text{gel}}}{V_{\text{unit}}}$$  \hspace{1cm} (S15)

In the same fashion we can also estimate the fraction of inaccessible volume due to only the PEG linkers as

$$\Phi_{\text{PEG}} = \frac{m_{\text{PEG}}}{m_{\text{tot}}} = \frac{n_{\text{PEG}} M_{\text{PEG}}}{m_{\text{tot}}} \frac{V_{\text{app}}}{V_{\text{sol}}^{\text{gel}}} \approx \frac{V_{\text{PEG}}}{V_{\text{unit}}}$$  \hspace{1cm} (S16)
where we use the values for the number \( n_{\text{PEG}} \) and the molar mass \( M_{\text{PEG}} \) of the PEG linkers given in Table ?? of the Methods Section. The factor of \( V_{\text{app}}/V_{\text{gel}}^{\text{sol}} \) accounts for the fact that only \( V_{\text{app}} = 1 \, \mu\text{L} \) of the total volume of prepared gel solution \( V_{\text{gel}}^{\text{sol}} \) are actually placed on the gel spot for the experiments. The total mass of the hydrogel \( m_{\text{tot}} \) is estimated from the measured hydrogel volumes by using the water mass density (cf. also Section S6). Combining eq. (S13), eq. (S14), and eq. (S15) from above gives the following expression for volume fraction occupied by hydration water

\[
\frac{V_{\text{hyd}}}{V_{\text{unit}}} = 1 - \Phi_{\text{gel}} - K_{\text{gel}} e^{\beta(\Delta F_{\text{dex}} + \Delta F_{\text{PEG}})} - 3\pi \left(\frac{r}{l}\right)^2 + \frac{32}{3} \left(\frac{r}{l}\right)^3 \tag{S17}
\]

The hydration water of eq. (S17) in principle binds to the entire hydrogel, meaning the hPG hubs and the PEG linkers. For a rough estimate, we neglect the presence of the hPG hubs and assume \( V_{\text{hyd}} \) is the volume of water molecules hydrating only the PEG linkers. We can compute the fraction of hydration water per unit PEG volume from eq. (S16) and eq. (S17) as

\[
\frac{V_{\text{hyd}}}{V_{\text{PEG}}} = \frac{n_{\text{hyd}} v_{\text{w}}}{n_{\text{PEG}} v_{\text{PEG}}} = 1 - \Phi_{\text{gel}} - K_{\text{gel}} e^{\beta(\Delta F_{\text{dex}} + \Delta F_{\text{PEG}})} - \frac{3\pi}{\Phi_{\text{PEG}}} \left(\frac{r}{l}\right)^2 + \frac{32}{3} \left(\frac{r}{l}\right)^3 \tag{S18}
\]

where \( n_{\text{hyd}} \) is the number of hydration water molecules, \( v_{\text{w}} \) is their partial volume, \( n_{\text{PEG}} \) is the number of PEG linkers and \( v_{\text{PEG}} \) is the PEG linker partial volume. The ratio between the partial volumes of water and the PEG linkers is approximated by the ratio of their molar masses as

\[
\frac{v_{\text{w}}}{v_{\text{PEG}}} \approx \frac{M_{\text{w}}}{M_{\text{PEG}}} \tag{S19}
\]

with the water molar mass \( M_{\text{w}} = 18 \, \text{g/mol} \) and the molar mass of the respective PEG linker \( M_{\text{PEG}} \) (see Methods Section ??). From eq. (S18), we can now compute the number of hydration waters per PEG linker molecule as
In order to obtain the number of hydration waters per PEG-monomer we simply divide eq. (S20) by the respective number of PEG-monomers per linker $N_{PEG}$, which we obtain from the ratio of total linker mass $M_{PEG}$ and PEG-monomer mass $M_{mono}^{PEG} = 44 \text{ g/mol}$, as $N_{PEG} = \frac{M_{PEG}}{M_{mono}^{PEG}}$. The number of hydration waters per PEG-monomer can thus be obtained as

$$\frac{n_{hyd}}{n_{PEG}} = \frac{1 - \Phi_{gel} - K_{gel} e^{\beta(\Delta F_{dex} + \Delta F_{PEG})} - 3\pi \left( \frac{r}{t} \right)^2 + \frac{32}{3} \left( \frac{r}{t} \right)^3 \frac{M_{PEG}}{M_w}}{\Phi_{PEG}}$$  \hspace{1cm} (S20)

