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Molecular Recognition with Soft Biomaterials

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Abstract:

Biomacromolecules and engineered materials can achieve molecular recognition if they engage their ligand with properly oriented and chemically complementary moieties. Recently, there has been significant interest in fabricating recognitive soft materials, which possess specific affinity for biological analytes. We present a summary and evaluation of current recognitive materials for biosensing, drug delivery, and regenerative medicine applications. We highlight the impact of material composition on the extent and specificity of ligand adsorption, citing new theoretical and empirical evidence. We conclude with a guide for synthesizing and characterizing novel recognitive materials, as well as recommendations for ligand selection and experimental design.

1. **Introduction**

Molecular recognition occurs when intermolecular interactions impart specific affinity between a ligand and receptor. This process determines the functionality of any system that identifies or separates a target molecule from a complex solution. Researchers have designed materials capable of molecular recognition for many biomedical applications, including but not limited to: resins for biomolecule purification (1,2), recognition elements for biosensing (3,4), affinity-mediated scaffolds for tissue regeneration (5,6), and cell-targeted nanoparticles for drug delivery (7,8).

Biomaterials engineered to exhibit molecular recognition properties will bind and sequester their target molecule in physiological environments. This specific affinity can help a researcher overcome some seemingly paradoxical design challenges within the biomedical domain. For example, scaffolds for tissue regeneration must be macroporous, with a pore size of several microns, to promote cell infiltration and growth (9,10). However, they must also retain and deliver protein cytokines, which possess a hydrodynamic diameter of only a few nanometers.

If a hydrogel scaffold relies solely on physical restriction (i.e. mesh size) for drug retention and delivery, entrapped cytokine will diffuse and elute rapidly. However, by adding recognitive material components, the mechanism of cytokine release shifts from *diffusion-mediated to affinity-mediated* (5,11). In this revised scenario, the kinetics of cytokine release are determined by the equilibrium dissociation of cytokine from the recognitive moiety. Depending on the magnitude of cytokine-material affinity, this can lead to retention within the scaffold from days to weeks.

Recognitive biomaterials are also useful in the processing or analysis of biological fluids. For example, Culver *et al.* (12) demonstrated that a poly(N-isopropyl acrylamide-co-methacrylic acid) hydrogel, coated on the surface of silica gold nanoshells (AuNS) sensitively binds and detects lysozyme and lactoferrin in human tears. The mechanism of molecular recognition was Coulombic interaction between the hydrogel and protein, leading to effective binding of lysozyme and lactoferrin (cationic, high isoelectric point protein) and exclusion of anionic proteins. This general separation and detection (i.e. on the basis of charge) is especially effective for proteins that are present at high concentration in the fluid (i.e. 1 μM or more) and this platform is promising as a point-of-care biosensor.

As illustrated through these two examples, scientists can modulate ligand – hydrogel affinity in a number of ways, to meet the design specifications of diverse medical products. In this review and perspective, we first give a brief overview of the fundamental principles that explain the ability of hydrogels to recognize biological molecules. Then, we highlight some of the most recent applications of recognitive hydrogels, which are leveraging molecular recognition to improve protein biosensing, gene delivery, and wound healing. We conclude with a discussion of current challenges that face the field, offering our commentary and suggestions for future research. In particular, we want to emphasize the importance of stating problem-driven, quantitative design criteria when developing new recognitive hydrogels, and performing rigorous, biologically relevant assessments to demonstrate efficacy.

2. Fundamental Considerations

Contributions of Intermolecular Interactions to Recognition Characteristics:

Molecular recognition is an intermolecular phenomenon, and is governed by species' chemical potential and their mechanical or physical constraints. (13,14) In biological systems, molecular

recognition occurs when a moiety or epitope on a ligand is complementary to one on a receptor. The specificity of that interaction is governed by the uniqueness of the ligand or receptor binding domain, while the affinity is determined by the thermodynamic properties of the ligand-receptor binding event.

The physical and chemical properties of hydrogel biomaterials determine the extent to which they permit the diffusion of solutes and engage in high-affinity binding events. Exhaustive reviews, which detail the fundamentals of hydrogel swelling in physiological fluids and the thermodynamics of protein-polymer molecular recognition events, were published recently by Koetting *et al.* (15) and Clegg *et al.* (16) respectively. Here, we present a brief overview, but like to reference readers to those reviews for detailed thermodynamic analysis.

The two most important intermolecular interactions driving material-solute affinity are Coulombic and hydrophobic interactions. Coulombic interactions between charged ligands and receptors act over a long distance, relative to hydrophobic interactions or hydrogen bonding (16). However, because physiological fluids contain ions, interactions between charged ligands and receptors are screened (i.e. not felt to any significant extent) when the intermolecular distance is greater than the Debye length (17). The Debye length is a function of each species' formal charge, the dielectric constant of the solvent, the concentration of ionic species in solution and the temperature. In spite of charge screening, Coulombic interactions are critical to ligand-receptor binding, as the interaction of oppositely charged species is enthalpically favorable (18,19). Therefore, it is useful to tune charged moiety content of engineered materials to engage or repel charged biological ligands (i.e. small molecule ions, protein, DNA, RNA).

Material hydrophobicity is another design parameter which can be modulated to influence the extent and specificity of protein adsorption. Ligand interaction with a hydrophobic material can be both entropically and enthalpically favorable (20–22). When a hydrophobic material is submerged in physiological, aqueous fluids, water molecules form a fixed arrangement around it to minimize material-water interactions. When a ligand (such as a protein) adheres to the hydrophobic material, it liberates those water molecules, leading to a greater system entropy. So while hydrophobic interactions require ligand-receptor contact, and are therefore experienced only at very short distances, they contribute significantly to the affinity of the binding event.

An illustrative *in silico* study, which investigated the influence of the charge state of ligands and receptors on binding thermodynamics, was published by Baron *et al.* (22). The authors modeled the system as a spherical ligand, a corresponding hemispheric receptor (radius = 0.8 nm), and 1030 water molecules (**Fig. 1a**). They ran molecular dynamics simulations to investigate the entropic and enthalpic contributions to ligand-receptor association, given a set cavity and ligand charge. As shown in **Figure 1b,** in nearly all cases, the entropic contribution to the binding free energy (green bars) is negative, indicating a greater entropic state due to liberation of associated water. The only cases with significant enthalpic favorability (blue bars) are when the ligand and receptor are of opposite charge, or are both electrically neutral. Charged ligand binding to a neutral cavity was not favorable, in spite of the greatest entropic gain of all cases, due to the significant enthalpic cost of breaking water-water interactions.

