

REVIEW SUMMARY

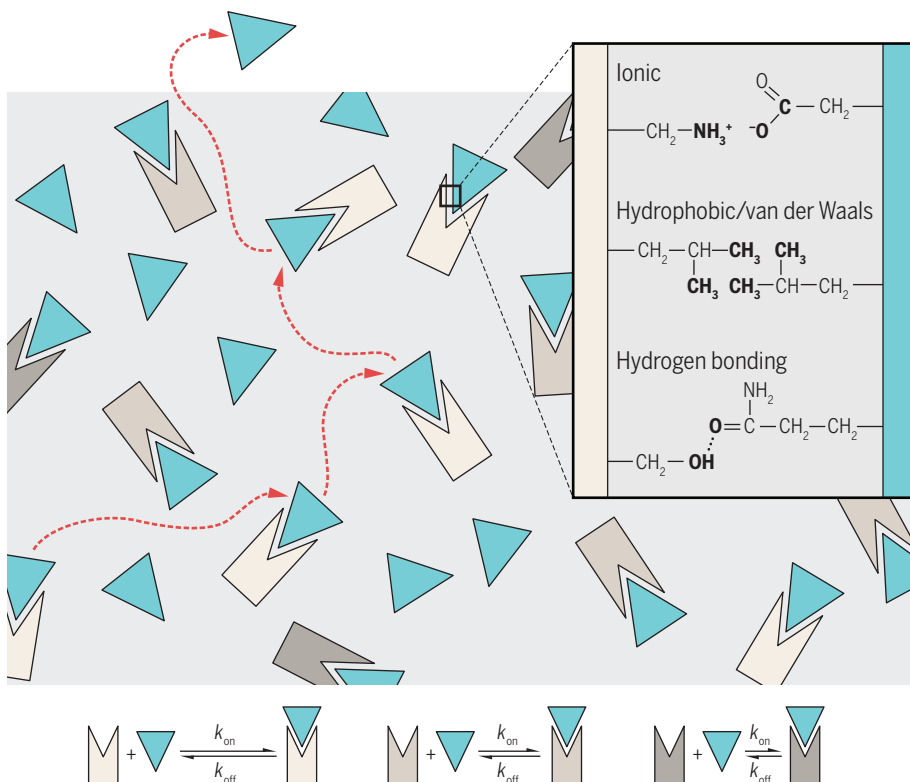
CHEMISTRY

Designer protein delivery: From natural to engineered affinity-controlled release systems

Malgosia M. Pakulska, Shane Miersch, Molly S. Shoichet*

BACKGROUND: Protein therapeutics constitute a multibillion-dollar market, yet their formulation and sustained delivery still pose a substantial challenge. Controlled release strategies developed for small-molecule drugs, such as microparticle encapsulation, typically involve organic solvents and harsh processing conditions that are detrimental to protein structure and function. Affinity-controlled release has emerged as an alternative strategy for the sustained and tunable release of protein therapeutics in a neutral aqueous environment, thus reducing protein loss and improving loading.

Affinity-controlled release depends on a preferred noncovalent interaction between a protein therapeutic and a binding ligand. This binding ligand can be another protein, a peptide, or an oligonucleotide. Typically, the binding ligand is covalently linked to a polymer matrix, such as a hydrogel. Soluble protein is added, and equilibrium is established between free protein and ligand-bound protein. Whereas free protein is able to diffuse from the system, bound protein cannot. This equilibrium is dynamic and changes in response to local conditions. The rate of protein release from the



Discovery of binding ligands for affinity-controlled release. Ligands (peptides, proteins, or oligonucleotides; shown as polygons) that bind a protein therapeutic (triangles) through noncovalent interactions (ionic, hydrophobic, van der Waals, or hydrogen bonding; indicated in bold) can be discovered in nature, selected from a library, derived through *in vitro* evolution, or designed computationally. By choosing ligands with different affinities, represented by the size of the equilibrium arrows (bottom), and covalently linking them to a polymer matrix such as a hydrogel, one can control the diffusive release (dashed arrows) of a protein therapeutic.

system is therefore governed not only by protein diffusivity and the concentration gradient, but also by the concentration of the binding ligand, the strength of the interaction, and the binding kinetics. The challenge lies in finding binding ligands that afford the desired release profiles.

ADVANCES: The earliest affinity-controlled release systems mimicked the extracellular matrix by using heparin to reversibly bind and control the release of various growth factors. Other natural interactions have since been used for affinity-controlled release, including albumin with small-molecule therapeutics and antibodies with cognate antigens. These systems have allowed for sustained release of protein therapeutics while maintaining protein activity; however, naturally occurring interactions are inherently limited in terms of available targets and binding strengths. *In vitro* selection and directed evolution are established

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techniques for isolation and engineering of binding partners against virtually any protein target. Harnessing these techniques for affinity-controlled release applications is now underway and has resulted in novel peptide-, protein-, and oligonucleotide-based binders for the sustained release of several growth factors.