With the values of $K_{gel} e^{\beta(\Delta F_{dex} + \Delta F_{PEG})}$, $V_{dex}/V_{unit} = 3\pi \left( \frac{r}{t} \right)^2 - \frac{32}{3} \left( \frac{r}{t} \right)^3$, $\Phi_{gel}$ and $\Phi_{PEG}$ for the two hydrogels from the main text, eq. (S21) allows us to estimate the number of hydration waters per PEG monomer for all measurements. Figure S1 shows the results of the calculation for each of the two hydrogels. Estimated values scatter around 8 water molecules per PEG monomer, with a slight dependence on the PEG linker length and dextran mass. Depending on the employed experimental method, values reported in the literature vary, ranging from 2 to 11 water molecules per PEG monomer.\textsuperscript{3–7} Additionally, an increase of the hydration waters per monomer has been observed, as a function of the polymerization degree.\textsuperscript{8} The values obtained from our estimates, all lie within the range of values reported in the literature, as indicated by the grey shaded area in Figure S1. This further corroborates our methodology and specifically the model for the free energy of eq. (??), since the estimate of eq. (S21) is based on this model.
Supplementary Figure S1: Estimated number of water molecules per PEG monomer \( n_{\text{hyd}}/n_{\text{mono}}^{\text{PEG}} \) from the obtained values of \( K_{\text{gel}} \) for the two hydrogels based on eq. (S21). The estimated values scatter around \( n_{\text{hyd}}/n_{\text{PEG}}^{\text{mono}} = 8 \) and agree with the range of values reported in the literature indicated as the grey shaded area, ranging from \( n_{\text{hyd}}/n_{\text{PEG}}^{\text{mono}} = 2 \) to \( n_{\text{hyd}}/n_{\text{PEG}}^{\text{mono}} = 11 \).
S4 Permeability Coefficient

The definition of the permeability coefficient $P$ is, as stated in the main text:

$$P(z_1, z_2) := \frac{J}{c(z_1) - c(z_2)} \tag{S22}$$

with the stationary flux $J$ and the equilibrium concentrations on both sides of the barrier $c(z_1)$ and $c(z_2)$. From the generalized diffusion equation (??) in the main text, one obtains the stationary flux for the case of $\partial c(z, t)/\partial t = 0$ as

$$J = D(z)e^{-\beta F(z)} \frac{\partial}{\partial z} (c(z, t)e^{\beta F(z)}) \tag{S23}$$

as a function of the diffusion constant $D(z)$ and the free energy landscape $F(z)$ across the barrier. After rearranging eq. (S23) and integrating from one side of the barrier from $z_1$ to the other $z_2$, we obtain the following relation

$$J \int_{z_1}^{z_2} \frac{e^{\beta F(z)}}{D(z)} \, dz = c(z_1, t)e^{\beta F(z_1)} - c(z_2, t)e^{\beta F(z_2)} \tag{S24}$$

We now assume that the free energy value is the same on both sides of the barrier and additionally set it to zero as reference so that $F(z_1) = F(z_2) = 0$ and thus

$$J \int_{z_1}^{z_2} \frac{e^{\beta F(z)}}{D(z)} \, dz = c(z_1, t) - c(z_2, t) \tag{S25}$$

which, in combination with eq. (S22), gives eq. (??) used in the main text

$$\frac{1}{P} = \int_{z_1}^{z_2} \frac{e^{\beta F(z)}}{D(z)} \, dz \tag{S26}$$
**S5 Molecular Mass Distributions of PEG linkers and Dextran Molecules**

