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The particle in the spider's web: transport through biological hydrogels

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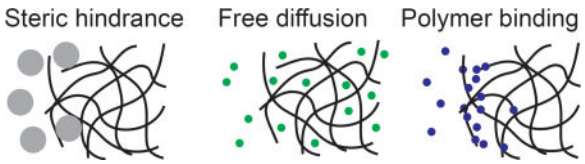
Abstract

Biological hydrogels such as mucus, extracellular matrix, biofilms, and the nuclear pore have diverse functions and compositions, but all act as selectively permeable barriers to the diffusion of particles. Each barrier has a crosslinked polymeric mesh that blocks penetration of large particles such as pathogens, nanotherapeutics, or macromolecules. These polymeric meshes also employ interactive filtering, in which affinity between solutes and the gel matrix controls permeability. Interactive filtering affects the transport of particles of all sizes including peptides, antibiotics, and nanoparticles and in many cases this filtering can be described in terms of the effects of charge and hydrophobicity. The concepts described in this review can guide strategies to exploit or overcome gel barriers, particularly for applications in diagnostics, pharmacology, biomaterials, and drug delivery.

Graphical abstract

Transport through biological hydrogels is an important biological process and of fundamental importance for drug delivery.



1. Introduction: hydrogels are ubiquitous selective barriers in biology

Biological hydrogels, which are composed of hydrated polymer networks, are found throughout every domain of life. For example, both archaea[1] and bacteria can form biofilms, or microbial aggregates surrounded by secreted extracellular polymeric substances (EPS) that act as a protective barrier and create microenvironments within which microbes thrive and adapt to harsh conditions.[2,3] In eukaryotes, hydrogels have evolved to fulfill an

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astonishingly diverse set of functions. For example, the nuclear pore is a barrier formed from crosslinked intrinsically disordered proteins called nucleoporins, which control the passage of macromolecules between the nucleus and the cytoplasm.[4,5] One of the largest hydrogels in the body is the mucus gel (Fig. 1a) that lines all wet epithelia and protects the underlying cells against toxins, pollutants, and invading pathogens.[6–10] Another example of a hydrogel-based barrier is the extracellular matrix (ECM) surrounding cells within tissues. ECM provides mechanical stability but also forms a selective barrier that regulates transport of signaling molecules secreted by the cells (Fig. 1b).[11–16]

The permeability of biological hydrogels poses a challenge for biomedical development. For example, biofilms are involved in the majority of infections in developed countries; they form on medical implants and wounds, cause middle-ear infections and gingivitis, and more.[17] One driver of antibiotic resistance in biofilms is binding of antibiotics to EPS, which sequesters them and/or reduces their penetration into the biofilm. In humans, the ECM is an obstacle to drug delivery, as its mesh prevents large nanoparticles from penetrating deep into tissue or tumors.[18–24] Mucus similarly controls the permeability of nanoparticles,[25,26] but can also limit diffusion of small molecules such as antibiotics.

Biological hydrogels are complex molecular assemblies with context-dependent properties. For biofilm EPS, the structure of the matrix varies widely across species, strains, and environments, complicating the characterization of EPS components. However, biofilm EPS commonly contains extracellular DNA and various types of polysaccharides.[2] One well-studied biofilm is that formed by *Pseudomonas aeruginosa*, an opportunistic pathogen that can cause biofilm-associated infection in wounds[27] or in the lungs of patients with cystic fibrosis (CF).[28] Interestingly, even this single species produces anionic (alginate), cationic (Pel),[29] and neutral (Psl)[30] polysaccharides in differing amounts depending on context and bacterial strain.[31]

Fibrous structural proteins such as collagens, elastin, fibronectin, and laminins, together provide much of the structural integrity of ECM. ECM also contains high levels of polyanionic glycosaminoglycans (GAGs) and proteoglycans (proteins with densely grafted GAGs) that give ECM a high net negative charge density. Structural components of ECM are covalently crosslinked to varying degrees, regulating matrix stiffness.[11]

In human mucus, the main gel-forming components consist of a family of large, intrinsically disordered secreted glycoproteins called mucins. The main secreted mucins are MUC2, MUC5B, and MUC5AC, although other secreted mucins can be present in smaller amounts as well.[7] Mucins have alternating regions of small, globular hydrophobic domains and highly glycosylated, primarily anionic (due to sialic acid and sulfation) unstructured regions.[7,32,33] Other components of mucus include lipids, soluble proteins and peptides, and nucleic acids.[32] Mucus composition can vary depending on disease state, and in this review we highlight CF, a genetic disease in which improper ion balance results in pathologically thick, dehydrated mucus.[34] The presence of necrotic neutrophils in CF airways results in high levels of free DNA and actin filaments in lung mucus,[35] increasing the mucus' viscoelasticity[32] and playing important roles in binding to cations. More detailed reviews can be found on composition of biofilms,[2,36] of the ECM,[11,24,37] and of mucus.[6,7,32,38,39]

While biological hydrogels are distinct in terms of their locations and molecular compositions, they share certain common principles that govern selective filtration. The goal of this review is to address the general principles that apply to the permeability of many types of biological hydrogels.

Broadly speaking, the transport of a solute through a gel is controlled by the solute's size, its interactions with the components of the gel, or a combination of the two (Fig. 2).[40] Size, or steric, filtering is a universal feature of biological gels, which have a polymeric mesh size that constrains the diffusion of large particles (Fig. 2a). Pure steric filtering is an important component of hydrogel selectivity, but it is also crude because it only selects based on one parameter. Filtering based on chemical interactions between solutes and gel components is also a common feature of biological gels. Depending on their chemistry, the gel components interact with solutes across gradients of chemical properties including charge and hydrophobicity, thereby differentially affecting diffusion.[40] A lack of interactions enables unhindered diffusion (Fig. 2b), while certain weak and diffuse solute-gel interactions can facilitate penetration of the solute into the gel without slowing transport (Fig. 2c). Binding to hydrogels, on the other hand, reduces effective solute diffusivity and hinders penetration (Fig. 2d). In drug delivery applications, facilitated transport and reduced effective diffusivity from binding can be combined to optimize drug delivery.[21,41]

2. Size-dependent filtration

2.1 Length scales and hydrogel mesh

Size effects are important for the transport of viruses, bacteria, eukaryotic cells, particulate pollutants, nanoparticles, and any other particle on the same length scale as the gel mesh. In this section, we discuss size filtering while assuming that all particles are inert to (do not interact with) gel components. To a first approximation, steric interactions are quite simple, corresponding in the macroscopic world to the fact that an elephant, but not a fly, can be stopped by a chain-link fence, while a tennis ball can pass through with some prodding.

In biological hydrogels, the microscopic mesh is formed by entangled and crosslinked polymers. The distance between adjacent links in the chain-link fence corresponds to the mesh size; objects much smaller than the mesh size diffuse at a rate corresponding to the viscosity of the interstitial fluid (the fluid between fibers; Fig. 3a), objects on the order of the mesh size are obstructed but not completely stopped (Fig. 3b), and objects much larger than the mesh size are trapped (Fig. 3c). This steric barrier is important for mucus because it blocks and/or traps large pollutants and potential pathogens, thus allowing some mechanism, such as mucociliary clearance, to clear the invading particle before it can reach the epithelium.[8] Mesh size is also an important consideration for nanoparticle design, as it sets a maximum possible size for a nanoparticle that must penetrate a hydrogel barrier. The mesh sizes of mucus, biofilm EPS, and ECM vary and are on the order of 10-1000nm.[19,20,23,24,42–46] Note that measurements of mesh size may be incorrect if the particles used to probe the mesh size interact with the gel;[47] we discuss adhesive interactions in detail in Section 4. The nuclear pore is filled with intrinsically disordered proteins called nucleoporins that form a mesh with a mesh size ~2.5-5 nm in size that excludes proteins and

protein complexes larger than 30-100 kDa, unless they are chaperoned by a nuclear transport receptor (NTR).[48,49]