Influence of Solute Transport on Molecular Recognition by Hydrogels: Up to this point, our conceptual and simulation examples focused on *ligand binding to a single receptor*, where the ligand and receptor have known physical properties (i.e. charge, hydrophobicity). However, hydrogel biomaterials usually possess an interconnected nanoscale or microscale pore

structure, which presents a multitude of sites for ligand adsorption. Ligand adsorption within a hydrogel mesh is determined by both the affinity interactions between the ligand and polymeric backbone and the size-exclusion characteristics of the hydrogel network (23). A protein can only diffuse through a hydrogel if the mesh size (i.e. distance between crosslinked polymer chains) is greater than the protein's hydrodynamic diameter (15).

Recently, Axpe *et al.* (24) published a new predictive model for solute diffusion in hydrogels. Their probabilistic model describes three distinct regimes of solute diffusion, which depends on the relationship between the size of the solvent, solute, and mesh, and makes the simplifying assumption that the solute and gel to not interact. As shown in **Figure 1c**, when the solute and solvent are similar in size, and both are much smaller than the gel's mesh size, the solute passes through the network according to free volume theory. According to the free volume theory, developed by Lustig and Peppas (25,26), these small molecule solutes pass through free voids that are dynamically present between molecules of polymer and solvent. Thus, the gel acts as a molecular sieve. When the solute is much larger than the water, but also much smaller than the gel mesh size, the solute passes through the mesh alongside the entrapped water. In this case, the gel mesh size, rather than the free volume, becomes the limiting parameter to solute flux. In the final case, where the mesh size is similar to the solute hydrodynamic diameter, solute efflux is physically restricted by the crosslinked polymer chains (24).

Typically, biomedical hydrogels possess a mesh size substantially greater than the solute (i.e. protein, nucleic acid) diameter. Therefore, strategies are necessary to increase solute retention to either capture a target molecule (i.e. biosensing) or retain payloads for sustained delivery (i.e. drug delivery, regenerative medicine). The most common approach, which is the focus of this perspective, is to overcome this is tradeoff by increasing the intermolecular interactions between the gel and solute. However, physical approaches to payload retention have also been explored, and can be quite useful for specific applications.

One example of a physical approach is to fabricate supramolecular, solute-loaded structures, such as nano- or micro-particles, and to physically embed the structures within a macroporous hydrogel (27,28). With this strategy, delivery is controlled by the mesh size, degradation, or equilibrium association of solute with the nano or micro-particle, rather than the bulk hydrogel. Common examples of biomedical hydrogels which employ this strategy are composite hydrogel containing nanocrystals or nanoclays, which have demonstrated significant utility for regenerative medicine applications (29,30).

In summary, solute retention or elution from a hydrogel is determined by the physical properties of the gel, the size of the solute, and the solute-gel interaction. Scientists can use various design strategies to modulate the effective solute diameter, hydrogel mesh size, and the solute-gel affinity. In the following section, we will review some illustrative examples of recognitive material design for biomedical applications.

3. Application of Molecular Recognition in Biosensing, Drug Delivery, and Regenerative Medicine:

In this section, we provide a survey of recent studies that have used recognitive materials and devices for biomedical applications. As the potential applications are expansive, this survey is by no means exhaustive, but rather intended to illuminate the importance of molecular recognition within the biomedical domain.

Biosensing: Biosensors must contain recognitive and transducing elements, so that changes in the concentration of a solute of interest lead to a detectable signal. Recognitive polymers have been applied to recognize and signal the presence of biomarkers of all sizes, from small molecules to whole cells. One of the most common examples of small molecule sensing with recognitive materials is the glucose oxidase – polymer system. Our lab and others have used this system extensively. For example, Podual *et al.* fabricated poly(diethylaminoethyl methacrylate-g-ethylene glycol) networks, where glucose oxidase and catalase were immobilized. When these networks were exposed to glucose, gluconic acid was generated by the pendant enzyme, leading to a reduction in the local pH and network swelling (31). This swelling was dynamic and reversible, allowing repeated exposure to, and sensing of, glucose solutions.

Polymeric sensors have been developed for sensing other biologically relevant small molecules. For example, Zhang *et al.* generated polymer-modified carbon nanotubes that through composition alone exhibited selective affinity for riboflavin (32). Upon binding riboflavin, the nanotube-polymer structure fluorescence exhibited a red shift (near infrared region), enabling real time detection of riboflavin. When these nanosensors were taken up by macrophages, they acted as a real-time intracellular riboflavin sensor.

Further research has led the development of polymeric biosensors for proteins and cells. Similar to small molecule sensing, it is possible to recognize proteins by carefully tuning the composition of polymers, such that they possess an optimized quantity of complementary functional groups. These recognitive polymers can be used to differentiate proteins with a range of physicochemical characteristics (4). In an effort to fabricate materials with selective affinity for proteins and cells, researchers have explored more sophisticated synthesis methods such as protein imprinting and template-guided synthesis.

For example, Rana *et al.* developed a group of green fluorescent protein – conductive polymer complex, which was capable of classifying healthy, cancerous, and metastatic cells (33). When the conductive polymers and GFP were complexed in solution via electrostatic interactions, they formed a Förster Resonance Energy Transfer (FRET) pair. Polymer interaction with mammalian cell surface, which differed between cell types, resulted in a change in the fluorescent signal. By using multiple polymeric receptors, the authors were able to generate unique signal fingerprints for 16 different cell types. They used the sensor system to classify unknown samples with 95% accuracy.

Drug delivery: Within the drug delivery field, molecular recognition characteristics are leveraged to achieve a number of design functions. For example, modulating the intermolecular interactions between a drug and its carrier can lead to enhanced payload loading, as well as sustained release kinetics (34,35). Modification or degradation of the carrier, as a result of a molecular recognition event, can further be a mechanism for intelligent or responsive drug delivery (36–40). Through the rational design of ligand-conjugated nanomaterials, the ligand-receptor interaction can also be leveraged for targeted delivery (41,42).