OUTLOOK: Many opportunities exist for the discovery or design of binding ligands for affinity-controlled release. Computational techniques can help to identify protein backbones that have geometric and electrostatic complementarity to a target, reducing the screening required to isolate lead variants. Selection conditions can be tailored to isolate intermediate-strength binders, or iterative rounds of *in vitro* evolution can provide a series of related variants with a spectrum of affinities for a target. Competition selections can ensure selectivity for simultaneous yet independent release of multiple proteins from their corresponding binding ligands. On-demand affinity-controlled release has yet to be explored, but structure-switching aptamers and computational design of allosteric regulator sites show potential. These techniques, coupled with concurrent advances in accurate high-throughput measurement of binding constants, will allow for the creation of libraries of binding partners with various affinities for each target therapeutic. Such a standardized yet versatile controlled release strategy has the potential to improve reproducibility and accelerate optimization of protein delivery systems. ■

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Designer protein delivery: From natural to engineered affinity-controlled release systems

Malgosia M. Pakulska,¹ Shane Miersch,² Molly S. Shoichet^{1,3*}

Exploiting binding affinities between molecules is an established practice in many fields, including biochemical separations, diagnostics, and drug development; however, using these affinities to control biomolecule release is a more recent strategy. Affinity-controlled release takes advantage of the reversible nature of noncovalent interactions between a therapeutic protein and a binding partner to slow the diffusive release of the protein from a vehicle. This process, in contrast to degradation-controlled sustained-release formulations such as poly(lactic-co-glycolic acid) microspheres, is controlled through the strength of the binding interaction, the binding kinetics, and the concentration of binding partners. In the context of affinity-controlled release—and specifically the discovery or design of binding partners—we review advances in *in vitro* selection and directed evolution of proteins, peptides, and oligonucleotides (aptamers), aided by computational design.

Molecular medicine has allowed us to find and characterize the fundamental molecular or genetic causes of many diseases and injury pathologies. Although small-molecule drugs remain valuable, protein-based drugs have emerged as a way to correct such pathologies using innate processes. Examples include the use of insulin to treat diabetes (1), antibodies against vascular endothelial growth factor (VEGF) to treat hyperactive vasculature in wet age-related macular degeneration (2), and erythropoietin to treat anemia (3). The protein drug market was worth \$151.9 billion in 2013 and is expected to reach \$222.7 billion by 2019 (4). This includes proteins that directly serve a therapeutic purpose (e.g., insulin, antibodies), protein vaccines (e.g., the HPV vaccine), and protein-based diagnostics (e.g., technetium-labeled antibodies for imaging) (5).

Despite the commercial availability of protein drugs, their formulation and delivery still pose substantial challenges (6). Most protein drugs are currently administered by repeated injections and, to the best of our knowledge, only one sustained release protein formulation has been clinically approved (Nutropin Depot, Alkermes and Genentech) (7, 8). With protein activity dependent on tertiary or even quaternary structure, sustained-release strategies developed for small-molecule drugs, such as encapsulation within poly(lactic-co-glycolic acid) microspheres, have proven difficult to translate. These microspheres

are typically prepared using a double-emulsion solvent evaporation process, where organic solvents and shear stress can cause protein instability and consequent loss of function. Affinity-controlled release is an alternative sustained-release strategy that is attractive for protein drugs, because the components can usually be mixed together in a neutral aqueous environment with low losses and high loading.

Affinity is a general term used to refer to a preferred noncovalent interaction (electrostatic, hydrophobic, and/or van der Waals) between two binding partners, such as protein-protein, protein-peptide, or protein-polymer interactions. Affinity-controlled release takes advantage of these interactions to slow the diffusion of a drug from a

release system. This is usually achieved by immobilizing a drug-binding ligand within a polymer matrix, often a hydrogel.

The challenge in affinity-controlled release is finding the right binding partners to achieve the desired release profile. Although many naturally occurring binding partners exist, the discovery and engineering of novel binding partners through advances in *in vitro* selection, directed evolution, and computational design have presented new possibilities for controlling biomolecule release using affinity (Fig. 1). A recent review of binding interactions used for affinity-controlled release (9) is a useful accompaniment to this review. Whereas the previous review focuses on the affinity-controlled release systems that have been used to date, this review aims to show how binding partners can be discovered or designed in order to make affinity-controlled release a convenient strategy for sustained drug release.

Characterizing binding partners for affinity-controlled release

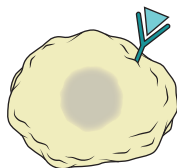
Affinity itself is not a novel concept: Scientists have been exploiting affinity between molecules for decades in enzyme-linked immunosorbent assay, immunostaining, and purification techniques, among others. To be successful, these laboratory techniques rely on strong and specific interactions that are often considered to be nearly irreversible. All affinity interactions, however, exist in equilibrium. This dynamic equilibrium is what makes affinity binding useful for controlled biomolecule release.

In a simple bimolecular affinity system, two molecules associate at a rate of k_{on} to form a complex and dissociate at a rate of k_{off} (Fig. 2). The ratio of these two rates determines the ratio of free and bound species at equilibrium and is defined as the equilibrium dissociation constant, K_d (Eq. 1).

$$K_d = \frac{[\text{freespecies}]_{eq}}{[\text{boundspecies}]_{eq}} = \frac{k_{off}}{k_{on}} \quad (1)$$