Note that biological gels are often heterogeneous, with a distribution of mesh sizes. For example, some biofilms contain channels that allow efficient passage of large particles to cells deep in the biofilm.[46,50] In mucus, nanoparticles often show a broad diffusivity distribution in particle tracking experiments, suggesting that some nanoparticles are trapped while others diffuse nearly freely.[32,45,51] One important question is whether the regions that allow free diffusion are connected enough to allow efficient penetration through macroscopic mucus layers, or whether these regions are simply large water-filled pores surrounded by impassable polymeric barriers. Answers to this question are somewhat conflicting, but overall it appears that mucoinert particles pass through macroscopic mucus barriers.[52–54]

On a related note, collagen fibrils in ECM are often aligned with each other; this directionality of the mesh drives anisotropic diffusion behavior that may be important for transport in ECM.[55,56] The importance of diffusion anisotropy is unclear in other gels, although filamentous bacteriophage can drive liquid crystallization of *P. aeruginosa* biofilms, suggesting the potential for anisotropic transport in that context.[57] Finally, in contrast to a chain-link fence, in gels thermal motion and structural dynamics play a role in controlling transport.[58] Gel polymers and physical crosslinks constantly rearrange, with local deformations allowing large particles to escape steric barriers and caging effects.[59–63]

2.2 Modulation of gel structure by transporting particles

The transport of a particle through a gel may also be promoted by the solute directly interfering with the gel's component polymers or crosslinks, thus changing the mesh size. For example, attaching the mucolytic protein papain to nanoparticle surfaces somewhat enhanced penetration of the nanoparticles through intestinal mucus (Fig. 3d).[64,65] Treatment of CF lung mucus with *N*-acetyl cysteine, a disulfide bonding reducer, likewise facilitated transport by increasing the mesh size,[66] although surprisingly treatment with recombinant DNase (used in CF to degrade DNA in mucus) did not improve transport.[67] An elegant example of crosslink interference may occur in the nuclear pore (discussed in detail in Section 5), in which transporters reversibly disrupt physical crosslinks, allowing fast transport and rapid self-healing of the pore structure (Fig. 3e).[68] This mechanism of reversibly disrupting physical crosslinks has not, to our knowledge, been found in any other biological system, but it presents an intriguing mechanism for biology and engineering applications. Finally, mucin-binding particles may sequester mucin strands, reducing the concentration of gel strands elsewhere in the gel. This reduction increases the effective mesh size and thus enhances transport of other, non-mucin binding nanoparticles.[69,70]

Living cells also modulate gel structure as they move. Many types of human cells degrade and/or secrete various ECM elements in tightly regulated ways, with degradation sometimes necessary for cell motility.[71] Improper regulation of ECM structure, including overexpression of ECM-degrading matrix metalloproteinases or the collagen crosslinking factor LOX, has been associated with numerous types of cancer.[37] Similarly, biofilm EPS is constantly remodeled by bacteria.[42] Bacterial motion disrupts the gel structure,[72] while lytic

enzymes such as alginate lyase are both used by bacteria and have potential as anti-biofilm treatments.[73,74] The stomach pathogen *Helicobacter pylori* has also been shown to facilitate its motion by secreting high levels of ammonia into stomach mucus; the local increase in pH deprotonates carboxylic acid groups in mucin, which reduces intermolecular hydrogen bonding and hydrophobic interactions while increasing electrostatic repulsion between mucin strands. *H. pylori* is able to easily swim through this locally weakened gel.[75]

2.3 Techniques to analyze particle transport

Experimental assays of particle transport generally require the particles to be fluorescently labeled or radiolabeled. From there, one common technique to measure transport is direct visualization of bulk particle transport into or through a gel, followed by qualitative analysis[76] or quantitative fitting of concentration timecourses or spatial profiles as the particles penetrate the gel.[77,78] Bulk transport visualization methods are simple and easily visualized, but they are generally unable to measure heterogeneity or other structural details. Two related techniques for analyzing particle transport in gels are fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS).[55,79] In FRAP and FCS, the gel is pre-incubated with fluorescently labeled particles. In FRAP, a region of the gel is photobleached and the recovery of fluorescence in the bleached spot is fit to models that calculate diffusion parameters.[80] In FCS, the fluorescence autocorrelation function within a small gel region is measured and fit.[79] With FRAP and FCS, observing and fitting parameters for subdiffusion may be possible using appropriate fitting procedures.[81–84] Subdiffusion refers to diffusion behavior in which the mean squared displacement of individual particles scales with t^{α} for $\alpha<1$, rather than with t^{1} as in standard diffusion; subdiffusion or transient subdiffusion is commonly observed in gel transport studies and can impact important parameters such as passage time through a gel.[85,86]

An alternative to bulk transport analysis is single particle tracking (SPT), in which the thermal motion of individual particles is recorded and analyzed.[87] SPT easily identifies both subdiffusion and heterogeneity in gel samples, which is useful for identifying subpopulations of particles that diffuse relatively unhindered.[32] This technique's main weakness is that diffusion out of a microscope's focal plane renders it difficult to track individual particles over tens of microns, which means that unlike in bulk diffusion or FRAP experiments, direct measurements of penetration through physiologically sized gels is not feasible.[54]

Finally, nanoparticle dispersal has been imaged *in vivo* for a variety of mucus systems as well as brain ECM. These assays do not provide quantitative measurements of diffusion parameters, but nonetheless serve as a valuable bridge between in vitro studies and translation to the clinic.[47,52,88,89]

3. Interactive filters

3.1 Overview: effects of chemical interactions on gel penetration and retention

Biological hydrogels selectively interact with foreign particles based on the solutes' chemical properties. These interactions often lead to reversible binding, which may be

desirable, undesirable, or neutral depending on context. Since reversible binding improves retention within a gel, it may enable longer drug action, as in mucoadhesive drug delivery systems.[9,90] Similarly, binding of native immune factors to cervical mucus may promote their retention at high concentration and prevent microbial penetration through the cervical mucus plug during pregnancy, which could lead to intrauterine infection and preterm labor.[39] On the other hand, reduced diffusivity may slow the penetration of a gel (Fig. 2d), impacting the oral bioavailability of drugs or microbial killing by antibiotics. Similarly, binding can sequester a molecule and decrease its effective concentration. Finally, diffusivity could simply be a tunable regulatory parameter, neither intrinsically "good" nor "bad." Whatever the effect of gel binding, unlocking the forces underlying it is crucial to understanding the function of native systems and optimizing drug function.

As with nanoparticles, bulk diffusion assays, FRAP, and FCS are commonly used to measure the diffusion coefficients of small solutes such as drugs and proteins.[46,91,92] SPT is more difficult for smaller solutes because of the difficulty inherent in resolving individual molecules with fluorescence microscopy; this technique is rarely if ever used to study diffusion of proteins or smaller molecules in gels.

To clarify what exactly is meant by slowing solute penetration and improving retention, we briefly present the standard model for diffusion of a solute with first-order reversible binding to a gel. Under the assumptions that 1) binding and unbinding are fast, such that the solute reaches local equilibrium between bound and free states at each point, 2) bound solute is immobile, 3) binding sites within the gel are far from saturated, and 4) binding to the gel does not disrupt gel structure, we can define the *effective* diffusivity D_{eff} as:

$$D_{eff} = D_F (1 + N_T / K_D)^{-1} \quad (1)$$

where D_F is the diffusion coefficient of the free solute in the gel (which may be lower than the diffusivity in water, due to steric constrains or increased interstitial fluid viscosity), N_T is the total binding site density in the gel, and K_D is the dissociation constant.