In a recent example, Rouet *et al.* (43) fabricated Cas9 ribonucleoprotein (RNP) conjugates, which achieved cell-specific gene editing through a molecular recognition event. The ASGPr ligand (trimer), which was linked to the Cas9 RNP through via a disulfide bond, promoted specific cellular uptake by hepatocytes **(Figure 2a)**. Through co-administration of the ASGPr-Cas9 RNP conjugate and an endosomolytic peptide, the authors achieved selective cell uptake and editing of a target gene (EMX1). Their work is a great example of leveraging molecular recognition for advanced, translational drug delivery.

In another example, Tian *et al.* fabricated surface-engineered exosomes for the targeted delivery of curcumin to treat cerebral ischemia (44). The authors isolated exosomes from cultured mesenchymal stem cells, modified the exosome surface with an adhesive c(RGDyK) peptide, and loaded curcumin post-fabrication. Exosomes, as well as control treatments, were administed via tail vein injection in a mouse ischemia model. While exosomes alone minimally decreased the expression of inflammatory markers in the tissue lesion region **(Figure 2b)**, exosomes loaded with curcumin inhibited inflammation significantly. This study nicely demonstrated how molecular recognition properties of engineered nanomaterials can enhance the efficacy of therapeutic agents.

Regenerative medicine: The retention or depletion of bioactive molecules can help form a suitable cell microenvironment for tissue engineering and regenerative medicine applications. For example, Abune *et al.* (45) fabricated macroporous poly(ethylene glycol) (PEG) scaffolds with pendant DNA aptamers for vascular endothelial growth factor (VEGF) or fibroblast growth factor (bFGF) **(Figure 3a)**. These aptamer-functionalized scaffolds bound and retained VEGF of bFGF, exhibiting superior cytokine retention for regenerative purposes. *In vitro*, the retention of both VEGF and bFGF promoted HUVEC cell migration **(Figure 3b)**. When scaffolds containing the cytokine retained for more than three days were implanted in the chorioallantoic membrane of chicken embryos, they successfully promoted vascularization **(Figure 3c)**.

Numerous similar approaches have been taken to form a regenerative niche via growth factor-sequestering biomaterials. For example, Grier *et al.* (46) applied similar logic in the formation of collagen-glycosaminoglycan scaffolds, which were modified with beta-cyclodextrin groups. The beta-cyclodextrin groups engaged in guest-host interactions with either BMP-2 or transforming growth factor (TGF) beta, leading to growth factor sequestration, sustained release, and guided differentiation of mesenchymal stem cells.

In a related approach, researchers have demonstrated that implanted biomaterials can bind and retain endogenous cytokine for regenerative purposes. For example, Lee *et al.* (47) used peptide amphiphile nanofibers with a bone morphogenic protein 2 (BMP-2) binding peptide for bone regeneration. These nanofibers retained exogenous BMP-2, and were able for facilitate bone healing without the administration of recombinant cytokine.

In another recent study, Li *et al.* fabricated an ECM-mimetic hydrogel sponge comprised of eletrospun fibers of a polysaccharide (EUP3) and gelatin (48). The EUP3 possessed a platelet-derived growth factor-BB (PDGF-BB) motif, enabling the binding and retention of PDGF-BB *in situ* **(Figure 3d)**. In a mouse wound model, the scaffold successfully retained endogenous PDGF-BB **(Figure 3e)**. They facilitated wound healing by recruiting endothelial cells, promoting vascular maturation, and increasing cell proliferation.

4. Challenges and Progress in Research on Protein Recognition

As proven through the previous series of examples, the ability to recognize biomolecules in solution is paramount to the function of biosensors, drug delivery vehicles, and scaffolds for regenerative medicine. The gold standard for molecular recognition is the monoclonal antibody. Monoclonal antibodies bind their antigen with a high degree of affinity and specificity. Because of these advantageous recognitive properties, antibodies are used clinically for a number of applications including targeted cell therapy and diagnostic sensing. Other biological molecules, such as peptides and aptamers, also can exhibit molecular recognition characteristics if they are sufficiently complementary to an epitope on their target. These lower molecular weight

recognitive molecules are also useful for diverse medical applications, as we discussed in the previous section.

These natural recognitive molecules, however, can be costly to produce and are sensitive to their environment. They denature under heating, as well as in acidic or basic solutions. For point-of-care biosensing, it is advantageous for the system to be robust to environmental conditions and exhibit long-term stability without a cold chain (12,34). To achieve this design goal, researchers have studied synthetic systems, which mimic natural molecular recognition processes, for biomedical applications.

Molecular Recognition via Molecular Imprinting: Mimicking natural molecular recognition within synthetic systems, however, has proven a formidable challenge. One popular approach to generating synthetic receptors, which has been studied within the Peppas lab for many years, is molecular imprinting. In molecular imprinting, a pre-polymer solution of monomers and crosslinking agents are incubated with a ligand (here termed the template) and allowed to self-assemble. Following sufficient assembly time (typically 30 minutes), the polymerization is initiated, forming a crosslinked network around the template molecules. Through a series of washing steps, the template is removed, leaving behind nanoscale voids that are complementary to the template (**Figure 4d**) (49–54).

In the early days of molecular imprinting research, some major successes were achieved. For example, Hilt, Byrne, and Peppas fabricated molecularly imprinted polymers (MIPs) which recognized glucose (55). These glucose-imprinted polymers successfully bound more glucose than their non-imprinted analogues. Further the MIPs excluded galactose, a sugar that differs from glucose only by the position of a hydroxyl group, when incubated with glucose and galactose simultaneously (56). The authors posited that these MIP systems could have utility as controlled release systems, where the increased solute-gel affinity imparts an extended release profile (57,58), or as recognition elements within cantilever-based biosensors (59,60).

There were many trends which emerged from about a decade of work on small molecule imprinting within MIPs. The first was that a high degree of crosslinking, at times as high as 80 mole percent of the pre-polymer feed, was necessary to achieve significant recognition. Additionally, the choice of solvent was important – as the use of a polar, aprotic solvent improved the MIPs molecular recognition properties, as it promoted hydrogen bonding between the template and functional monomers during the self-assembly step. These trends presented a significant design challenge, as the field shifted from small molecule MIPs to protein MIPs.

In protein imprinting, dilute monomer concentrations and aqueous solutions are necessary to prevent template denaturation prior to synthesis (61). Additionally, a much lower degree of crosslinking is necessary, so that the hydrogel mesh size is greater than the protein template's hydrodynamic diameter. There have been some limited successes, where protein MIPs have exhibited recognition characteristics, particularly when proteins are imprinted on thin film surfaces (62–64). Protein imprinting in bulk hydrogels, a method analogous to that which was successful in small molecule imprinting, has consistently increased the gels' adsorption capacity, but not specific affinity, for the template (65–67).