The dextran molecular mass distribution is characterized using gel permeation chromatography (GPC), the results of which are presented in Table S1. GPC measurements are performed on an *Agilent* device (1100er series) with a PSS Suprema column (pre-column, 1x with pore-size of 30 Å, 2x with poresize of 1000 Å, all of them with a particle size of 10 µm), with pullulan as calibration standard, and ethylene glycol as internal standard. As solvent, H$_2$O with 0.1 M NaNO$_3$ is used. For the characterization of the PEG linker mass distribution, matrix assisted laser desorption ionization (MALDI) on a *Bruker Ultraflex* II is performed. The obtained values are shown in Table S2. From the number average molecular mass $M_n$ and the weight average molecular mass $M_w$ the polydispersity index $PDI$ is determined as

$$PDI = \frac{M_w}{M_n}$$ \hspace{1cm} (S27)

The dextran molecules show a considerable dispersion regarding the molecular mass, while the PEG linker mass distribution is rather uniform.

**Table S1: Results obtained from GPC measurements of the different dextran molecules.**

| $M_{\text{dex}}$ [kDa] | $M_w$ [kDa] | $M_n$ [kDa] | $PDI$ |
|------------------------|-------------|-------------|-------|
| 4                      | 3.55        | 2.32        | 1.53  |
| 10                     | 9.55        | 5.55        | 1.72  |
| 20                     | 16.5        | 9.42        | 1.75  |
| 40                     | 35.7        | 19.5        | 1.84  |
| 70                     | 61.9        | 50.1        | 1.24  |

**Table S2: Molecular weights and polydispersity of the PEG linkers as measured in MALDI experiments.**

| $M_{\text{PEG}}$ [kDa] | $M_w$ [kDa] | $M_n$ [kDa] | $PDI$ |
|------------------------|-------------|-------------|-------|
| 6                      | 6.29        | 5.90        | 1.07  |
| 10                     | 11.3        | 11.3        | 1.00  |
S6 Hydrogel Volume Reconstruction

The hydrogel volume is determined using confocal laser scanning microscopy as follows. The hydrogels are first equilibrated using PBS buffer, followed by injection of the $M_{\text{dex}} = 70\text{ kDa}$ FITC-labeled dextran and recording of 3D dextran concentration profiles using confocal microscopy (covering an imaged volume of $2,304 \times 2,304 \times 0.5\text{ mm}^3$). The permeation measurements (shown in the main text) reveal that this dextran is too large to penetrate the hydrogel, so that the hydrogel can be identified in these 3D concentration profiles based on an exclusion of FITC-labeled dextran (i.e., absence of FITC fluorescence; see Figure S2). In order to quantify the hydrogel volume, the (2D) hyperplane at which the FITC intensity has dropped to 50% of its bulk value is determined using home-written scripts in Matlab (MathWorks, Natick, MA). This hyperplane indicates the positions, at which the point spread function of the confocal microscope is equally filled by the FITC-dextran bulk solution and either the hydrogel or the supporting substrate, and therefore allows for extraction of the exact locations of the substrate and hydrogel interface within the sample. Furthermore, as the substrate is a flat glass slide, the hydrogel-substrate interface is extracted from this data by first fitting a plane to the regions corresponding to the substrate-bulk interface (i.e., in regions far away from the hydrogel spot) and by interpolating the position of this plane underneath the hydrogel. This procedure allows for extraction of the entire hydrogel boundary, allowing determination of the hydrogel volume by numerical integration. This yields volumes of $V_{\text{tot}}^{h\text{PG-G6}} = 0.42 \pm 0.03\mu\text{L}$ and $V_{\text{tot}}^{h\text{PG-G10}} = 0.31 \pm 0.04\mu\text{L}$ for the two hydrogels.
Supplementary Figure S2: In these experiments, the hydrogels have been equilibrated using PBS buffer (as described in Methods Section ?? of the main text) followed by application of a $M_{\text{dex}} = 70$ kDa dextran solution. The dextran is too large to penetrate into the hydrogel as evidenced by using confocal fluorescence microscopy to record multiple sample sections (at different z-heights as indicated in (a)). In these images, the hydrogel appears as a black circle, the radius of which decreases with increasing distance to the glass interface (located at the z-height $z_{\text{bot}}$). The bright areas correspond to FITC-dextran solution in the bulk. These images can be used to extract the position of the glass interface and hydrogel in space. A 3D representation and a side view of this hyperplane are given in (b) and (c), respectively, showing that the hydrogels possess a semi-elliptical shape with radii being on the order of 1050 µm (major axis) and 150 – 210 µm (minor axis). This figure shows a representative measurement for the hPG-G6 gel. The scale bars in (a) correspond to 500 µm.
S7 Fitting Procedure for FCS Measurements