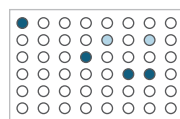
Natural

- ECM-integrin
- Receptor-ligand
- Protein-protein
- Antibody-antigen



Selection and screening

- Phage, yeast, bacterial, or ribosomal display



In vitro evolution

- Affibodies
- Anticalins
- AdNectins
- Aptamers



Computation

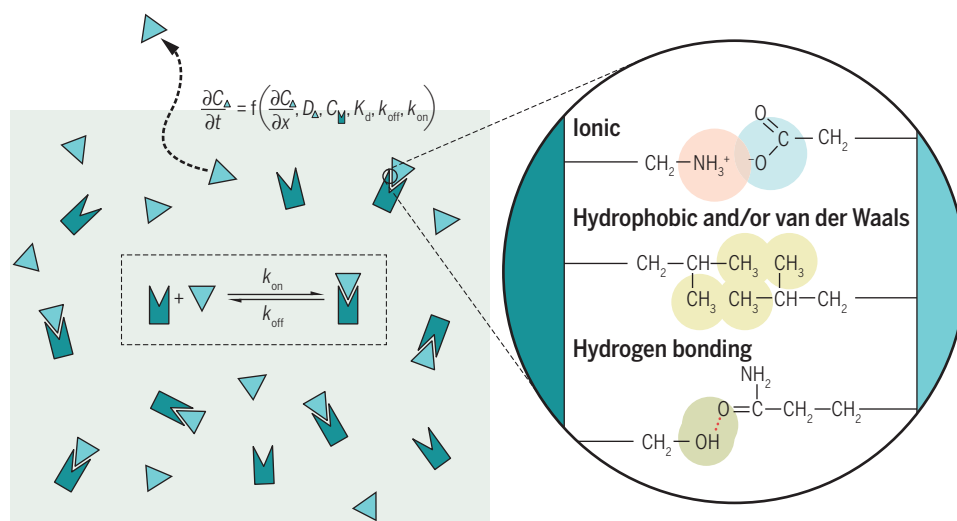
- Stochastic
- Deterministic
- Empirical
- Physics-based



Fig. 1. Interactions suitable for affinity-controlled release can be discovered or designed in different ways. Naturally occurring interactions such as antibody-antigen or receptor-ligand binding (shown on the left) can be exploited for affinity-controlled release. Members of a protein or peptide library can be selected and screened for their ability to bind a target using display methods (e.g., phage, yeast, bacterial, or ribosomal) or yeast two-hybrid systems. *In vitro* selection can be combined with diversification steps (e.g., random mutagenesis) in multiple iterative rounds to obtain *in vitro*-evolved ligands that bind a specific target. Computational methods can be used to design sequences and structures that bind a target.

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Fig. 2. Schematic of an affinity-controlled release system. A protein therapeutic (triangle) binds reversibly to an immobilized binding ligand (polygon) within a polymer matrix via electrostatic (ionic or hydrogen-bonding), hydrophobic, and/or van der Waals interactions. Diffusive release [the change in concentration (C_{triangle}) over time (t); dashed arrow] is governed by the protein concentration gradient (dC/dx), its diffusivity within the matrix (D), the concentration of the immobilized binding ligand (C_{polygon}), the equilibrium dissociation constant (K_d), and the binding kinetics (k_{on} and k_{off}).



The rate of release from an affinity-controlled system is governed by both diffusion and the association-dissociation reaction (Fig. 2). Generally speaking, the stronger the interaction (smaller K_d) and/or the higher the concentration of the immobilized binding partner, the slower the release. In-depth analysis of how individual factors affect affinity-controlled release rates and release profiles can be found in (10–12).

To fully characterize a binding pair for affinity-controlled release, K_d and either k_{on} or k_{off} must be known. The most commonly used methods for quantifying affinity binding interactions are isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and spectroscopic methods, although only SPR and QCM provide kinetic data (13, 14).

ITC is the most common approach for obtaining thermodynamic data for molecular interactions, but it provides no information about kinetics and is limited to solution-based equilibria (15). SPR and QCM are both powerful measurement methods because they give binding information in real time and provide kinetic as well as equilibrium data (16, 17); however, they require the immobilization of one of the components, potentially affecting the binding interaction (18).

Spectroscopic methods include fluorescent correlation spectroscopy (19), fluorescence anisotropy (20, 21), and Förster resonant energy transfer (FRET) (22). These methods can also provide insight into the nature of the binding site and the distance between fluorescent tags on the two bound molecules (14), but the presence of fluorophores or fluorescent tags can change the environment around the binding site, possibly resulting in inaccurate measurements. Spectroscopic methods usually have low sensitivity, requiring strong binding ($K_d < 10^{-6}$ M) or high concentrations (millimolar range) to be effective. Table 1 summarizes the characteristics of these techniques.

These current experimental methods to measure binding constants are time-consuming. For tunable release rates based on variable binding strength, we need methods to measure K_d and

kinetics in a high-throughput manner so that we can create libraries of molecules with a range of K_d for the same drug target.

Several advances are being made in instrumentation for high-throughput screening of binding constants. Miniaturization of ITC instruments has decreased the sample size and measurement time required, although measurements are still performed in series. Array calorimetry, in which multiple measurements are performed in parallel, is an emerging technology but is not yet available commercially (23). SPR microscopy (SPRM) is essentially SPR with a charge-coupled device detector array, offering high spatial resolution for each binding event (24, 25). This can be coupled with patterned microarrays of biomolecules or microfluidics (26) for a high-throughput method of measurement. Similarly, a QCM sensor array coupled with microfluidics could be used for high-throughput QCM measurements (27).

Spectroscopic methods can be combined with microfluidics for massively parallel measurements of binding kinetics. Geertz *et al.* (28) used mechanically induced trapping of molecular interactions in a microfluidic platform to simultaneously analyze the kinetics of transcription factors binding to their fluorescently labeled DNA ligands—a total of 223 unique interactions. These interactions have a range of K_d values and tend to have high association rates and short half-lives, making them challenging to measure using standard techniques (28, 29).

An additional challenge is quantifying the interaction of biomolecules immobilized to a polymer matrix, as is often the case in affinity-controlled release systems. In this sense, measurement methods where one of the molecules is immobilized, such as SPR and QCM, may best emulate the release conditions if the method of attachment is the same (30). Other methods include indirect measurement of binding constants by fitting experimental data to adsorption or diffusion models for molecules within the gel (31). For example, the binding constant for a protein immobilized to a gel was measured by observing

the diffusion of its binding partner after wet-stamping it on specific sites on the gel surface (32). Indirect methods such as this, however, are not high-throughput, and SPRM coupled with microfluidics may be the current option that best mimics the affinity-controlled release environment.