A recent study by Culver *et al.* demonstrated the impact of protein templating of hydrogels' molecular recognition characteristics (66). The authors fabricated thin hydrogel coatings, which were imprinted with lysozyme, on solid nanoparticle supports. As shown in **Figure 4a**, lysozyme imprinting increased the gels' adsorption of lysozyme. By the metric of imprinting factor, which is the protein bound by the imprinted polymer normalized to the non-

imprinted polymer under the same conditions, molecular recognition was achieved. However, fitting a Langmuir isotherm to the data revealed that, although the maximum lysozyme adsorption was influenced by molecular imprinting, the equilibrium adsorption constant was not. Therefore imprinting increased the number of binding sites for lysozyme, but not the affinity of lysozyme for the gel. This conclusion was validated by incubating the MIPs and NIPs with a number of model proteins **(Figure 4b)**. The MIPs bound more of each protein than the NIPs, consistent with the hypothesis that the MIP possessed more protein binding sites than the NIP. When the MIP protein adsorption data were plotted as a function of protein molecular weight and isoelectric point (**Figure 4c**), a more interesting trend emerged. Protein recognition, as measured by the total adsorption, was a function of the protein molecular weight and isoelectric point, not its similarity to the imprinting template.

Clegg *et al.* took this a step further, fabricating MIPs with three distinct proteins that were similar in molecular weight and isoelectric point (65). While each of the MIPs bound more protein than the NIP, there were no differences in the protein adsorption behavior between any of the MIPs. This further validated that the imprinting process increased the networks' ability to load protein without imparting specificity for the template.

Next Generation Protein-Recognitive Materials: While protein imprinting has failed to yield recognitive proteins with single-molecule specificity, research on protein imprinting revealed a useful conclusion. By carefully tuning the composition of materials, it is possible to generate materials with high (nanomolar) affinity for protein biomarkers. Applications of these protein recognitive materials are numerous, and these high affinity formulations do not require the laborious process of molecular imprinting.

In the last couple of years, numerous studies have demonstrated the utility of these protein-binding nanomaterials for diagnostic and therapeutic purposes. For example, poly(n-isopropylacrylamide) nanoparticles containing either an anionic or cationic co-monomer were used to successfully identify eleven model proteins, which possessed a range of molecular weight and isoelectric point. After measuring the adsorption of each protein to multiple formulations within multiple buffer conditions, it was clear that each protein had a unique binding profile. Through this differential sensing approach, 100% classification accuracy of the 11 model proteins was achieved (4).

Other research groups have also demonstrated the utility of protein recognitive polymers. For example, the Shea group recently fabricated nanoparticles with nanomolar affinity for vascular endothelial growth factor (VEGF) (68). When these nanoparticles were administered within Matrigel hydrogels to mice subcutaneously, the nanoparticles successfully sequestered VEGF from the physiological environment and inhibited scaffold vascularization (68,69). They have applied similar logic, generating alternate nanoparticle formulations that possess high affinity for siRNA (70) or peptide toxins (71–74). The Strano lab has also fabricated a number of polymer composite materials, based on the self-assembly of amphiphilic co-polymers on carbon nanotubes. By carefully tuning the polymer composition, they were able to recognize human fibrinogen (75) and insulin (76) in serum with high selectivity.

Selection of Model Proteins for Material Research and Development: As protein-specific polymer formulations are becoming more of a reality, it is necessary to revisit some standard research practices. Specifically, the complexity of model protein selection. Frequently, researchers will use a 'model' compound when designing new polymeric formulations for a

specific medical application. These model proteins are intended to reduce the total cost associated with the material development process, as individual proteins are very expensive. Typically, researchers determine the bulk characteristics of their biomarker of interest (i.e. its molecular weight, shape, and isoelectric point) and then select a model protein which possesses similar properties.

In practice, however, a very limited number of model proteins are used repeatedly. A summary of the physicochemical characteristics of four of the most common model proteins is given in **Figure 5**.

By example, and as shown in the figure, lysozyme (hen egg white) and cytochrome c (bovine heart) are two model proteins that are similar in molecular weight, isoelectric point, and geometry. Through those three measures, you would expect lysozyme and cytochrome c to be rather interchangeable. However, a detailed analysis of the solvent accessible surface area of the two proteins (**Figure 5, right**) reveals significant differences in their relative surface composition. Lysozyme's surface is rich in arginine, whereas cytochrome c's surface is rich in lysine. Lysozyme has more polar, uncharged residues than cytochrome c. Cytochrome c has more anionic residues than lysozyme. We could point out any number of additional, significant differences.

These differences in solvent accessible surface lead to significant differences in the affinity interactions between each protein and polymeric structures. This has been shown repeatedly for lysozyme (4,65,66,77–79). Yet, this type of protein interchange is a typical research practice, without regard for the proteins' solvent accessible surface composition. We understand that cost-effective practices are necessary in academic research, and that model proteins are a necessary tool. Our goal is to highlight the importance of performing detailed analyses of the similarities and differences between model and target proteins. The Protein Data Bank (PDB), Protein Data Bank in Europe Proteins Interfaces Structures and Assemblies (PDBePISA), and the ExPASy bioinformatics resource portal are just a handful of the resources available to perform these analyses free of charge. With these data collected, researchers will be able to precisely state the extent to which their model protein mimics their target biomarker, and therefore determine their confidence in the model validity.

5. Contemporary Methods for Research on Recognitive Hydrogels

In order to meet the need for recognitive polymeric systems in medical applications, scientists have developed a number of methods for synthesizing and characterizing new formulations. The purpose of this section is to survey the repertoire of tools available to scientists in the future, who are interested in fabricating new recognitive systems.

Methods for Synthesis and Fabrication: A schematic description, which highlights the tradeoff between complexity and ease of synthesis when fabricating recognitive polymers, is given in **Figure 6a**. The most common synthetic approach for fabricating recognitive polymers is free radical polymerization. The main advantage of free radical polymerization is the relative ease of synthesis, and the diversity of commercially available monomers. Free radical polymerization is typically employed to generate random co-polymers of functional acrylates, methacrylates, or acrylamide derivatives. There is a great diversity of ionizable monomers, which can impart pH-responsive behavior and engage in electrostatic complexation with analytes upon polymerization. Further, there are commercially available hydrophobic monomers, which

allow scientists to tune the hydrophobicity of their materials (i.e. by modulating the chain length and steric bulk of the hydrophobic moiety).