An accurate description of the dextran autocorrelation functions (ACFs) $G(\tau)$ required to use at least 2 components, which originate from the fluorescence emission of the FITC-labeled dextrans and, in addition, from residuals of free FITC molecules (i.e., not being conjugated to dextran). The FCS ACF $G(\tau)$ was therefore described using the equation

$$G(\tau) = T(\tau) \cdot (G_F(\tau) + G_{DF}(\tau))$$  \hspace{1cm} (S28)

in which $G_F(\tau)$ and $G_{DF}(\tau)$ give the contributions from free FITC molecules and FITC-labeled dextrans, respectively, while the term $T(\tau)$ accounts for triplet state dynamics according to

$$T(\tau) = \frac{(1 - \Phi_T + \Phi_T e^{-\frac{\tau}{\tau_T}})}{1 - \Phi_T}$$  \hspace{1cm} (S29)

with $\Phi_T$ denoting the fraction of molecules in the triplet state and $\tau_T$ the corresponding decay time of triplet states.$^{10}$

The contribution of the free FITC molecules was modeled using the theoretically derived ACF for free diffusion in 3D

$$G_F(\tau) = \rho_F \left(1 + \frac{\tau}{\tau_F}\right)^{-1} \left(1 + \frac{\tau}{\tau_F \kappa^2}\right)^{-\frac{1}{2}}$$  \hspace{1cm} (S30)

in which $\rho_F$ denotes the average number density of free FITC molecules in the confocal readout volume, $\tau$ the lag time of the ACF, $\kappa$ the ratio of axial $r_z$ to radial extension $r_{xy}$ of the confocal readout volume, and $\tau_F$ the decay time.$^{11}$ The value of $\tau_F$ was determined from calibration measurements on FITC molecules diffusing in buffer and $\kappa$ was fixed to 6,$^{12}$ so that only the density $\rho_F$ was a free parameter when fitting the component $G_F(\tau)$ to experimentally determined ACF.

Since the dextrans showed a log-normal size distribution, as observed in the GPC mea-
surements, the component of FITC-labeled dextrans of the ACF, $G_{DF}(\tau)$, was modeled by the superposition

$$G_{DF}(\tau) = \sum_i p_i G_{DF,i}(\tau)$$  \hspace{1cm} (S31)

using the log-normally distributed weights

$$p_i := \frac{1}{\tau_{DF,i} \sigma_{DF} \sqrt{2\pi}} e^{-\frac{(\ln(\tau_{DF,i})-\mu_{DF})^2}{2\sigma_{DF}^2}}$$  \hspace{1cm} (S32)

and the corresponding ACF contributions

$$G_{DF,i}(\tau) := \rho_{DF} \left(1 + \frac{\tau}{\tau_{DF,i}}\right)^{-1} \left(1 + \frac{\tau}{\tau_{DF,i} K^2}\right)^{-\frac{1}{2}}$$  \hspace{1cm} (S33)