Another important characteristic of a binding pair is specificity, especially in the context of the simultaneous release of multiple therapeutics. Interaction strength is often mistaken for high specificity, but these are different concepts (33). Interaction strength is the difference in free energy between bound and unbound states (ΔG), whereas specificity is the difference in the ΔG of binding between two different binding pairs ($\Delta\Delta G$).

Although we think of bimolecular interactions as single binding events, each one is actually made up of many individual, interdependent events. Specificity can be independent of affinity if there are individual interactions that confer no energy change from the unbound to the bound state, such as if the decrease in the free energy of the interaction is exactly balanced by the increase in free energy caused by the removal of water. These interactions would then contribute to the specificity of binding but not to the overall strength (affinity) of the interaction (34). Similarly, one might think a higher complementary surface area would result in higher specificity, but this is not always the case. A major reorganization upon binding could lead to higher energy costs to the molecule that outweigh the benefits of a larger complementary interface. An alternative binding partner with a less desirable interface but with no requirement for reorganization might therefore preferentially interact (35).

Affinity-controlled release inspired by nature

The noncovalent interaction between heparin in the extracellular matrix (ECM) and a series of growth factors serves as the inspiration for many of the affinity-controlled release systems pursued today. Within the ECM, heparin binds growth factors such as basic fibroblast growth factor

Table 1. Comparison of methods used to quantify affinity binding interactions. Data are from (13–22, 146). ΔH and ΔS are the enthalpy and entropy of binding, respectively.

Method	ITC	SPR	QCM	Spectroscopic methods
K_d range	10^{-3} to 10^{-9} M	10^{-3} to 10^{-12} M	10^{-4} to 10^{-10} M	$< 10^{-6}$ M
Thermodynamic data (ΔH , ΔG , ΔS)?	Yes	No	No	No
Kinetic data (k_{on}/k_{off})?	No	Yes	Yes	No
Immobilization?	No	Yes	Yes	No
Modifications (e.g., fluorescent tag)?	No	No	No	Yes*
High-throughput potential	Array calorimetry	SPR microscopy coupled with microfluidics	QCM sensor array coupled with microfluidics	Coupled with microfluidics

*Except for cases of innate fluorescence (e.g., tryptophan fluorescence).

(bFGF), VEGF, and nerve growth factor (NGF) with K_d values ranging from 10^{-6} to 10^{-9} M (36). The earliest examples of affinity-controlled release used albumin-heparin microspheres to control the release of adriamycin, a cytostatic agent (37), or heparin-Sepharose beads within alginate microcapsules to control the release of bFGF (38, 39). There are now numerous studies that have taken advantage of heparin for affinity-controlled release of bFGF (40, 41), VEGF (41, 42), NGF (43), neurotrophin 3 (43, 44), brain-derived neurotrophic factor (43), and bone morphogenic protein 2 (45), among others. However, the heterogeneity and promiscuity of heparin make it challenging to reproduce and control binding.

Binding to heparin is primarily mediated by electrostatic interactions with sulfate groups that are negatively charged at physiological pH. Other anionic polymers, such as sulfated alginate (46, 47) and gelatin (48, 49), have also been used for affinity-controlled release because of their potential for electrostatic interactions. Gelatin can be produced recombinantly with specific isoelectric points, thus increasing control over binding strength (50). Molecular imprinting is another method to generate affinity for a specific molecule within a polymer. Molecularly imprinted polymers are synthesized in the presence of the imprinting molecule, thereby retaining pockets that specifically recognize this molecule. Although molecular imprinting has been used for affinity-controlled release of small molecules (51), the synthesis of polymers that can specifically recognize proteins remains a challenge because of protein size and flexibility and the necessity for aqueous conditions (52). Additionally, once imprinted, a polymer must remain intact, limiting injectability and the potential for minimally invasive application.

Other molecules with known binding partners, such as albumin (53) and antibodies (54), have also been used for affinity-controlled release. Proteins that do not have well-known binding partners have been recombinantly expressed as fusions with a binding domain. For example, Jeon *et al.* sustained the release of a FGF-collagen binding domain fusion protein from collagen matrices for up to 7 days to promote angiogenesis in ischemic organs (55). Other recombinant strategies

have employed Src homology domain 3 and its binding partners to control the release of protein therapeutics such as bFGF, chondroitinase ABC, and insulin growth factor 1 fusion proteins from hydrogels for 7 days or more (56–58).

In vitro selection and directed evolution of binding peptides and proteins

If an appropriate binding interaction for a therapeutic protein of interest is not known, in vitro selection and directed evolution can be used to identify and optimize novel protein-protein or protein-peptide interactions for use in affinity-controlled release. The process of designing affinity partners proceeds through (i) the creation of a combinatorial DNA library by means of mutagenesis of a known peptide sequence or protein scaffold; (ii) the display of the peptide or protein variants using phage, bacteria, yeast, ribosome, or mRNA display; and (iii) in vitro selection of variants from the displayed library through repeated rounds of exposure to the target of interest, capture, elution, and amplification. The term “scaffold” here represents a protein backbone within which mutations can be made, rather than a biomaterial for tissue engineering applications. Selected variants can then be characterized for affinity, specificity, and stability and stratified for application and/or continued engineering of desired attributes using directed evolution (Fig. 3). Efforts in this field have resulted in the identification of a wide variety of novel interactions that could be exploited for affinity-controlled release applications, and a database of affinity peptides (MimoDB) now exists (59).