The main disadvantage of free radical polymerization is the stochastic nature of monomer incorporation. Monomer incorporation is determined by the pre-polymer feed, as well as the relative reactivity of the species (80,81). Depending on the reactivity of a monomer with itself, as opposed to co-monomers, it is possible to yield random or block co-polymer architectures. The relative incorporation of monomer species can also be heavily influenced by the extent of reaction, if there is a significant difference in reactivity. Therefore, it is important to carefully select monomers with suitable reactivity for one's recognitive polymers.

Controlled or living polymerizations are a great alternative to random free radical polymerization if a higher degree of control over polymer molecular weight is needed, or if sequential addition of monomers for block co-polymerization is desired. Two of the most common controlled polymerization techniques for biomedical polymers are reversible addition-fragmentation chain transfer (RAFT) and atom transfer radical polymerization (ATRP). There have been numerous studies, which detail methods for RAFT (82,83) or ATRP (84,85) in water, making it a suitable polymerization method for both recognitive and or molecularly imprinted polymers. Recent studies have even suggested that the use of controlled polymerization techniques enhances the effects of molecular imprinting (86,87).

In an ideal case, a scientist would be able to determine the incorporation of monomers on a single subunit level. This would mimic biological systems, where amino acids are incorporated into proteins with single molecule precision. Two recent approaches for generating highly ordered polymers are sequential click reactions and template directed assembly. Sequential click reactions are highly powerful in that they proceed to exceptionally high yield, and can be designed to proceed in water in the absence of catalyst. On the other hand, they are a bit limiting because they can only accommodate alternating monomer incorporation (88,89). So, while they are very useful for generating alternating co-polymers that mimic nucleic acid repeat structures (90), they are not useful for generating precise architectures of multifunctional co-polymer.

In template directed assembly, monomers are allowed to associate with a macromolecular template either prior to, or during, polymerization. Molecular imprinting was one example of a template directed polymerization. Another example is the formation of highly ordered polymers via self-assembly with complementary DNA. In this case, a parent DNA strand is fabricated and incubated with the pre-polymer solution for very precise self-assembly. While this strategy is currently exploratory, it has already been employed for generating sequence-controlled xenonucleic acids (91) as well as crosslinked polymeric mimics of the bacterial cell wall (92). In the future, these highly ordered polymers could be immensely useful for disease diagnosis and therapy.

The gold standard process for synthesizing highly ordered organic molecules is biosynthesis. Cellular machinery translates genetic information into macromolecules (i.e. RNA, protein) with single base precision. Further, the cell possesses the necessary enzymes and co-factors to facilitate macromolecular folding and assembly into functional structures. For this reason, many studies which synthesize active biomacromolecules in a host organism, and after purification repurpose the molecule(s) for a diagnostic or therapeutic application.

Biohybrid systems are an intersection or integration of natural and synthetic components. As shown in **Figure 6b**, there is an extensive repertoire of natural and synthetic materials that are applied to perform biomedical functions. Biohybrid structures take advantage of the precision of biological systems, as well as the tunability and stability of synthetic materials.

Further analysis of the content within **Figure 6b** reveals another important trend with current biohybrid systems. Current systems utilize the molecular recognition properties of the natural component (i.e. antibodies, peptides, aptamers) and a structure or scaffold of synthetic material (i.e. vesicles, fibers, networks, or particulates). They are all biomimetic in nature. They all actuate biological signal or signals.

As research efforts continue to advance the field of recognitive materials, we believe there will be two significant deviations from what is shown in **Figure 6b.** First, some synthetic recognition elements (e.g. sequence controlled polymers, high affinity protein-polymer pairs) will displace natural recognition elements for specific applications. Second, biohybrid systems will be used to perform functions that biological systems cannot. In these cases, constructs of natural and synthetic origin will perform useful non-native functions. Researchers investigating soft robotics and biological actuators, which we reviewed previously (16), are beginning to pioneer this new area.

Methods for Characterizing Protein-Polymer Interactions: The most common experiment for characterizing the molecular recognition characteristics of a new polymeric formulation is to quantify adsorption in a non-competitive environment and compute an adsorption isotherm. This method has some advantages, in that its simplicity is amenable to throughput and the output parameter, an association constant, can be readily compared between proteins and formulations. In many cases, however, the conditions of these adsorption experiments do not suitably mimic those of the intended application.

At a minimum, future studies must make an effort to mimic the competitive binding environment that the new material or device will experience when applied practically. This simulated environment should capture, at a minimum, the pH, ionic strength, temperature, and total protein concentration of the physiological fluid. Ideally, the simulated environment will not only match the native protein concentration level, but also contain physiologically relevant levels of key proteins which are similar to, and likely to compete with, the target protein.

Further, we should take apply our full repertoire of analytical tools to characterize the protein-material interaction. For example, in addition to determining an adsorption isotherm, one could perform a microscale thermophoresis experiment. In microscale thermophoresis, ligand and receptor molecules are allowed to interact, in a competitive or non-competitive environment, within a temperature gradient. Molecules will start to flow along the gradient, and that flow is opposed by diffusion. The spatial distribution of molecules within the temperature gradient is a function of many parameters, including properties of the solution (i.e. the temperature, dielectric constant of the medium, and Debye length) as well as the ligand-receptor complexes (i.e. the surface area, hydration entropy, and effective charge) (93). This method enables the quantification of thermodynamic properties of the ligand-receptor binding event in biologically relevant conditions, which confound the measurements obtained in other popular techniques (94,95).

Other powerful techniques for investigating ligand-receptor binding events are quartz crystal microbalance (QCM) (96–98) and surface plasmon resonance (SPR) (99,100) studies. Both QCM and SPR are highly sensitive techniques. They utilize gravimetric and optical sensing, respectively, where the change in signal is proportional to the sensor-bound mass. A major advantage of both QCM and SPR is that they are label-free, meaning neither the ligand or receptor must emit fluorescence. The one disadvantage of QCM and SPR, relative to microscale thermophoresis, is the need to immobilize the ligand to a solid surface. This bound conformation

is typically a non-native state. Furthermore, immobilization reactions can block epitopes on the ligand which participate in the natural binding event.