The parameter $\sigma_{DF}$, which determines the broadness of the log-normal distribution, was determined by matching the FCS-related polydispersity index ($PDI$), defined by

$$PDI_{FCS} = \frac{\sum_i p_i M_i^2}{(\sum_i p_i M_i)^2}$$  \hspace{1cm} (S34)

using

$$M_i \propto R_i^3$$  \hspace{1cm} (S35a)

$$R_i = \frac{k_B T}{6\pi \eta D_i}$$  \hspace{1cm} (S35b)

$$D_i = \frac{r_{xy}^2}{4\tau_{DF,i}}$$  \hspace{1cm} (S35c)

where the $PDI$ of the dextran mass distribution was determined using GPC. The parameters $\rho_{DF}$ and $\tau_{DF} = e^{\mu_{DF}}$ denote the average number density of FITC-labeled dextran molecules in the confocal readout volume and their average decay time, respectively, and were determined when fitting the component $G_{DF}(\tau)$ to the experimentally determined ACF.
Fitting the 2-component model therefore yields information about the number densities of free FITC molecules and FITC-labeled dextran molecules in the confocal readout volume ($\rho_F$ and $\rho_{DF}$, respectively) and their decay times $\tau_F$ and $\tau_{DF}$, which can be translated into diffusion coefficients using

$$D_F = \frac{r_{xy}^2}{4\tau_F}$$  \hspace{1cm} (S36a)

$$D_{DF} = \frac{r_{xy}^2}{4\tau_{DF}}$$  \hspace{1cm} (S36b)

and into hydrodynamic radii using the Stokes-Einstein relation.\textsuperscript{11}
Drifts in the Measured Fluorescence Intensity Data

The experimentally measured fluorescence intensity data displays a continuous drift in the signal in all recorded measurements, which is likely due to an automatic re-adjustment of the laser intensity in the used setup. An example of the observed drift in the raw unscaled signal is shown in Figure S3, recorded for $M_{\text{dex}} = 70$ kDa dextran molecules at the $hPG-G10$ interface. Even though almost no penetration of the large dextran molecules into the hydrogel is observed, the fluorescence intensity in the probed part of the bulk solution changes significantly over time. In order to obtain physical values for the dextran concentration, the measured profiles are being re-scaled during the fitting procedure. The obtained re-scaling factors for every measured concentration profile $\vec{f}$ decline over time, thus overcoming the constant increase of signal intensity due to the drift (see Figure S4A). Additionally, smaller changes in the fluorescence intensity are apparent. Since robust results are obtained by employing this re-scaling routine, this suggests that the entire information about the diffusion process is present in the relative shape of the concentration profiles.

The obtained re-scaling factor can additionally be used, to estimate the experimental bulk concentration $c_{\text{bulk}}$ far away from the hydrogel interface, based on the experimentally measured profiles alone, without using the numerically computed dextran distributions. The total amount of dextran in the system $C_{\text{tot}}$ is computed from the first concentration profile as $C_{\text{tot}} = \int_{-\infty}^{\infty} c(z, t=0) \, dz$, where $c(z, t=0)$ was approximated by $c_i^{\text{init}}$ according to eq. (??) in the main text. An average experimental concentration in the bulk region $\bar{c}_{\text{bulk}}(t_j)$ can then be estimated from the fitted re-scaling factors as

$$\bar{c}_{\text{bulk}}(t_j) = \frac{C_{\text{tot}} - \sum_{i=1}^{M} f_i \cdot c_i^{\text{exp}}(t_j) \cdot \Delta z_i}{z_{\text{top}}}$$

(S37)

Values for $\bar{c}_{\text{bulk}}(t_j)$ are shown in Figure S4B and are virtually constant for the exemplary measurement of $M_{\text{dex}} = 70$ kDa dextrans, as is expected due to the absence of penetration into the hydrogel.
Supplementary Figure S3: Raw fluorescence intensity data from experiments of $M_{\text{dex}} = 70$ kDa dextrans in combination with the $hPG-G10$ hydrogel. A significant change in the signal over time is observed in the probed part of the bulk solution, even though almost no penetration of the dextrans into the hydrogel is apparent. This drift in the experimentally measured signal is overcome by the numerically determined re-scaling factors.