The timescale for a solute to penetrate a gel (or equivalently, the timescale of escape from a gel) τ_{lag} is related to the D_{eff} by:

$$\tau_{lag} \sim L^2 / D_{eff} \quad (2)$$

where L is the length scale of the gel. These two equations show how increasing solute-gel binding strength or gel binding site density increases τ_{lag}, thus slowing penetration or improving retention.[77] Fig. 4a shows this effect in action, as the penetration of tobramycin (an aminoglycoside antibiotic) into a *P. aeruginosa* biofilm is hindered by binding of tobramycin to biofilm components.[76]

Given these results, it is no surprise that specific protein-ligand interactions are commonly exploited to regulate transport and retention within a gel, often for protective purposes or to control signaling.[12,93] For example, sialic acid-terminated O-linked oligosaccharide chains on secreted mucin molecules, particularly Muc5AC, act as decoy receptors for sialic acid targeting bacteria and viruses, including the influenza virus.[94] This interaction slows penetration of sialic acid targeting bacteria and viruses through the mucus layer to the vulnerable epithelium[95,96] and competitively inhibits binding to cell-associated sialic acid.[94] A mouse model with lung Muc5AC overexpression has increased resistance to influenza, illustrating this protective function of lung mucus.[97] In the ECM, binding of insulin-like growth factor (IGF) to IGF binding proteins limits IGF transport, thus helping to regulate intercellular signaling.[13] Sequestration of transforming growth factor-β in the ECM is another well-studied case showing the importance of binding to the gel matrix.[98,99] In addition to non-covalent protein-ligand interactions, covalent linkage to biological hydrogels is also possible; for example, certain mucoadhesive formulations use free thiol groups to form disulfide bonds to mucin.[100]

While Equations 1 and 2 describe the simplest effects of gel binding, interesting cases arise when the assumptions are violated. For example, saturation of gel binding sites results in enhanced penetration because there are fewer binding sites available per solute molecule to arrest diffusion.[76] In another example, Braga *et al.* showed that if bound solute is mobile with diffusivity D_B but the other assumptions hold, (we have changed the notation slightly to stay consistent with this review):[101]

$$D_{eff}=(1-f)D_F+f\,D_B \quad (3)$$

where f is the fraction of time a solute is bound (or equivalently, the equilibrium bound fraction of solute at equilibrium). It can be shown that $f = N_T/(N_T+K_D)$, so we recover Equation 1 in the limit of immobile bound solute (D_B=0). In the opposite limit of D_B=D_F, penetration is not slowed because D_{eff}=D_F. In the nuclear pore, the mobility of bound solute may help explain why weak binding of NTRs to nuclear pore components does not arrest passage.[102] On the applied side, Fig. 4b shows that penetration of the positively charged protein Avidin into articular cartilage ECM is facilitated by electrostatic interactions (Fig. 4b), potentially allowing Avidin to act as a nanocarrier for osteoarthritis drugs.[21,41] We emphasize the dramatic difference between Figures 4a and 4b: while tobramycin and Avidin both electrostatically interact with their respective gels, tobramycin penetration is inhibited with respect to a neutral counterpart, whereas Avidin penetration is facilitated. The difference arises because Avidin-ECM electrostatic interactions do not hinder Avidin diffusion, and in fact these interactions do not constitute binding at all in some sense; Avidin transport is best described using Donnan partitioning analysis.[77,103] Mechanisms of mobility for interacting solutes such as nuclear pore transporters and Avidin are related to the nanoscale energy landscape encountered by the solutes (Fig. 4c and 4d), and are described in Section 3.4.

Violation of criterion 4, that binding to the gel does not disrupt gel structure, also has interesting effects. For example, high concentrations of tobramycin induce precipitation of alginate gels; this disruption of barrier integrity facilitates tobramycin transport at high concentrations.[79] Also, reversible binding of particles to physical gel crosslinks could disrupt existing crosslinking (Fig. 3e), which could help overcome a steric barrier as discussed in Sections 2.2 and 5.

3.2 Biochemistry of reversible binding to the gel matrix

Reversible binding to biological hydrogels is important for molecules throughout the size spectrum;[92] even proton transport can be slowed by gastric mucus, which protects epithelial tissue from stomach acid.[105] Here, we discuss representative examples that illustrate the importance of various intermolecular forces, as well as several cases showing how modulating gel binding could deliver novel approaches to fighting infections.

The biochemistry underlying reversible binding to gels is difficult to study for several reasons. First, crystal structures of gel components do not usually exist since gel polymers are generally intrinsically disordered. It is also often not obvious to which component(s) of the gel a solute binds, since biological gels contain a complex mix of components.[76] Despite the lack of structural information, existing literature on gel binding and the composition of biological hydrogels yields useful predictions and conclusions about transport in gels; these predictions usually center on how charge and hydrophobicity influence diffusion. Other intermolecular forces such as van der Waals forces and hydrogen bonding are important, but their effect on binding is not easily predictable. In one case, research has shown that hydrogen bonding is important for mucoadhesion of hydrophilic polymers such as poly(acrylic acid).[90] In summary, analysis of a molecule's overall chemical properties may predict that the molecule will bind to *something* within a gel.

An instructive instance in which charge interactions dominate is *P. aeruginosa* lung infections in CF patients, which involve two biological gels: mucus and biofilm EPS. One primary class of antibiotics used to treat *P. aeruginosa* infections, aminoglycosides, are highly positively charged and thus bind to polyanions such as mucins, DNA, and actin, which are present at pathologically high levels in CF respiratory mucus. This binding impedes antibiotic penetration and inhibits activity.[74,106] The *P. aeruginosa* EPS acts as a second protective barrier for biofilm-associated cells. The EPS also contains DNA and, in the case of mucoid strains, high levels of the polyanionic aminoglycoside-binding alginate, although alginate surprisingly seems to not significantly hinder penetration.[107–109]

Although charge-mediated binding to polyanions is important for aminoglycosides and other cationic antibiotics such as colistin,[110] reversible gel binding does not seem to be a major factor in many other antibiotic treatments of biofilms;[111] for example, Fig. 4a shows that ciprofloxacin easily penetrates a model *P. aeruginosa* biofilm. In addition to slowing antibiotic penetration, biofilms have other resistance mechanisms including slow metabolism and hypoxia in the biofilm interior (which are related to reaction-diffusion dynamics of nutrients and oxygen, emphasizing the broad importance of diffusion in gels), and slow-dividing “persister cells.”[17,112,113] The various resistance mechanisms may be cooperative;

slowed penetration could allow more time for bacterial adaptations to antibiotic treatment.[114]

The importance of hydrophobic interactions in small molecule transport has mainly been studied in the context of oral drug bioavailability, which is partially dependent on the ability to diffuse through intestinal mucus to the epithelium. Many hydrophobic drugs such as paclitaxel, testosterone, and cinnarizine bind to exposed hydrophobic sites on mucin, lipids present in mucus, or both; binding slows penetration and renders intestinal mucus a barrier to absorption into the bloodstream.[115]

However, for many molecules it is not immediately clear whether hydrophobic or electrostatic interactions drive interactive properties. For example, cationic antimicrobial peptides (CAMPs), an important class of antibiotics and immune molecules, contain both cationic and hydrophobic residues; the charge, or the hydrophobicity, or both, could lead to gel binding. EPS-CAMP interactions are particularly important, as many antimicrobial peptides are under investigation as possible anti-biofilm treatments, but binding to EPS components often inhibits their effectiveness.[116–118] For example, the Deber group designed a set of synthetic anti-*P. aeruginosa* CAMPs and investigated their interaction with alginate.[119–122] While alginate is anionic, highly soluble, and contains no large hydrophobic domains, alginate blocked the penetration of synthetic anti-*P. aeruginosa* CAMPs when a hydrophobicity threshold was exceeded.[121] Furthermore, they showed that this interaction was mediated by a combination of electrostatic and hydrophobic interactions with alginate.[119]

Similarly, both synthetic and natural CAMPs have been shown to bind mucins, DNA, and F-actin, and stronger binding correlates with greater inhibition of CAMP activity against pathogenic bacteria in saliva and CF sputum.[123–126] CAMPs are also investigated as anti-cancer treatments, and unsurprisingly can be inhibited by ECM: the GAG heparan sulfate inhibits antitumor CAMPs.[127]

3.3 The effect of nanoscale heterogeneity on solute-gel interactions

While knowing the composition of a gel gives an overall sense of the gel's net charge and helps predict how solutes behave, it is also important to understand how nanoscale molecular heterogeneity, or spatially varying chemical properties, tunes solute-gel interactions. Heterogeneous surfaces are commonly found in gel-related molecules: protein surfaces and antimicrobial peptides contain charged, neutral hydrophilic, and hydrophobic residues, and larger objects such as viruses have complex surfaces (Fig. 5a).[128] In addition, the gels themselves often have locally varying properties that render simplistic models of gel charge problematic. For example, despite the net negative charge of ECM, both positively and negatively charged nanoparticles and liposomes have reduced diffusivity in reconstituted ECM gels compared to neutral hydrophilic particles. The observation that anionic particles are slowed just like cationic particles is probably due to alternate patches of negative and positive charge in the gel that bind positive and negative charges, respectively, on the particles.[129,130]

As an example of the effect of nanoscale heterogeneity on the solute side, when Li *et al.* examined the diffusion of two peptides, with the same near-neutral net charge but a different arrangement of these charges, these peptides diffused differently into a reconstituted mucin gel. The block-charge peptide interacted weakly with mucin, while the alternating-charge peptide did not (Fig. 5b).[131] This result is indicative of how variations in molecular structure beyond simple net charge affect transport properties in gels, which could have applications for drugs whose penetration is inhibited by gel binding.