Directed evolution offers a powerful means of modifying a protein to obtain desirable properties. These methods begin with a lead binding candidate and involve multiple iterative rounds of randomization, selection against a target under evolutionary pressures to eliminate undesirable clones, and characterization of variant properties (Fig. 3). Although examples of the directed evolution of antibodies are numerous (60, 61), their larger size, more complex architecture, and high binding affinities limit their utility for affinity-controlled release. Increasingly, smaller protein scaffolds (62, 63) are being designed for use as protein therapeutics, diagnostics, and imaging

agents (62–64). Each step in the in vitro selection and directed evolution process is described below in the context of affinity-controlled release.

Combinatorial libraries and randomization

The purpose of randomization is to sample as large a sequence space as possible in order to identify a variant with desired properties, while considering limitations imposed by the display platform and the approach to randomization.

For short peptides, randomization can be achieved by direct chemical synthesis using solid-phase split-mix synthesis. In this approach, a different amino acid is coupled to each pool of nascent peptide; the pools are then mixed and split again. The process is repeated, resulting in an exponential increase in the number of peptide variants with each round (65). Split-mix synthesis has been used to rationally design a combinatorial library of peptides to bind VEGF. In this instance, the amino acids were chosen to mimic the VEGF binding region of heparin and included four sulfated residues. Peptides that bound VEGF were selected using a microbead-based binding assay with fluorescently modified VEGF and characterized by SPR. The strongest resulting binding peptide bound VEGF with a K_d of 3.1×10^{-6} M (66). Although arguably simpler given the lack of intermediate organisms (e.g., bacteria or phage), this type of approach is limited to small peptides, which may not provide enough diversity to achieve a desired interaction.

Alternatively, for longer peptides and proteins, combinatorial libraries for use in in vitro selection can be generated by means of mutagenesis. Mutagenesis can be achieved by random position methods [(error-prone polymerase chain reaction (PCR)], mutagenic oligonucleotide-based methods [hard and soft randomization, custom codon (67), and di- and tri-nucleotide codons (68–70)], or recombination-based block-shuffling methods (71). Although error-prone PCR is a popular method, only one to three mutations are introduced per gene at random (72, 73), leaving little room for rational design. In contrast, mutagenic oligonucleotide-based methods offer more refined control over the position and type of mutations that are introduced, which can help to limit

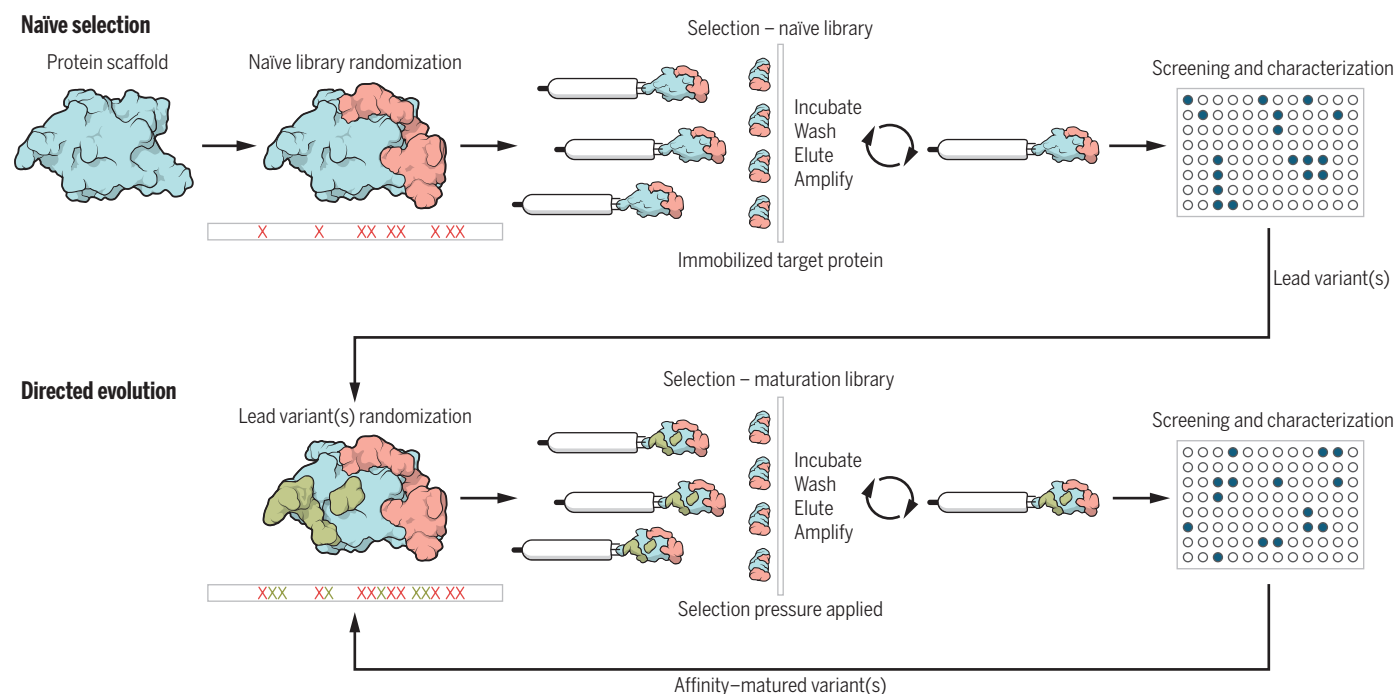


Fig. 3. Naïve selection and/or directed evolution can be used to find and optimize novel binding partners for a target.