Supplementary Figure S4: A: Set of re-scaling factors $\vec{f}$ for every measured concentration profile obtained from numerical analysis of experimental data from $M_{\text{dex}} = 70$ kDa dextrans at the $hPG-G10$ hydrogel interface. Decreasing re-scaling factors counteract the drift observed in the raw experimental data. Additionally, peaks in the re-scaling factor distribution are observed, counteracting shorter fluctuations in the fluorescence intensity. B: Average bulk concentration $c_{\text{bulk}}$ computed according to eq. (S37) for the same measurements. The bulk concentration remains constant in this measurement, since almost no dextran penetrates into the hydrogel.
S9 Analytical Solution for Two-Segment System

Simplifying the hydrogel-water setup as a two-box system with piece-wise constant values of the free energy and diffusion constant in the two regions allows for an analytical solution of the diffusion problem. The modeled system with the corresponding boundary conditions is sketched in Figure S5A.

We solve the following diffusion equation in each of the two segments

\[
\frac{\partial}{\partial t} c(z, t) = D(z) \frac{\partial^2}{\partial z^2} c(z, t) \tag{S38}
\]

where the diffusion constant \( D(z) \) has a different value in each of the two regions

\[
D(z) = \begin{cases} 
D_0, & \text{if } 0 \leq z \leq z_{\text{int}} \\
D_1, & \text{if } z_{\text{int}} < z \leq z_{\text{bot}} 
\end{cases} \tag{S39}
\]

as does the free energy \( F(z) \), which we set to zero in the left segment as reference

\[
F(z) = \begin{cases} 
F_0 = 0, & \text{if } 0 \leq z \leq z_{\text{int}} \\
F_1, & \text{if } z_{\text{int}} < z \leq z_{\text{bot}} 
\end{cases} \tag{S40}
\]

At the interface \( z_{\text{int}} \), the flux needs to be continuous due to mass conservation, while the jump in the free energy leads to a jump in the concentration profile \( c(z = z_{\text{int}}, t) \). This defines the boundary conditions at \( z_{\text{int}} \) as

\[
\lim_{z \searrow z_{\text{int}}} D_0 \frac{\partial}{\partial z} c(z, t) = \lim_{z \nearrow z_{\text{int}}} D_1 \frac{\partial}{\partial z} c(z, t) \tag{S41a}
\]

\[
\lim_{z \searrow z_{\text{int}}} c(z, t) e^{-\beta F_1} = \lim_{z \nearrow z_{\text{int}}} c(z, t) \tag{S41b}
\]

Since we are modeling a closed system, the edges at \( z = 0 \) and \( z = z_{\text{bot}} \) are reflecting
boundaries with
\[ \frac{\partial}{\partial z} c(z = 0, t) = 0 \]  
\[ \frac{\partial}{\partial z} c(z = z_{\text{bot}}, t) = 0 \]  

Initially, the diffusors are only present in the left segment, modeling the bulk solution. This defines our initial condition as

\[ c(z, t = 0) = \begin{cases} 
  c_0, & \text{if } 0 \leq z \leq z_{\text{int}} \\
  0, & \text{if } z_{\text{int}} < z \leq z_{\text{bot}} 
\end{cases} \]  

We now solve eq. (S38) by means of Laplace transformation. To this end, we use the single sided Laplace transform in time, defined as \( \hat{f}(s) := \int_0^\infty f(t)e^{-st}dt \), where \( s \) is the complex variable in Laplace space \( s = \sigma + i\omega \). This converts the partial differential equation (S38) into an ordinary differential equation of second order

\[ \left[ s - D(z) \frac{\partial^2}{\partial z^2} \right] \hat{c}(z, s) = c(z, t = 0) \]  

The general solution of eq. (S44) for the two regions reads

\[ \hat{c}(z, s) = \begin{cases} 
  a_1e^{\lambda_0 z} + a_2e^{-\lambda_0 z} + \hat{c}_p, & \text{if } 0 \leq z \leq z_{\text{int}} \\
  a_3e^{\lambda_1 z} + a_4e^{-\lambda_1 z}, & \text{if } z_{\text{int}} < z \leq z_{\text{bot}} 
\end{cases} \]  