For example, the Smyth group covalently attached a 5kDa polyethylene glycol (PEG) chain to tobramycin and found that the PEGylated variant was more effective *in vitro* against *P. aeruginosa* biofilms than the unmodified antibiotic, likely due to reduced EPS binding. While the PEGylated tobramycin remained highly positively charged as is required for its mechanism of action, PEGylation sufficiently changed the context of that charge to reduce gel binding. However, the improvement in activity of the PEGylated tobramycin against biofilms was modest, because the PEG chain compromised the biological activity of the molecule.[132] One possible strategy to avoid reduced activity is to make the PEGylation reversible via hydrolysis, a strategy that has been investigated for gentamicin (another aminoglycoside) due to the potentially improved pharmacokinetic properties offered by PEGylation, but not studied in a biofilm context.[133] Reversible PEGylation may allow for gel penetration, as well as full activity of the hydrolyzed form.

An alternate strategy would be to minimally change the structure of tobramycin in order to reduce gel binding without compromising activity. Recent experiments have suggested that modifying hydrophobicity could achieve this aim, because hydrophobic interfaces can affect electrostatic binding.[134] We also note that immobilized charged groups on hydrophobic surfaces can tune hydrophobic interactions (Fig. 5c), which could be useful for changing the gel binding of partially hydrophobic solutes such as CAMPs .[135]

For a more fully developed example of the utility of preventing sequestration by gels, we consider the activity of lysozyme, a cationic antimicrobial protein, against *P. aeruginosa* in CF. Lysozyme is inhibited by F-actin,[136] mucin, alginate, and DNA. As with tobramycin, some positive charge is required for lysozyme's function; however, in a screen of charge-reduced variants, at least one variant reduced its inhibition by all of these polyanions while retaining full antimicrobial activity.[137,138] This mutant lysozyme also displayed superior antibiotic activity to wild type in a murine *P. aeruginosa* lung infection model.[139,140] There was substantial variation in *P. aeruginosa* killing by lysozyme both with and without the presence of polyanions throughout the screen, emphasizing that (as with the charged peptide assay in Fig. 5b[131]) net charge is not sufficient to determine the strength of electrostatic binding; context and charge placement are important as well.[137,138]

3.4 Mechanisms for mobility of interacting solute

We mentioned in section 3.1 that gel interactions need not reduce effective diffusivity if the bound particles remain mobile ($D_B \sim D_F$). We now present two mechanisms for fast diffusivity of interacting particles: mobility of the bound complex and high density of interaction sites. In the first mechanism, if a solute is bound to a site which itself is diffusing, then the solute is taken along for the ride with $D_B = D_{bound\ complex}$. Experimentally, Sprakel

et al. (2007) observed transient subdiffusion of a silica particle bound to a polymer network due to Rouse dynamics.[60]

Bound complex mobility would be most significant in weakly physically crosslinked gels comprised of flexible polymers because these properties allow fast polymer dynamics, and for larger solutes because small molecules diffuse more quickly than polymers ($D_F \gg D_{bound\ complex}$). Also, bound complex diffusion is faster on short timescales because crosslinks and entanglements limit range of motion over long timescales.[60] Binding events with fast off rates will therefore take greater advantage of bound complex mobility. Transport of NTRs through the nuclear pore neatly checks off each of these constraints: NTRs transiently bind weakly crosslinked and highly mobile nucleoporins. Thus, bound complex mobility helps explain why NTR binding to nucleoporins does not result in slow transport, as one would naively expect given Equation 1, and accurately predicts that strengthening NTR-nucleoporin binding should inhibit transport.[102,141,142]

To understand the high interaction site density mechanism, we consider an energy landscape interpretation of diffusion, in which interactions are represented by electrochemical potential wells (Fig. 4c, 4d). Escape of a solute from potential wells into interstitial fluid is slow, thus impeding diffusion (Fig. 4c), as with tobramycin penetration into biofilms. However, if the density of binding sites is high enough, then it is possible for a solute to transition from one free energy minimum to another without transitioning to bulk interstitial fluid (Fig. 4d). The classic example of this is sliding diffusion on DNA: certain DNA-binding proteins transition from free diffusion in the nucleus or cytoplasm to one-dimensional sliding along DNA upon nonspecific DNA binding.[143–145]

A similar effect likely takes place for the diffusion of Avidin (a positively charged 60kDa protein) in articular cartilage ECM (Fig. 4b), in which proteoglycans allow full three-dimensional mobility. High densities of proteoglycans and GAG chains combined with weak nonspecific interactions enable cations such as Avidin to freely move along and between GAGs (Fig. 4d). In this case, since Avidin moves between interaction sites it does not make sense to refer to “binding” to any particular site (Avidin actually also has true diffusion-arresting binding sites within ECM, for which the term “binding” does make sense).[21] The partition coefficient between the gel and a bath in this limit can be quantitatively predicted via Donnan equilibrium analysis, which assumes a constant potential throughout the gel (green dotted line in Fig. 4c and 4d).[77] Note a subtlety of language found in the literature: partition coefficient of a solute between two phases is usually defined as the concentration ratio of the solute in each phase at equilibrium, but in studies of diffusion in biological hydrogels, the partition coefficient often refers only to the partitioning of freely diffusing, unbound solute between the phases.[77] We define partitioning here in the latter sense.

Finally, differential partitioning into hydrogels can be driven by forces other than Donnan equilibrium. Steric interactions are an obvious case, as they drive partitioning of large solutes toward phases with larger mesh sizes. Aqueous two-phase systems (ATPSs) consisting of phase-separated dextran and PEG gels differentiate biomolecules based partly on their hydrophobicity.[146,147] Similarly, partitioning into membrane-less organelles such as Cajal bodies, P bodies, and P granules—which are likely formed via phase separation,

sometimes including a sol-gel transition—is a key feature of cellular organization.[148,149] Finally, transport through the nuclear pore depends in part on a combination of hydrophobic and electrostatic partitioning effects.[102,150]

4. Selective transport of objects on the order of mesh size

4.1 Adhesive interactions and polyvalent trapping

When large particles are not inert with respect to the gel matrix, even low-affinity binding tends to dramatically slow particle diffusion because the particles bind multivalently to the gel (Fig. 6a).[128] For example, while hydrophobic interactions moderately reduce the diffusivity of hydrophobic drugs in mucus, polystyrene beads are almost completely trapped because they form many hydrophobic bonds simultaneously. Similarly, aminated beads bind to polyanions in mucus, generally reducing their diffusivity relative to carboxylated polystyrene beads.[43,44] Carboxylated beads in turn diffuse far more slowly in mucus than do PEGylated beads, due either to hydrophobic interactions resulting from incomplete coverage by carboxyl groups or to interactions with patches of positive charge on mucin molecules (similarly to the description of ECM, as described in Section 3.3).[51,129,151] PEGylated beads also diffuse more quickly than cationic and anionic nanoparticles in biofilms; the relative diffusivities of the charged nanoparticles depend on the species.[44,46,152]