For illustration, regions of a protein scaffold displayed on the surface of a phage (depicted in white) are colored corresponding to mutations (a series of X's) shown in the primary sequence below the structure. Scaffold protein or peptide candidates are randomized to generate naïve libraries for use in *in vitro* selection against immobilized target proteins. Target-binding variants are captured

and enriched from libraries in repeated rounds of selection, then characterized and ranked to identify lead variants. Successive rounds of protein or peptide lead randomization and selection under affinity-modifying pressures (antigen or competitor concentration, binder valency, and temperature) enable exploration of possible binding interfaces. By directing the evolution of binding properties, a system suitable for tunable affinity-controlled release can be achieved.

the number of nonfunctional variants in the library. Random position and oligonucleotide-based approaches are often viewed as complementary.

When developing randomization schemes, it is important to strike a balance between the theoretical diversity that can be introduced and the limitations of the display platform. For instance, hard randomization using NNN oligonucleotides (where N represents equal proportions of A, C, G, and T) in nine positions could create 20^9 or 5.12×10^{11} unique clones, approaching the limit of what can be captured with some display methods (discussed in the next section). The simple use of NNK or NNS oligonucleotides (where K indicates an equal proportion of G and T, and S indicates an equal proportion of C and G) still permits all 20 amino acids, while reducing the number of nonfunctional clones by eliminating two of the three stop codons (67). Redundancy in the genetic code may also result in various degrees of amino acid bias. Combinatorial approaches such as di- and tri-nucleotide block precursors (68, 69, 74) for oligonucleotide synthesis can further reduce or eliminate redundancy but may be prohibitively expensive for some laboratories. Novel combinatorial strategies are being devised to generate both cost-effective and nondegenerate mutagenic primers and have been assisted by computational efforts (75–77). Investigators should consider the type or scope of mutations incorporated and the potential forms of bias in each approach

when developing selection and evolution strategies for a particular application (74).

For identification of novel binding pairs, numerous protein scaffolds have been shown to be amenable to randomization and the development of *de novo* binding interfaces (78–81). These efforts have been aided by the existence of structural information that can facilitate rational engineering choices regarding (i) the types of mutations that are likely to be tolerated by various regions and structures and (ii) the identification of residues and regions that contribute critically to binding interactions when randomizing the binding surface. Additionally, computational techniques such as protein docking can aid in identifying scaffold proteins with shape and electrostatic complementarity for a target (discussed below) (82). Currently, the majority of these engineered proteins function as antagonists, blocking interactions by binding receptors or soluble factors. If these molecules were immobilized in a polymer matrix, they could instead act as a depot for their selected binding partner and prolong therapeutic action. For example, a VEGF antagonist, Angiocal, is designed to treat solid tumors (83); however, one could reverse this strategy and use it for affinity-controlled release of VEGF, which has been studied as a therapeutic in numerous tissue-regeneration applications, including cardiac repair after infarction or heart failure (84). Thus, the same technology that is used to sequester

VEGF could be leveraged to create a simple, well-defined, tunable VEGF delivery system.

Display platforms

Display platforms translate the genetic information in the combinatorial library to peptides and proteins, effectively coupling genotype and phenotype, for subsequent exposure to the target of interest and the isolation and characterization of binding clones.

Phage display using a filamentous phage, such as M13, f1, or fd, is one of the most widely used display methods because of its high phage titers, library diversity (10^9 to 10^{11} variants), and easy access to encoding genes (85, 86). The option of displaying proteins as either a fusion with pIII (monovalent) or pVIII (polyvalent) phage coat protein may be particularly useful for affinity-controlled release applications, because avidity effects during polyvalent display can promote the isolation of weaker binders. Peptides (87, 88) and small and mid-size homo- or heteromeric proteins—such as fibronectin (Adnectin) domains (81), three-helical bundles (89, 90), anticalins (79), and atrimers, among others (62, 80)—have been successfully selected and engineered from phage-displayed libraries. Recent reports describe the use of a commercial phage-displayed peptide library to find short peptide sequences that bind heparin or NGF for subsequent use in affinity-controlled release; identified peptides were coupled

to fibrin matrices for controlled release of heparin and NGF (91) or NGF alone (92), with the rate of release being dependent on the strength of the interaction (91).

Cell-surface display methods are attractive because of their potential for coupling with fluorescence-activated cell sorting for high-throughput analysis and separation. Efforts to develop bacterial cell-surface display have demonstrated display levels sufficient to observe enrichment of binding variants (93), library sizes ranging from 10^9 to 10^{10} (94), and suitability for use in isolating constrained peptides (95, 96) and in antibody affinity maturation (97). Despite these efforts, challenges associated with presentation of displayed peptides and proteins on the outer membrane have thus far limited the utility of this approach (95, 96). Additionally, bacteria are not capable of eukaryotic posttranslational modifications that are sometimes required for proper protein folding and function.

Yeast surface display is a eukaryotic alternative to bacterial display, circumventing the challenges faced during heterologous protein expression. The use of a variety of strains of yeast and approaches to membrane anchoring have been demonstrated (98, 99); however, the Aga2p fusion system in *Saccharomyces cerevisiae* remains the most widely used (100). Library variants are expressed as an N- or C-terminal fusion with a secreted Aga2p subunit, which is then captured by means of disulfide linkages to a plasma membrane-anchored Aga1p subunit. One of the primary limiting factors of yeast surface display is library size, which usually ranges from $\sim 10^7$ to 10^9 variants, although increased functionality due to eukaryotic expression may offset this limitation. Yeast surface display has been successfully used to isolate and engineer peptides (101), protein scaffolds (102), single-chain antibody fragments (scFvs) (103), and T cell receptors (104).