where we define \( \lambda_i := \sqrt{\frac{s}{D_i}}, i = 0,1 \) and \( \hat{c}_p := \frac{c_0}{s} \). The coefficients \( a_i \) of eq. (S45) are determined by solving the system of linear equations obtained by Laplace transforming the boundary conditions of eq. (S41) and eq. (S42) and substituting the general solution (eq. (S45)). After some algebra, the solution to the posed problem is obtained as
\[ \hat{c}(z, s) = \begin{cases} \hat{c}_p \frac{K \tanh(\lambda_1(z_{\text{bot}} - z_{\text{int}})) [\cosh(\lambda_0 z_{\text{int}}) - \cosh(\lambda_0 z)] + \sinh(\lambda_0 z_{\text{int}}) \sqrt{\delta}}{K \tanh(\lambda_1(z_{\text{bot}} - z_{\text{int}})) \cosh(\lambda_0 z_{\text{int}}) + \sinh(\lambda_0 z_{\text{int}}) \sqrt{\delta}}, & \text{if } 0 \leq z \leq z_{\text{int}} \\ \hat{c}_p \frac{K \cosh(\lambda_1(z_{\text{bot}} - z)) \tanh(\lambda_0 z_{\text{int}}) \sqrt{\delta}}{K \sinh(\lambda_1(z_{\text{bot}} - z_{\text{int}})) + \tanh(\lambda_0 z_{\text{int}}) \cosh(\lambda_1(z_{\text{bot}} - z_{\text{int}})) \sqrt{\delta}}, & \text{if } z_{\text{int}} < z \leq z_{\text{bot}} \end{cases} \]

where \( \delta := \frac{D_0}{D_1} \) and \( K := e^{-\beta F_1} \).

The solution in Laplace space (eq. (S46)) is then transformed into real space by use of the Mellin integral

\[ c(z, t) = \frac{1}{2\pi i} \int_{s=\sigma-i\infty}^{s=\sigma+i\infty} \hat{c}(z, s) e^{st} ds = \frac{e^{\sigma t}}{2\pi} \int_{-\infty}^{+\infty} \hat{c}(z, \sigma + i\omega) e^{i\omega t} d\omega \quad (S47) \]

where the last integral was solved numerically through the inverse discrete Fourier transform.

Figure S5B shows a comparison of the analytical solution and the numerical model for an exemplary parameter set of \( z_{\text{int}} = 100 \ \mu \text{m}, \ z_{\text{bot}} = 300 \ \mu \text{m}, \ c_0 = 1 \ \text{mg/L}, \ D_0 = 50 \ \mu \text{m}^2/s, \)
\( D_1 = 100 \ \mu \text{m}^2/s \) and \( F_1 = 0.5 \ k_B T \), mimicking a slight immobilization and repulsion in the right segment, as observed for the smaller dextrans in the experiments (see main text). The stationary state is reached faster in the approximate system compared to the actual measurements in the main text, due to the much smaller \( z \)-dimension. Perfect agreement between the numerical model and the analytical solution is obtained.
Supplementary Figure S5: Approximation of the dextran hydrogel setup as a two-segment system, which can be solved analytically. A: Two-segment system with different diffusion constants and a jump in the free energy at the interface $z_{int}$. The used boundary conditions are also indicated. B: Comparison of the numerical model and the analytical solution of the system explained in A, with values for the parameters of $z_{int} = 100 \, \mu m$, $z_{bot} = 300 \, \mu m$, $c_0 = 1$, $D_0 = 50 \, \mu m^2/s$, $D_1 = 100 \, \mu m^2/s$ and $F_1 = 0.5 \, k_B T$. 

\[
\begin{align*}
D_0 \frac{\partial}{\partial z} c(z_{int} - 0, t) &= D_1 \frac{\partial}{\partial z} c(z_{int} + 0, t) \\
\frac{\partial}{\partial z} c(0, t) &= 0 \\
\frac{\partial}{\partial z} c(z_{bot}, t) &= 0 \\
D_0 &= c(z_{int} - 0, t) e^{-\beta F_1} = c(z_{int} + 0, t) \\
F_0 &= 0 \\
F_1 &= 0.5 \, k_B T.
\end{align*}
\]
S10 Error Estimate for Numerical Analysis