Low molecular weight (2-5 kDa) PEG coatings are particularly effective at reducing adhesive trapping for two reasons. First, PEG is neutral yet hydrophilic, thus precluding strong electrostatic or hydrophobic interactions.[51] Second, high-density surface-grafted PEG forms a brush that physically resists the adsorption of gel components,[153] while the low PEG molecular weight precludes adhesion to gel components due to chain entanglement.[51] Neither of these reasons is gel-specific, which explains why PEGylation seems to be fairly universally applicable to promoting diffusion in biological hydrogels: PEGylated particles effectively penetrate many types of mucus,[51–53,89,154,155] biofilms,[44] and ECM.[47,156,157] In addition, recently Maisel *et al.* (2016) recently showed that PEG molecular weights as high as 40 kDa can be used for mucus penetration, with higher molecular weights requiring extremely high PEGylation density to reduce entanglements.[158]

In addition to uniform surface coatings such as PEGylation or carboxylation, heterogeneous particle surfaces have been investigated in the context of viral diffusion in mucus. Viruses such as human papilloma virus (HPV), Norwalk virus, herpes simples virus 1 (HSV-1), and human immunodeficiency virus (HIV) quickly penetrate cervical or cervicovaginal mucus under pH-neutral conditions in the absence of antibodies,[91,159,160] likely because these viruses are densely coated with both positive and negative charges (Fig. 4a), yielding overall hydrophilicity without having any uniformly charged domains large enough for electrostatic interactions.[128] This explanation is corroborated by the charged peptide diffusion assay, in which the alternating-charge peptide had weaker interactions with mucin than the block-charge peptide (Fig. 4b).[131] Also, in contrast to influenza, these viruses do not specifically bind sialic acid; as mentioned in Section 3.1, sialic acid binding hinders influenza transport and this may be compounded by multivalency (Fig. 6b). At any rate, some non-sialic acid dependent viruses are unable to penetrate the mucus barrier: adenoviruses and adeno-associated viruses (AAVs) used as gene therapy vectors diffuse thousands of times more

slowly in CF sputum than in water,[161] potentially explaining why the many gene therapy trials for CF employing adenoviruses or AAVs have all failed. Interestingly, mutating an AAV to reduce heparin binding enhanced penetration through CF sputum, highlighting the potential for rational design of gene delivery vectors to enhance transport.[162]

The mucosal immune system exploits polyvalency by employing antibody-based trapping of viruses. For example, anti-HSV-1 immunoglobulin G (IgG) located in cervical mucus can prevent HSV virions from penetrating the mucus and infecting cells. HSV trapping occurs because the Fc region of IgG binds mucin with low affinity; while this binding does not significantly hinder free IgG transport, when many IgG molecules coat one HSV virion, the weak polyvalent binding to mucins adds up to an interaction that is strong enough to nearly completely trap the virion (Fig. 6c).[160] Recent results suggest antibody trapping may even be more important than sialic acid-mediated trapping for preventing influenza penetration.[163] It should be noted that strong antibody binding to mucus and/or membrane-bound mucins may or may not occur;[164–166] however, theoretical treatments have shown that weak antibody-mucin binding is sufficient for trapping.[167,168]

Finally, exogenously added anti-PEG IgG and IgM appear to be able to trap PEGylated nanoparticles in mouse cervicovaginal mucus via the same polyvalent trapping mechanism as for viruses.[169] As mucosal anti-PEG antibodies may be common in the human population but are probably not present in animal mucus models, antibody-mediated trapping (and immunogenicity of PEG in general[170]) is a potential hurdle for clinical applications of PEGylated nanoparticles.[169]

4.2 Implications of gel penetration studies for nanoparticle design

Nanoparticles are used for a myriad of functions in medicine[171] including gene therapy,[53] antimicrobial[172] and anticancer treatments,[173] insulin delivery for diabetes,[174] and sustained drug release,[175] and many of these contexts require diffusion through a gel layer discussed in this review. Proper surface functionalization is therefore key to developing effective therapeutics. Two major lessons emerge from the studies of PEGylation, uniformly charged nanoparticles, and viruses described above. First, even weak adhesive interactions can trap a particle if there is a high density of binding sites. Of particular note is the ability of the immune system to generate antibodies against essentially all foreign substances, which means that antibody trapping should be considered wherever an immune response could be present.

Second, a good strategy across gel types to avoid both hydrophobic and electrostatic binding is to have a dense hydrophilic but overall neutral coating, which has been implemented in two different ways: neutral hydrophilicity and dense opposite charges. In addition to PEGylation, N-(2-hydroxypropyl) methacrylamide polymer (pHPMA)[174,176,177] and poly(vinyl alcohol) (PVA)[178] were recently tested as alternative mucoinert coatings, with some success. A dense coating of opposite charges has shown effectiveness in multiple different contexts, including viruses (as discussed above), self-assembled nanoparticles comprised of distinct ratios of polycationic chitosan and polyanionic poly(acrylic acid) (also in mucus),[26,179] zwitterionic dilauroylphosphatidylcholine monolayer-coated nanoparticles (in intestinal mucus),[180] and liposomes with zwitterionic dipalmitoyl phosphatidylcholine

membranes (in biofilms and respiratory mucus as an inhaled treatment carrying amikacin, an aminoglycoside; currently in clinical trials).[172] Not all of these coatings provide ideal protection against gel interactions, and in particular polymer brushes form a useful physical adsorption barrier in addition to a biochemical one. Yu *et al.* found that while a zwitterionic liposome diffused faster than polystyrene nanoparticles in cervical mucus, PEGylating the liposome still significantly increased its diffusivity.[181]

The utility of promoting gel transport is application dependent, and gel adhesion may be unavoidable or even desirable.[9] Mucoadhesive drug delivery systems are useful for promoting long contact times with mucosal surfaces, thus allowing for sustained drug release, particularly on surfaces with slow turnover.[9] Cationic liposomes may have increased contact time with biofilms and promote sustained antibiotic release, and their affinity with bacterial membranes gives them increased antibacterial efficacy.[175] Furthermore, even slowly diffusing nanoparticles will eventually penetrate a gel depending on the timescale of treatment, and even if the particles themselves do not penetrate, released free drug likely will.

Several groups have designed nanoparticles that change properties over time in order to exploit both fast initial diffusion and the favorable properties of cationic particles (gel adhesion and/or cell penetration). Two approaches have been used: 1) a dissociable mucoinert coating with a cationic core,[174] and 2) alkaline phosphatase-mediated phosphate cleavage that increases overall surface charge over time, thus switching from a mucopenetration to charge-mediated trapping.[182,183]

5. The nuclear pore: a case study in transport at the molecular level

Having illustrated the main factors that control transport behavior in biological polymeric matrices, we now discuss the nuclear pore in detail as a case study. Nuclear pore translocation encompasses every concept discussed above and has been studied in molecular detail, which is not possible for complex heterogeneous macroscopic gels such as mucus, biofilms, and ECM.

The nuclear pore complex has a diameter of approximately 100nm, and the central channel is filled with intrinsically disordered proteins called nucleoporins. These nucleoporins contain many instances of short hydrophobic repeats called FG repeats, which likely act as crosslinkers. The nuclear pore inhibits nuclear translocation of proteins larger than roughly 30-100kDa and even some smaller proteins such as histones.[48,184] However, NTRs efficiently carry large cargo through the nuclear pore.[49] This transport ability of NTRs derives in large part from exposed hydrophobic patches on NTR surfaces, which have hydrophobic and/or **π-π** interactions with the FG repeats on nucleoporins.[68] The mechanism by which these interactions mediate selective transport is still under debate, and multiple models exist.

In the selective phase model, the nuclear pore is represented as an FG repeat-crosslinked hydrogel whose pore size determines the size cutoff above which inert particles cannot penetrate the hydrogel.[102] NTRs locally dissolve the gel by binding FG repeats and

disrupting the crosslinks, allowing for penetration of large objects (Fig. 3e).[68] While individual FG crosslinks are weak and highly dynamic,[185,186] even transient crosslinks may contribute to barrier function, and these transient crosslinks may be dissolved by hydrophobic NTR regions. Brownian dynamics simulations suggest that while FG crosslinks are dynamic, at each point in time the crosslinks percolate through the center of the pore, forming a hydrogel in some technical senses.[187] Purified FG-rich nucleoporin domains self-assemble into hydrogels with transport selectivity similar to that of the nuclear pore.[68]

The other main family of models, the virtual gate and/or entropic barrier models, notes that there is a free energy barrier to entering the nuclear pore because nuclear pores are extremely small relative to the size of the nucleus or cytoplasm.[188] Thus, entering the pore imposes a loss of translational entropy and corresponding increase in free energy; entropic exclusion by nucleoporins causes an even greater increase in free energy.[185,186] In these models, NTR binding to FG repeats functions like Donnan partitioning: it locally increases the concentrations of NTR near and inside the nuclear pore, without immobilizing the NTRs due to fast unbinding from FG repeats.[141,142] This local enrichment of NTRs overcomes the barrier to nuclear pore entry and thus enables effective transport of NTR-cargo complexes.[189] Note that the virtual gate and selective phase models are not mutually exclusive; Donnan-like transport facilitation effects, entropic exclusion, and dissolution of FG repeat crosslinks may all be important for nuclear pore transport *in vivo*.