In contrast to the above platforms that rely on efficient DNA transformation and cellular propagation for display, ribosomal and mRNA display methods offer a fully in vitro alternative. Without the need for living cells, library sizes of 10^{12} to 10^{14} variants are frequently achieved. These two approaches are similar in that they generate mRNA transcripts and protein variants from the DNA library by in vitro transcription and translation. However, the means by which the translated protein variants are coupled to their encoding transcript for purification and use in selections differ (105). Ribosomal or mRNA display methods have been successfully used in the selection and evolution of various types of binders, including high-affinity cyclic peptides (106), serum-stable peptides (107), scFvs (73), and a growing body of protein scaffolds (78, 108), which could conceivably be used for tunable affinity-controlled release.

Screening strategies for affinity-controlled release

To promote the isolation of variants with desired properties, it is critical to optimize selection con-

ditions, including the method of display, target concentration, presence of competitors, and time and temperature of incubation, among others. For example, off-rate selections with a long incubation period and a large excess of a soluble binding competitor enabled the isolation of a peptide-binding scFv clone with a K_d of ~ 5 pM (73). This and other screening strategies for drug development are usually designed to isolate the strongest binders; however, for affinity-controlled release, a range of dissociation constants from the millimolar to the nanomolar scale would be desired. To achieve this, selection conditions favoring intermediate binders, such as high target concentrations and polyvalent display, may be favorable. Strategies that provide a progressive improvement in affinity, such as iterative rounds of in vitro evolution, would enable the isolation of a series of related variants with a spectrum of affinities that could then be explored for tunable affinity-controlled release.

Competition selections with soluble target competitors are a common method of applying selective pressure to enhance affinity by removing lower-affinity binders; similar approaches can also enhance the specificity of binding variants for a target. Enhanced specificity would be important for the simultaneous affinity-controlled release of multiple therapeutic proteins. This strategy was successfully demonstrated in the isolation of a variant of a serine protease inhibitor, ecotin, that bound plasma kallikrein with a K_d of 11 pM but had equilibrium dissociation constants for five related proteases (FXa, FXIa, FXIIa, MT-SPI, and plasmin) that were four to seven orders of magnitude higher (109). The versatility of this strategy was further demonstrated by varying screening conditions to allow the isolation of other ecotin variants with specificities for other proteases.

Another strategy that is relevant for affinity-controlled release is the engineering of bispecificity in a protein scaffold. Nilvebrant *et al.* engineered the albumin-binding domain 3 of the streptococcal protein G both for high-affinity binding of ErbB2 and tunable binding to human serum albumin (110). Many of the novel protein scaffolds that have been described recently are below the renal filtration cutoff of 60 kDa and are rapidly cleared from circulation (80). Because it is an abundant and long-lived serum protein, binding to albumin can reduce the rate of clearance. Similarly, engineering a small protein scaffold to bind both a polymeric vehicle and a therapeutic target could provide tunable affinity-controlled release of the therapeutic from the vehicle. In this case, the engineered protein would act as an intermediate between the delivery vehicle and the therapeutic, allowing the same vehicle to be used for the delivery of a range of therapeutics.

Directed evolution of oligonucleotides (aptamers)

Aptamers are short oligonucleotides that derive their name from the Latin word “aptus,” meaning “to fit.” They are selected by directed evo-

lution to bind a specific target using a process termed SELEX (systematic evolution of ligands by exponential enrichment). Similarly to the directed evolution of proteins described above, the process begins by subjecting a random sequence pool of RNA or DNA to a binding assay with a target. The sequences that bind are selected, amplified by PCR, diversified, and resubjected to the same binding assay. This process is repeated until all the RNA or DNA ligands can bind to the target with high affinity (111, 112). Theoretically, this method can be used to generate high-affinity ligands for any therapeutic protein target.

Aptamers have several advantages over antibodies, including no requirements for animals or cells, ease of synthesis, and the ability to select for binders under nonphysiological conditions (113); however, they suffer from short serum half-lives (114). Nevertheless, aptamers are being investigated for a variety of applications (115). Aptamer microarrays and photo-cross-linkable variants (116) allow screening for the presence of protein targets in diagnostic applications (115, 117). Aptamers can also act as therapeutics, and at least one aptamer-based therapeutic is currently in clinical use (118). In drug delivery, aptamers are being investigated for their potential to enable targeted drug delivery to specific tissues, especially in cancer (119–121).

The use of aptamers for affinity-controlled release has emerged within the past 5 years. Aptamer affinity can be tuned by selection conditions, but most selection methods are designed to isolate high-affinity binders. An alternative approach is to select for a high-affinity aptamer and mutate the essential nucleotides to obtain a library of affinities. This technique has resulted in aptamers that bind platelet-derived growth factor (PDGF)-BB with affinities ranging from 11 to 350 nM. Conjugation of these aptamers to a series of hydrogels—agarose, poloxamer, and polyethylene glycol (PEG) diacrylate—resulted in the tunable release of PDGF-BB (122, 123). High-throughput, parallel measurements of aptamer binding constants have generated large libraries of aptamers for a specific target (124).

Aptamers have also been evolved to bind non-protein targets such as peptides, small molecules, or nucleic acids (115). For example, aptamers selected to bind tetracycline with high affinity were conjugated to a PEG hydrogel, which increased tetracycline loading relative to unmodified gels. This resulted in increased and prolonged antibiotic release and ultimately decreased bacterial proliferation between 12 and 72 hours after treatment (125).

Structure-switching aptamers (SSAs) that are capable of controlled cargo release in response to allosteric binding of a specific target molecule have recently been reported (126). These SSAs selectively released a fluorescent cargo in response to allosteric binding of adenosine triphosphate (ATP) through a conformational change. The selection procedure involved isolating sequences that selectively unbound from a column containing the cargo in the presence of ATP. Such

ligands could provide a method for triggered affinity-controlled release.