In order to determine confidence intervals for the fitted parameters of $D_{\text{sol}}$, $D_{\text{gel}}$ and $\Delta F_{\text{gel}}$, the values are varied from the optimum until the agreement with the experimental data is 50% worse than for the optimal parameter values. Figure S6 shows an exemplary analysis of the fitted parameters influence on the error. All parameters are varied independently, meaning that the error is always computed while keeping all other parameters fixed at their optimal values. Also, the fitted values for $d_{\text{int}}$ and $z_{\text{int}}$ are not changed but kept at their optimum. It is apparent that increasing the fitted diffusion constants does not affect the agreement with the experimental data as strongly as a decrease (see Figure S6A and B). Changing the free energy difference influences the numerical error $\sigma$ more symmetrically, meaning increasing $\Delta F_{\text{gel}}$ has the same influence on the error as decreasing it.

![Graphs showing error estimation](image)

Supplementary Figure S6: Error estimation of the fitted values for $D_{\text{sol}}$ (A), $D_{\text{gel}}$ (B) and $\Delta F_{\text{gel}}$ (C) for measurements of $M_{\text{dex}} = 40$ kDa dextran molecules diffusing into the hPG-G10 hydrogel. Fitted optimal values for the parameters are indicated by dotted lines, while a 50% change in $\sigma$ is shown by the dashed black line. A larger value of the diffusion constants does not affect the agreement with the experimental data as strongly as a smaller value.
References

(1) Rubinstein, M.; Colby, R. H. *Polymer Physics*; OUP Oxford, 2003.

(2) Netz, R. R.; Andelman, D. *Physics Reports* **2003**, *380*, 1–95, DOI: 10.1016/S0370-1573(03)00118-2.

(3) Huang, L.; Nishinari, K. *Journal of Polymer Science, Part B: Polymer Physics* **2001**, *39*, 496–506, DOI: 10.1002/1099-0488(20010301)39:5<496::AID-POLB1023>3.0.CO;2-H.

(4) Shikata, T.; Takahashi, R.; Sakamoto, A. *The Journal of Physical Chemistry B* **2006**, *110*, 8941–8945, DOI: 10.1021/jp060356i.

(5) Kaatze, U.; Gottmann, O.; Podbielski, R.; Pottel, R.; Terveer, U. *The Journal of Physical Chemistry* **1978**, *82*, 112–120, DOI: 10.1021/j100490a025.

(6) Bieze, T. W. N.; Barnes, A. C.; Huige, C. J. M.; Enderby, J. E.; Leyte, J. C. *The Journal of Physical Chemistry* **1994**, *98*, 6568–6576, DOI: 10.1021/j100077a024.

(7) Žwirbla, W.; Sikorska, A.; Linde, B. B. *Journal of Molecular Structure* **2005**, *743*, 49–52, DOI: 10.1016/j.molstruc.2005.02.019.

(8) Branca, C.; Magazù, S.; Maisano, G.; Migliardo, F.; Migliardo, P.; Romeo, G. *The Journal of Physical Chemistry B* **2002**, *106*, 10272–10276, DOI: 10.1021/jp014345v.

(9) Diamond, J. M.; Katz, Y. *The Journal of Membrane Biology* **1974**, *17*, 121–154, DOI: 10.1007/BF01870176.

(10) Widengren, J.; Rigler, R.; Mets, Ü. *Journal of Fluorescence* **1994**, *4*, 255–258, DOI: 10.1007/BF01878460.

(11) Rigler, R.; Elson, E. S. *Fluorescence Correlation Spectroscopy: Theory and Applications*; Springer Series in Chemical Physics; Springer Berlin Heidelberg, 2012.
(12) Rüttinger, S.; Buschmann, V.; Krämer, B.; Erdmann, R.; MacDonald, R.; Koberling, F.

*Journal of Microscopy* 2008, *232*, 343–352, DOI: [10.1111/j.1365-2818.2008.02105](http://dx.doi.org/10.1111/j.1365-2818.2008.02105).