6. Conclusion and Future Perspectives:

Scientists are leveraging the molecular recognition properties of biomaterials to achieve a range of diagnostic and therapeutic functions. In this review, we have described in detail how these systems are being used to detect analytes, sequester drugs, target cells, or facilitate a regenerative niche. In addition to generating useful technologies for biomedical applications, research efforts on recognitive biomaterials have characterized polymer-solute interactions, developed new methods for synthesizing highly ordered polymers, and invented new quantitative assays for future research.

We believe that recent studies have also illuminated several areas of need, which researchers should carefully consider when developing new recognitive material systems. First, *we need clear design specifications for recognitive biomaterials*. When recognitive biomaterials move from the realm of exploratory research to translational medical solutions, scientists and engineers will need to justify that additional material components or design complexities lead to superior functionality. We will need to clearly define the design specifications (i.e. sensitivity, time to response, relative affinity for competitive solutes, treatment duration, etc.) for specific applications in order to design the most suitable solutions. This means that translational research on recognitive materials will need to go hand-in-hand with fundamental explorations of disease biology.

Further, when new recognitive biomaterials are developed, we need to ensure sufficient *biological rigor within the experimental design*. In addition to *in vivo* models, it is important to engineer controlled and relevant *in vitro* systems. By reconstructing biologically relevant competitive environments for molecular recognition studies, we will improve the field's understanding of ligand-material interactions in physiological fluids. We will uncover new mechanisms for high affinity ligand-material interactions, and this further understanding will inform next-generation designs.

Finally, we need to be *thorough and quantitative in our material characterization.* One shortcoming of some recent studies, which demonstrate the function of recognitive polymers for sensing, drug delivery, or regenerative medicine, is a lack of system characterization. While it is important that recognitive systems perform their diagnostic or therapeutic function, from a research and development point-of-view it is equally important that we understand the underlying mechanisms and confounding factors.

As described throughout this review, molecular recognition is critical to the precise functionality of biological systems, and materials with recognitive properties have proven useful for medical applications. Most research efforts in this area have focused on mimicking natural recognition events with engineered sensors or therapeutic devices. In the future, highly ordered synthetic materials and biohybrid systems will enable advanced functionality, which not only mimics but also augments biological function. We believe there is a vibrant future for recognitive biomaterials. Studies that use advanced synthesis protocols, sensitive characterization methods, and physiologically relevant experimental conditions will catalyze the advancement of the field.

7. Acknowledgements:

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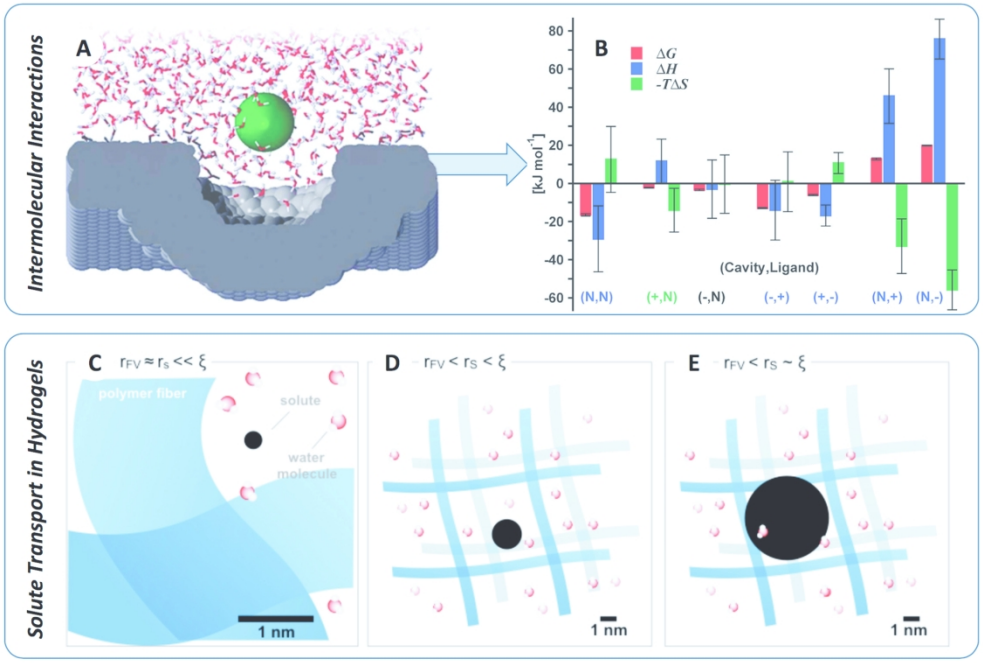

Figure 1: Solute Interactions with Functional Networks. (a) A ligand and cavity of set ionization (neutral, negative, positive) were allowed to interact in water. (b) The enthalpic and entropic contributions to the Gibbs free energy were computed for each ligand-cavity interaction. Water actively contributed to the thermodynamics of the ligand-receptor interaction becasuse of water-water, water-ligand, and water-cavity interactions. Adapted with permission from (22), available from https://pubs.acs.org/doi/abs/10.1021/ja1050082. Copyright (2010) American Chemical Society (ACS). Further permissions related to this figure should be directed to ACS. (c-e) Schematic representation of the diffusion of solutes with a range of diameter in hydrogels (rFV = radius of free volume, rs = solute radius, ξ = mesh size). Adapted with permission from (24), available from https://pubs.acs.org/doi/abs/10.1021/acs.macromol.9b00753. Copyright (2019) ACS. Further permissions related to this figure should be directed to ACS.