Computational design

Computational design of protein-protein interactions has the potential to generate binding partners for any therapeutic protein of interest. Although identification of new protein-protein interactions remains difficult and computationally intensive, completely new protein folds and topologies have been generated (127), as have enzymes with activities not found in nature (128) and proteins with entirely new binding interfaces (129–131).

Computational protein design has two main steps: (i) sampling of the conformational and sequence space and (ii) scoring of the resulting protein candidates (132, 133). The pool of all possible conformations and sequences is initially reduced by starting with a fixed backbone conformation and/or through the a priori definition of fixed residues at the binding interface (“hotspots”) (130). The chosen backbone conformation can be based on an existing three-dimensional (3D) structure or on a de novo design (127). Molecular docking simulations are often used initially to determine which backbone conformations exhibit reasonable geometric compatibility with the target of interest (134, 135). The sequence-conformational space is then sampled within these restrictions using stochastic (e.g., Monte Carlo) or deterministic (e.g., dead-end elimination) (136) search algorithms. In designing a protein that binds a specific target, the focus is on optimization of the residues at the interface. Resulting candidates are filtered by scoring algorithms that can be knowledge-based (relying on empirical data collected from protein databases) or physics-based (derived from physical principles).

Computational design has not been used directly for affinity-controlled release to date, but it has been successfully used to design novel protein-protein (82, 131) and protein-small molecule (130) binding pairs. Tinberg *et al.* (130) designed a protein that could bind the steroid digoxigenin (DIG), which is the deglycosylated form of digoxin, a cardiac glycoside used to treat heart disease (137). Digoxin has a narrow therapeutic window and could benefit from a controlled release strategy. Five predefined hotspot interactions, including hydrogen bonds to the polar groups on DIG and hydrophobic packing interactions among the steroid ring system, were placed at geometrically compatible sites in a set of scaffold protein structures. The rest of the binding site amino acids were then optimized for binding affinity and protein stability using RosettaDesign. Designs were scored based on interface energy, solvent-exposed surface area, binding orientation, shape complementarity, and binding site preorganization. Seventeen candidates were chosen for experimental testing, and the tightest binder bound DIG with a K_d of 12.2 μ M, determined by ITC. Directed evolution was then used to increase the binding affinity even further (130), yet even the initial affinity

would be useful for affinity-controlled release. Computational design could also lead to more sophisticated affinity-controlled release systems. For example, a pH-dependent immunoglobulin G (IgG) binding protein has been generated using hotspot-guided computational protein interface design (138). This protein has a K_d for IgG of 4.0 nM at pH 8.2 but only 3.8 μ M at pH 5.5. If this protein was conjugated to a polymer scaffold, it could provide triggered affinity-controlled release of IgG upon exposure to a low-pH environment, such as that encountered in tumor tissues (139–141).

Despite this progress, many challenges still remain in the computational design of protein-protein interfaces. The extreme diversity of protein interfaces makes it difficult to predict energetically important interactions, especially because many proteins undergo substantial reorganization upon binding that may not be captured in traditional lock-and-key binding models (132, 142). Solvent effects must also be considered, including energetically unfavorable desolvation of polar residues at the binding interface or the presence of explicit water molecules that are directly involved in binding interactions (143). Except in the case of truly de novo design, computational methods rely on 3D structures of homologous proteins as a starting point; thus, limited structural information can present a bottleneck. Global efforts in structural genomics (144) and advances in homology modeling methods (145) are accelerating determinations of macromolecular structure, thereby increasing the number of available structures and opening this bottleneck in the computational design of binding partners.

Future outlook on affinity-controlled release

Affinity-controlled release provides significant advantages over traditional sustained drug-release strategies and is likely to be widely adopted in the drug delivery community. The elimination of organic solvents and high shear forces, which are typically used for protein encapsulation in synthetic water-insoluble polymers, enables higher active therapeutic loading for the same amount of polymer vehicle. Although there is still a certain amount of trial and error required to obtain the desired release profile from a protein encapsulated within polymeric micro- or nano-particles, mathematical modeling of affinity-controlled release has defined the variables important to control release profiles: For a known dose and characteristic diffusion length, the required K_d and immobilized ligand concentration can be calculated (12).

Advances in computational design, in vitro selection, and directed evolution offer new ways to discover or design suitable binding partners for affinity-controlled release. Combining these methods will further increase efficiency. The use of computational techniques to identify scaffolds with geometric and electrostatic properties that are complementary to a target could reduce the upfront empirical screening required to isolate lead variants. Alternatively, in silico methods could also help identify critical binding interactions that

can rationally guide mutagenesis and randomization. High-throughput measurement methods for K_d and association rates would then be needed to create libraries of binding partners for a variety of therapeutics.

To effectively treat complex diseases, combinatorial drug strategies will probably be required. Ideally, a single delivery system could be designed to release multiple therapeutics, each with its own independent release profile. This is the next frontier for affinity-controlled release systems and requires affinity interactions that are specific and nonpromiscuous. For affinity-controlled release applications of multiple therapeutics, it will therefore be important to perform competitive selections to determine specificity and ensure independent control of the release rate.

On-demand drug release, using stimulus sensitivity, presents another opportunity for affinity-controlled release. Although systems such as structure-switching aptamers or computationally designed pH-dependent binding proteins have shown promise, this area is open to innovation.

Affinity-controlled release has great potential to be expanded in scope and provide fully customized delivery solutions. We envision a modeling program that would suggest a binding ligand from an existing library based on the desired release profile and protein therapeutic. This ligand would then be tested for affinity-controlled release of the protein therapeutic after covalent attachment to a well-defined polymeric vehicle.

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