Finally, the fact that hydrophobic interactions with FG repeats are required for transport through the nuclear pore does not mean that charge is not important as well. Our group has found that nucleoporins and NTRs tend to have complementary net charges (positive and negative respectively), thus implying that charge assists in the recruitment and translocation of NTRs;[150] this hypothesis is supported by theoretical[189] and structural[141] analysis.

6. Outlook

We have taken a whirlwind tour through the forces and concepts that guide diffusion through ECM, mucus, biofilm EPS, and the nuclear pore. In all of these systems, size filtration prevents the penetration of overly large particles via a steric barrier; in turn, size filtration can be modulated by gel cleavage or reversible crosslink disruption. Interaction filtering enables more precise tuning of gel selectivity. For particles below the mesh size, the nuclear pore displays little selectivity; diffusion in the ECM is mainly determined by electrostatics; both hydrophobic and electrostatic interactions are important in mucus; and electrostatic interactions are certainly important in biofilms, while hydrophobic interactions may or may not be important depending on the species and environment. Electrostatic interactions can have multiple effects that facilitate or repress particle penetration depending on whether the interactions constitute binding or Donnan partitioning; these interactions are useful for optimizing drug delivery. Similar diffusion rules apply to biological hydrogels that have not been discussed in great detail here. For example, in the vitreous humor, cationic nanoparticles diffuse more slowly than anionic nanoparticles and PEGylation enhances transport.[190–192]

Chemical selectivity acts on the nanoscale, where small variations in the spatial arrangement of charge tune gel binding affinity.[131] Furthermore, the relationship between charge-charge and hydrophobic interactions is not fully understood, and the chemical heterogeneity of biological gels lends the problem an additional layer of practical uncertainty. These difficulties notwithstanding, the cases of PEGylated tobramycin[132] and charge-reduced lysozyme[137–140] suggest that engineering drugs with hydrogel interactions in mind can improve efficacy. Engineering drug interactions with biological gels may also lead to new functional biocompatible hydrogels, sidestepping the need for complex synthetic formulations. For example, complexing LL-37 (a CAMP) with a calcium alginate hydrogel reduced the toxicity of LL-37 toward mammalian cells and allows for sustained LL-37 release kinetics.[193] Covalently crosslinked methacrylated bovine submaxillary mucin hydrogels also showed sustained release kinetics of both polymyxin B (a cationic antibiotic) and paclitaxel (a hydrophobic cancer drug).[194]

For large particles, polyvalent weak interactions with the gel matrix can lead to adhesion and trapping. Synthetic nanoparticles, and probably some viruses as well, use net-neutral hydrophilic coatings to avoid trapping and promote mucus penetration.[128] On the other hand, weak interactions may facilitate gel penetration through transient crosslink dissolution. This mechanism may occur in the nuclear pore and reconstituted gels formed from nucleoporin.[68] Studies of the nuclear pore's unique transport properties may lead to new innovations; for example, nuclear pore-mimicking nanopores have been developed with novel potential biotechnology applications.[195]

Given the importance of gel permeability in living systems, it may serve as a biomarker for disease states, particularly for mucosal diseases in which mucus is easily accessible. For example, we showed that cervical mucus stratifies reproductive states, distinguishing between pregnant women at high and low risk for preterm birth via both rheological and permeability-based assays ([196] and submitted; Fig. 1a depicts the visual difference between mucus from high- and low- risk patients). Overall, understanding particle transport in biological gels will lead to powerful applications and innovations in diagnostics, biomimetic gels, pharmacology, and nanomedicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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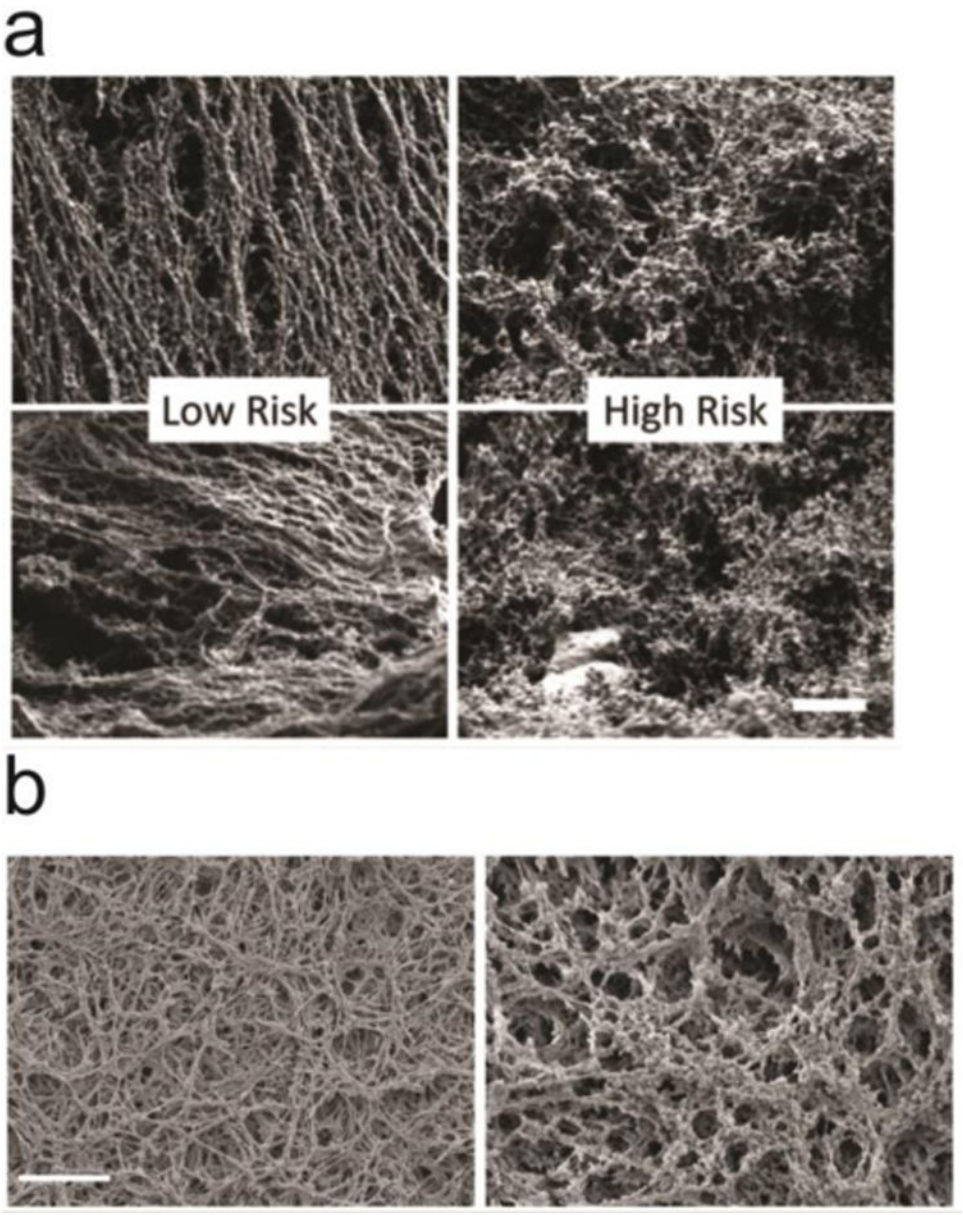

Figure 1.