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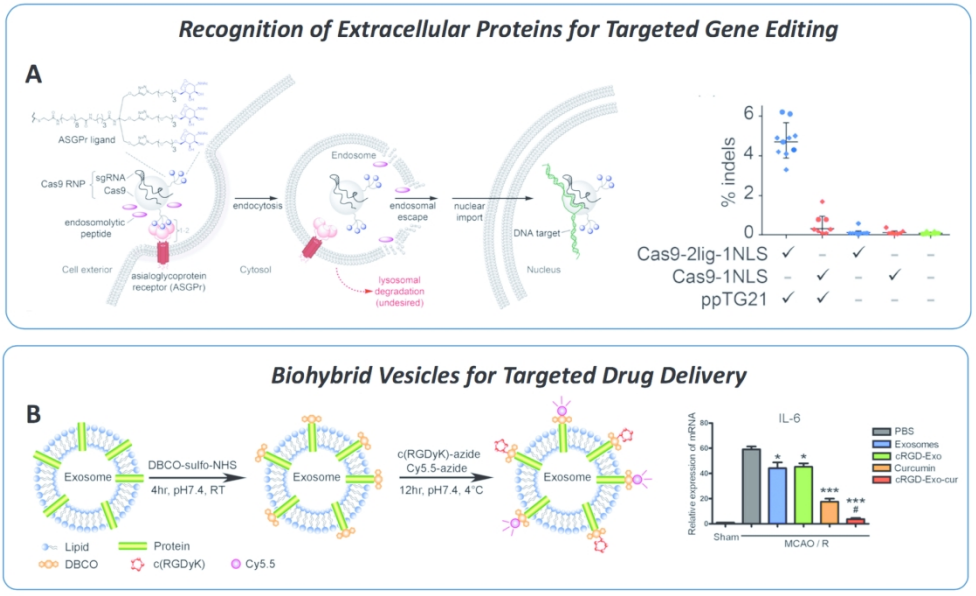

Figure 2: Recognitive biomaterials for drug delivery applications. (a) Nanoscale complexes of ligand-functionalized CAS9 ribonucleoprotein, guide RNA, and an endosomolytic peptide were successfully delivered to liver cells, where they edited a target gene in a cell-specific manner. Adapted with permission from (40). Copyright (2018) American Chemical Society. (b) Exosomes decorated with an adhesive c(RDGyK) peptide enhanced the delivery of curcumin to reduce inflammatory markers in a mouse model of ischemic injury. Adapted with permission from (41). Copyright (2019) American Chemical Society

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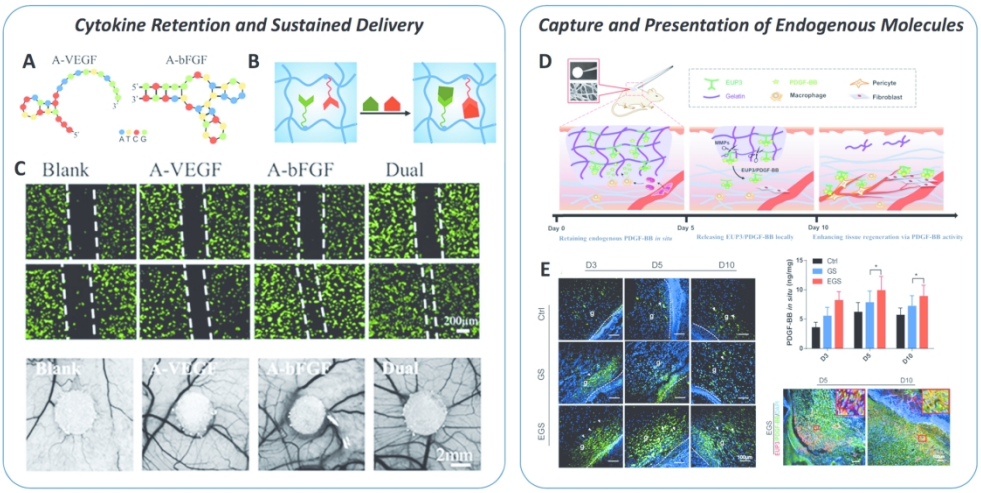

Figure 3: Recognitive biomaterials for regenerative medicine applications. (a) Hydrogel scaffolds with pendant VEGF and bFGF aptamers successfully retained the soluble growth factor for more than three days, (b) promoted cell mobility in vitro, and (c) enhanced tissue vascularization in vivo. Adapted with permission from (42). Copyright (2019) American Chemical Society. (d-e) Electrospun hydrogel sponges composed of gelatin and the EUP3 polysaccharide were capable of retaining endogenous PDGF-BB through high affinity PDGF-EUP3 interactions. Cytokine retention resulted enhanced wound healing in a full-thickness wound mouse model (Images: blue = DAPI nuclear stain, green = fluorescent PDGF-BB). Adapted with permission from (45). Copyright (2017) Elsevier.

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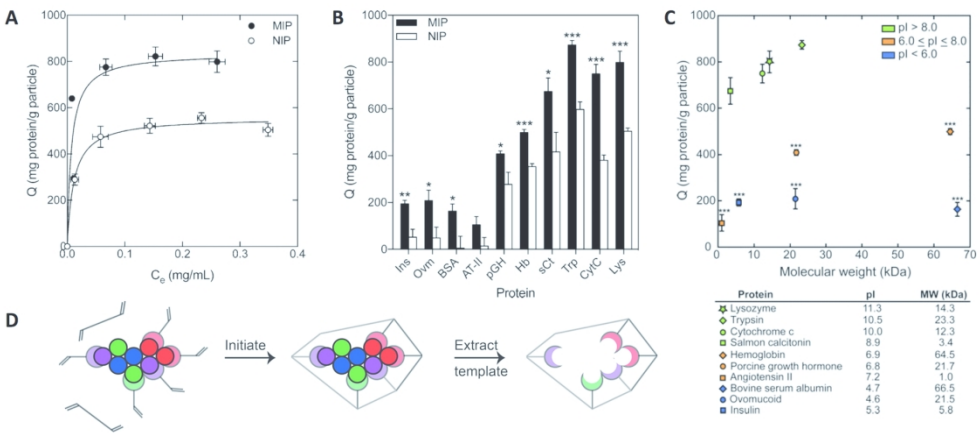

Figure 4: Protein adsorption behavior of molecularly imprinted polymers. (a) Molecularly imprinted polymers (MIPs) bound more of their template (lysozyme) than control non-imprinted polymers (NIPs), but no difference in the association constant was observed. (b) Lysozyme imprinting increased MIPs' adsorption of all tested proteins, indicating that imprinting induced a change in network structure rather than specific affinity. (c) The MIPs' composition imparted specificity for high isoelectric point biomarkers, as well as exclusion of low isoelectric point proteins. This indicated that the polymer composition, rather than the imprinting process, determined protein-polymer affinity. Adapted with permission from (63). Copyright (2016) American Chemical Society. (d) Descriptive schematic for the protein imprinting process. MIPs are formed through the self-assembly and polymerization of functional monomers around a protein template. Following template extraction, nanocavities remain within the network. Adapted with permission from (46). Copyright (2017) American Chemical Society