Scanning electron microscopy of biological hydrogels. a) Cervical mucus samples from pregnant patients at low or high risk for preterm birth. Scale bar: 200nm. Reprinted with permission from Critchfield *et al.* (2013).[196] Copyright (2013) PLOS. b) Reconstituted basal lamina ECM gels. Scale bar: 25μm. Reprinted with permission from Arends *et al.* (2015).[197] Copyright (2015) PLOS.

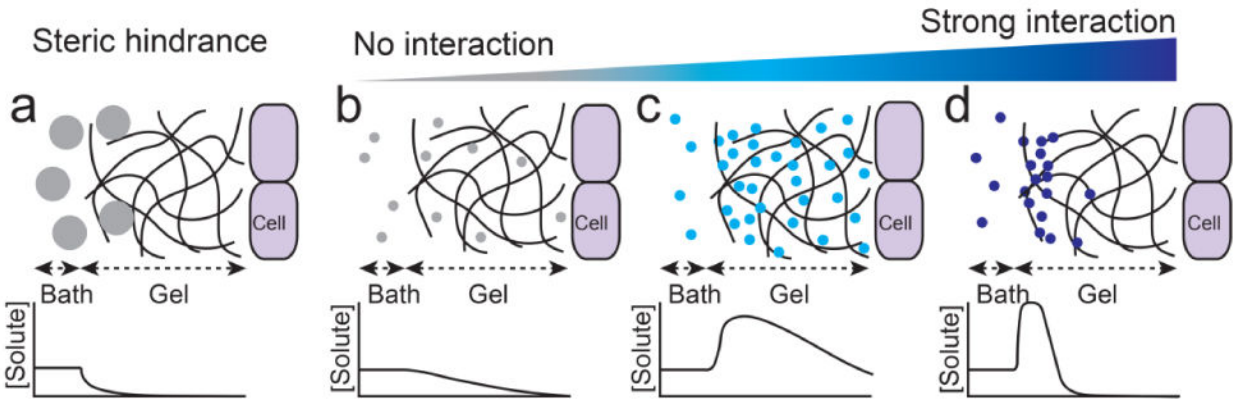

Figure 2.

Effects of steric hindrance and chemical interactions on gel penetration. a) Particles above the mesh size are unable to penetrate the gel, even if they do not interact with the gel. b) Small inert particles penetrate gels. c) Under some conditions (see section 3.4 for details), weak interactions with gel polymers can enhance partitioning into the gel and subsequent penetration. The schematic assumes that the bath is fixed at a constant solute concentration. d) Binding to the gel causes enrichment of solute at the interface but slowed gel penetration.

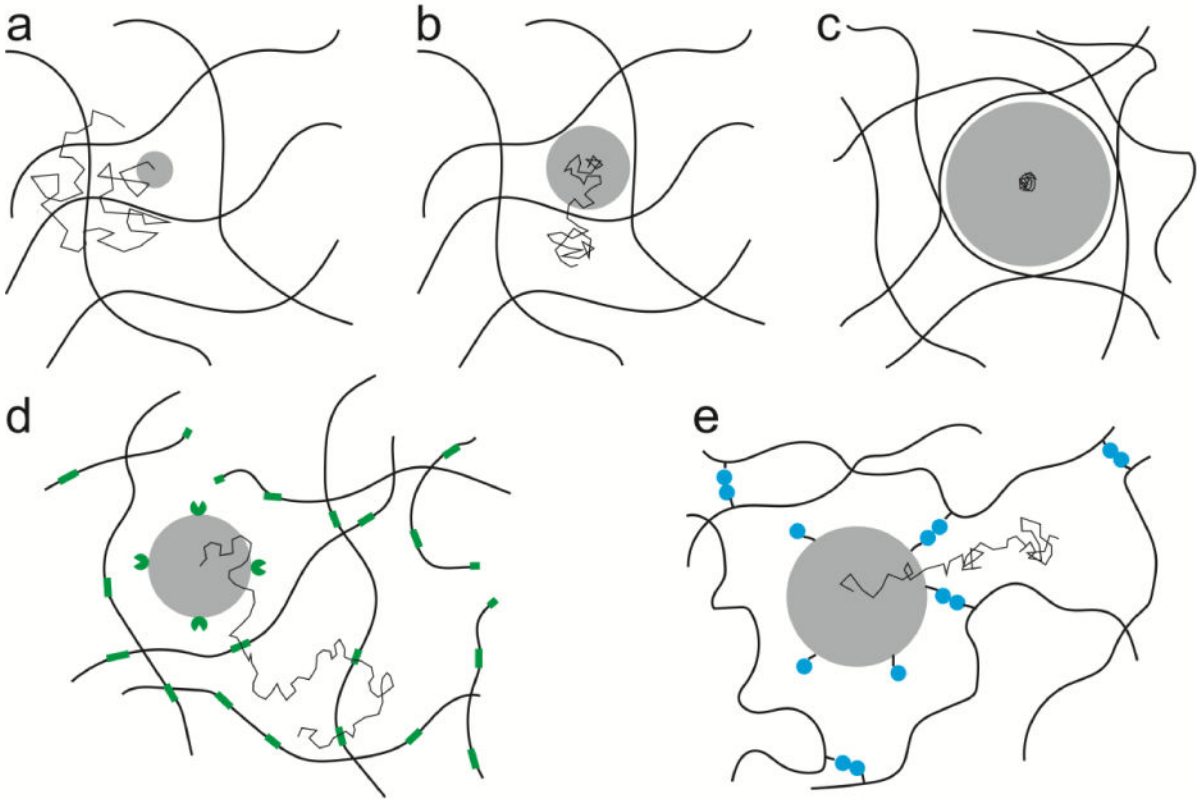

Figure 3.

Steric effects on diffusion in gels (a-c) and mechanisms to modulate steric hindrance (d-e). Thin lines represent thermal motion of the particle. a) Particles smaller than the mesh size diffuse freely in interstitial fluid. b) Particles on the order of the mesh size have significant steric hindrance but eventually penetrate gels. c) Large particles are trapped. d) Particles that cleave gel polymers may diffuse more quickly. Notched green circles attached to particle are lytic enzymes; green segments of gel polymer are substrates for the enzymes. e) Particles may reversibly disrupt gel crosslinks (blue-blue contacts), allowing enhanced diffusion without irreversibly degrading the gel.

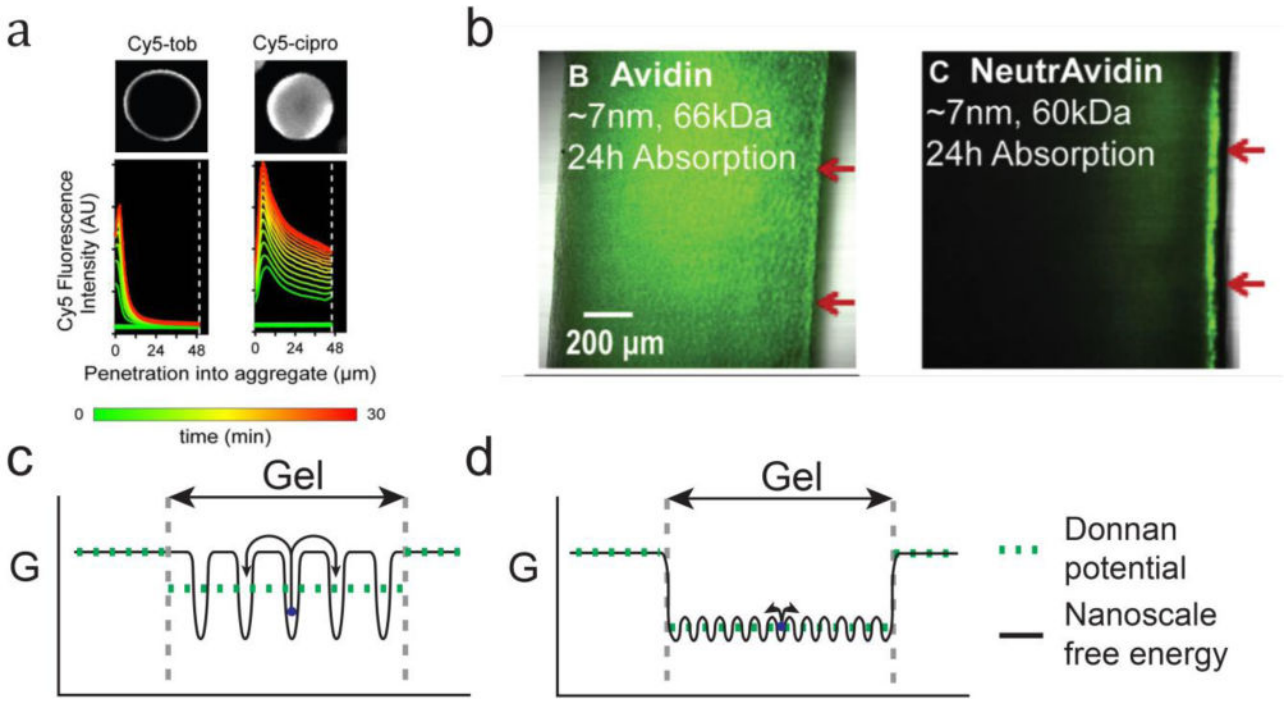

Figure 4.