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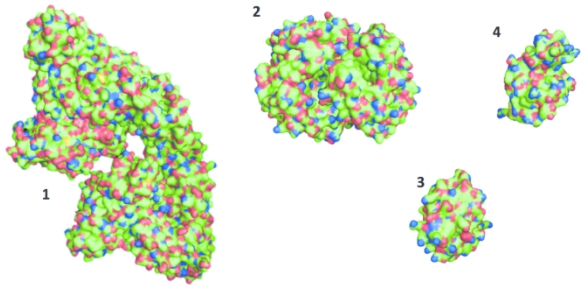

Protein	BSA[1]	Hemoglobin[2]	Cytochrome c[3]	Lysozyme[4]
Host / Source	Bovine	Bovine blood	Bovine heart	Chicken egg white
Molecular Weight (kDa)	66.5	60.2	12.4	14.3
Isoelectric Point	4.7	6.8	10.5	11.35

Amino Acid	LYS	CYT C	HGB	BSA	
LYS	7.3%	32.9%	14.8%	15.7%	Cationic
ARG	22.9%	1.2%	5.8%	4.4%	
HIS	0.6%	2.2%	11.8%	3.3%	
ASP	7.0%	2.9%	7.5%	10.8%	Anionic
GLU	1.8%	14.3%	6.2%	17.9%	
PHE	1.5%	2.5%	2.8%	2.0%	Aromatic
TYR	2.0%	1.7%	2.1%	2.0%	
TRP	3.5%	0.3%	0.3%	0.2%	
SER	4.7%	1.4%	6.6%	3.4%	Polar
THR	5.4%	4.2%	5.7%	7.1%	
CYS	1.6%	1.0%	0.0%	2.4%	
PRO	3.0%	3.7%	7.1%	5.7%	
ASN	15.3%	5.2%	1.0%	2.8%	
GLN	3.8%	5.4%	1.0%	4.9%	
GLY	7.8%	8.5%	4.5%	0.9%	Non Polar
ALA	3.3%	3.4%	9.7%	6.9%	
VAL	3.2%	2.5%	5.4%	3.7%	
LEU	3.5%	1.1%	7.5%	4.5%	
MET	0.0%	1.7%	0.1%	0.3%	
ILE	1.8%	3.8%	0.0%	1.0%	

Figure 5: Model protein identification and composition analysis. (left) Most researchers look at the molecular weight and isoelectric point of low-cost model proteins, and select one for their study that is similar in size and charge to a biomarker of interest. Here, we show the surface projection, molecular weight, and isoelectric point of four of the most common model proteins, lysozyme (LYS), cytochrome c (CYT C), hemoglobin (HGB) and, bovine serum albumin (BSA) (green = carbon, blue = nitrogen, red = oxygen). (right) Solvent accessible surface analysis reveals differences in the relative composition of each protein. Lysozyme and cytochrome c, for example, which are very similar in molecular weight and isoelectric point, are significantly different in surface composition. Protein surface composition will influence protein-polymer interactions (blue = low, relative to model protein group, red = high, relative to model protein group).

171x88mm (300 x 300 DPI)

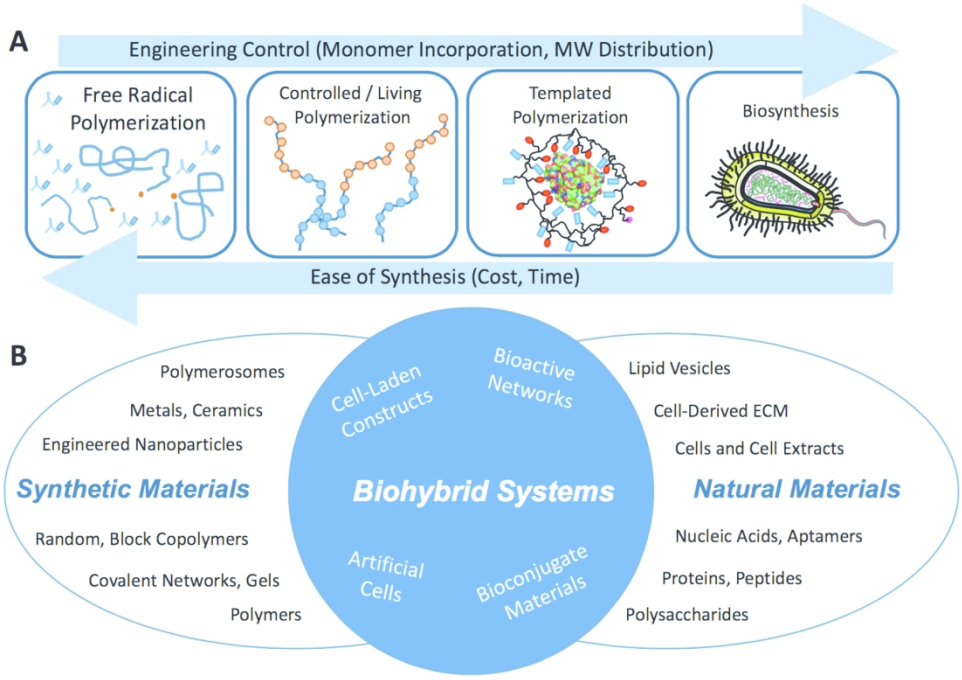

Figure 6: Synthesis of recognitive soft biomaterials. (a) There is a tradeoff between engineering control over polymer synthesis (i.e. influence over monomer incorporation and molecular weight distribution) and ease of synthesis. When designing a new recognitive biomaterial, it is important to consider the extent to which synthesis complexity improves the material or device's functionality. (b) Natural and synthetic materials are each useful for molecular recognition applications. Biohybrid systems typically combine the structural integrity and environmental responsiveness of synthetic materials with the specific activity of biomacromolecules.

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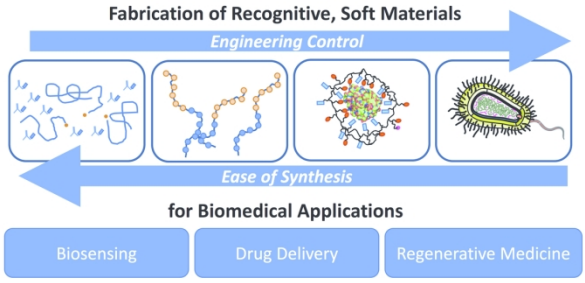
This review and critique provides fundamental considerations and practical suggestions for fabricating new polymeric biosensors, drug delivery vehicles, and scaffolds for tissue regeneration with tunable molecular recognition properties.



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