Distinction between "binding" and "partitioning." a) Penetration of Cy5-labeled (fluorescent) tobramycin and ciprofloxacin into *P. aeruginosa* biofilms. Plots represent quantified timecourses of penetration, with representative images shown above plots. Cy5-tobramycin penetrates the gel less extensively than Cy5-ciprofloxacin. Adapted with permission from Tseng *et al.* (2013).[76] Copyright (2013) Society for Applied Microbiology and John Wiley and Sons Ltd. b) Penetration of Avidin and a neutral Avidin variant (NeutrAvidin), both fluorescently labeled with fluorescein isocyanate (shown in green), into bovine articular cartilage. Positively charged Avidin penetrates more than NeutrAvidin. Reprinted with permission from Bajpayee *et al.* (2014).[21] Copyright (2014) Elsevier. c, d) Schematic of difference between a and b in terms of nanoscale free energy landscape. c) Cy5-tobramycin binds specific targets and must escape binding energy wells to continue diffusing, meaning that electrostatic potential is not constant on the nanoscale. d) Avidin diffuses in nearly uniformly charged cartilage; thus, binding energy wells are minimal and a Donnan treatment of electrostatics is appropriate.

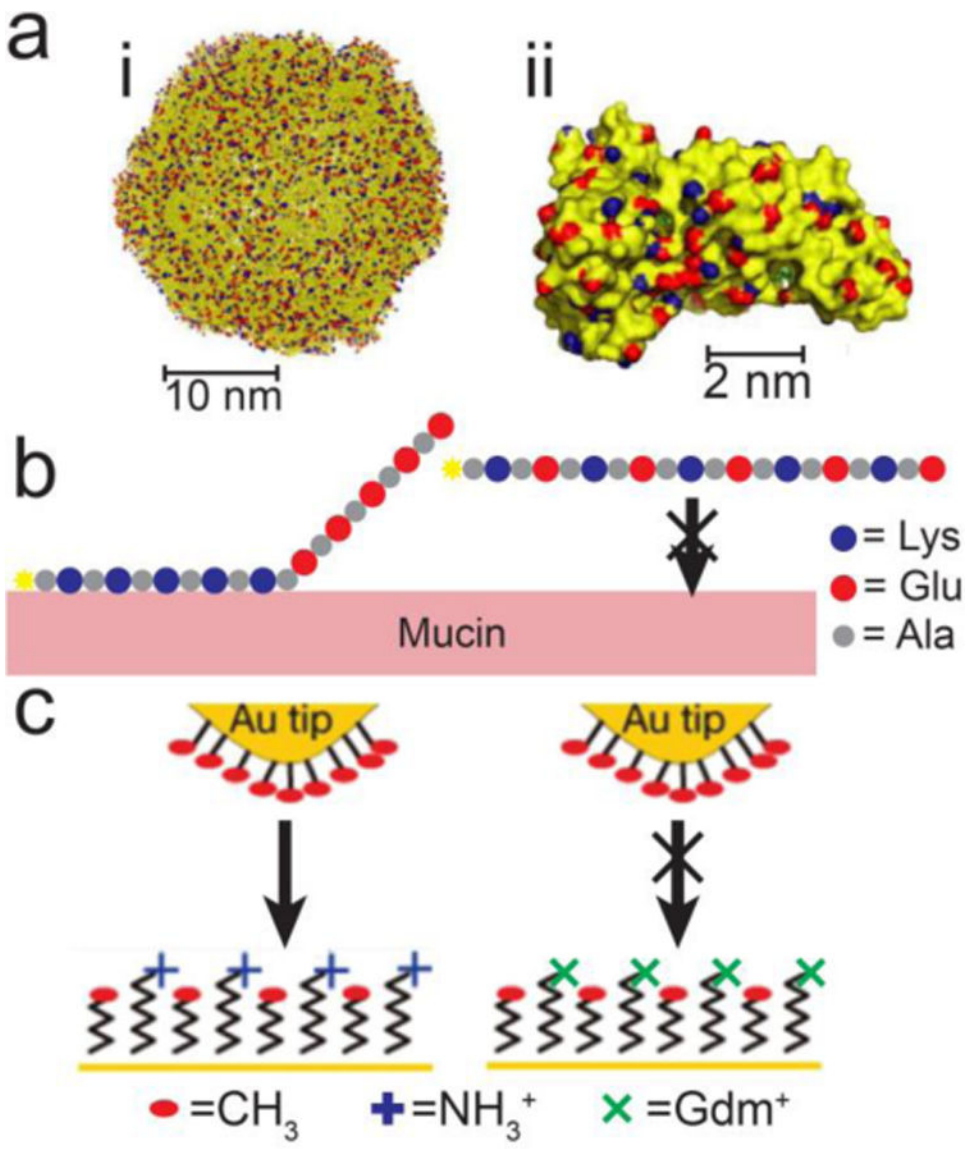

Figure 5.

Nanoscale heterogeneity of solutes and effect on gel diffusion. a) Surfaces of (i) human rhinovirus (PDB: 2rm2) and (ii) human albumin (PDB: 2bxi), with positive charges in blue and negative charges in red. Reprinted with permission from Cone (2009),[128] (Copyright (2009) Elsevier). Rhinovirus and albumin are densely coated with opposing charges. b) Two fluorescently labeled peptides with the same net charge but different spatial arrangement. The “block” peptide at left interacts weakly with mucin while the “alternating” peptide at right does not;[131] schematic shows potential mechanism for this difference. c) Effect of immobilized charges on nearby hydrophobic interactions, probed by measuring the adhesion of a hydrophobically functionalized gold (Au) atomic force microscopy tip adhesion to surface monolayers. Amine (NH_3^+) groups strengthen hydrophobic interactions between these hydrophobic surfaces, while guanidinium (Gdm^+) groups weaken or eliminate them. Adapted by permission from Macmillan Publisher Ltc: Nature (Ma *et al.*, 2015).[135] Copyright (2015).

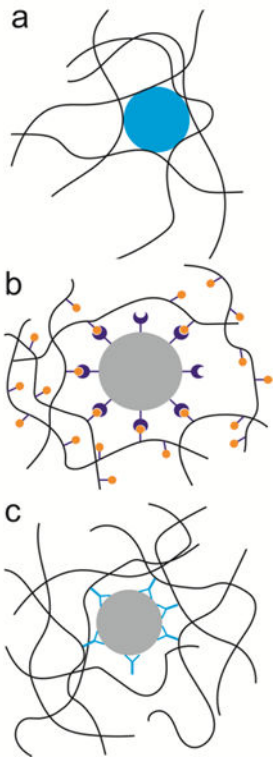

Figure 6.

Mechanisms of polyvalent trapping in gels. a) Non-specific interactions (hydrophobic, electrostatic, etc) with gel can trap a particle, even if individual interactions are weak. b) Binding to decoy receptors (such as sialic acid) that are present on gel polymers can trap a particle. c) Gel-binding antibody bound to an otherwise inert particle mediates trapping.