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Perspective

Polyvalency: Recent Developments and New Opportunities for Chemical Engineers

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Introduction

Polyvalency refers to the simultaneous binding of multiple ligands on one entity to multiple receptors on another.[1] Polyvalent interactions are ubiquitous in nature, with examples including the attachment of viruses to target cells, bacteria to cells, cells to other cells, and the binding of antibodies to pathogens.[1] A major advantage of polyvalent interactions in a biological context is that they can be collectively much stronger than the corresponding monovalent interactions. Polyvalency also enables control over the strength of an interaction by varying the number of interacting receptor-ligand pairs. In this article, I review recent developments in polyvalency and discuss the numerous opportunities for chemical engineers to make contributions to this exciting field, whose applications include drug discovery, tissue engineering, and nanofabrication.

Thermodynamics of Polyvalent Interactions

A simple thermodynamic analysis illustrates the ability of polyvalency to strengthen inherently weak interactions. The free energy change for a noncooperative polyvalent interaction involving the simultaneous binding of N ligands to N receptors ($\Delta G_{poly,N}$) is N times the free energy change for the corresponding monovalent interaction. Consequently, $K_{poly,N} = (K_{mono})^N$, where $K_{poly,N}$ and K_{mono} represent the association constants for the polyvalent and monovalent interactions, respectively, illustrating the significant enhancement in affinity that can be provided by polyvalency. For instance, for a noncooperative trivalent interaction (Figure 1), $\Delta G_{poly,3} = 3^* \Delta G_{mono}$ and $K_{poly,3} = (K_{mono})^3$. Rao et al.[2] have designed a trivalent system for which the free energy change for a trivalent interaction is approximately three times the free energy change for the corresponding monovalent interaction; this system is one of the most stable organic receptor-ligand pairs involving small molecules with an association constant greater than 10^{16} M, and a higher affinity than biotin-avidin, which is one of the strongest interactions known in biological systems.

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The free energy change ($\Delta G_{poly,N}$) involves both enthalpic and entropic contributions. As a first approximation, the enthalpy change for a polyvalent interaction, $\Delta H_{poly,N}$, can be taken to be the sum of the enthalpy changes for N monovalent interactions. In principle, the value of $\Delta H_{poly,N}$ could be higher than this estimate; the value would, however, be lower than this estimate if the binding of one ligand to its receptor interfered with the next binding event.[1] The entropy change for a polyvalent interaction, $\Delta S_{poly,N}$, contains contributions due to changes in translational, rotational, and conformational entropies, and changes in the entropy of the solvent (water). The loss in translational and rotational entropy on binding is weakly dependent on the mass of a species, and is to a first approximation, equal for a polyvalent and monovalent interaction.[1] Since these entropic penalties are paid primarily during the first binding event

$$|\Delta S_{trans+rot,poly,N}| \sim |\Delta S_{trans+rot,mono}| < N^* |\Delta S_{trans+rot,mono}| \quad (1)$$

Polyvalent interactions can, therefore, be entropically enhanced, ie, the free energy change for a second binding event can have a greater magnitude than the free energy change for the first binding event. These entropic gains may, however, be partially or completely offset by the loss in conformational entropy on polyvalent binding. Consequently, polyvalent interactions may be noncooperative (additive), positively cooperative (synergistic), or negatively cooperative (interfering).[3] However, even for a negatively cooperative interaction — one in which the binding of a second ligand to a second receptor occurs with a less favorable free energy than the binding of the first ligand to the first receptor — polyvalency may still allow the strength of an interaction to be enhanced by several orders of magnitude.

Polyvalent Inhibition: A Biomimetic Approach to Drug Design

The concept of polyvalent enhancement of the affinity of interactions can be used to design potent inhibitors — polyvalent molecules that bind to a pathogen or toxin, and prevent the attachment of the pathogen or toxin to its target cell. Polyvalent inhibitors have been synthesized by attaching multiple copies of suitable ligands to a variety of scaffolds, such as linear

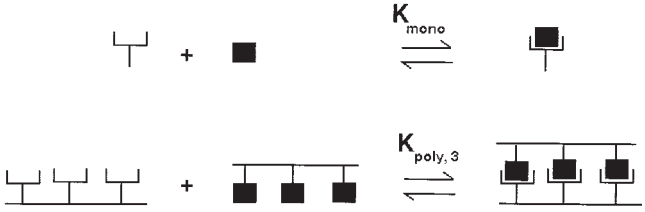

Figure 1. Monovalent and trivalent interaction, with corresponding association constants K_{mono} and, $K_{poly,3}$, respectively; for a noncooperative interaction, $K_{poly,3} = (K_{mono})^3$.

polymers, dendrimers, and liposomes,[1, 4] and have been used to target viruses and toxins. In previous work, we designed a polyvalent inhibitor of anthrax toxin that was effective *in vivo*.[5] Anthrax toxin — a protein toxin secreted by *Bacillus anthracis* — is responsible for the major symptoms and death caused by anthrax, and is, therefore, a key target for therapeutic intervention. Anthrax toxin consists of three components — the receptor-binding protein, termed protective antigen (PA), and the toxic enzymes lethal factor (LF), and edema factor (EF). PA is processed by proteases into a 63 kDa fragment, PA_{63}, that oligomerizes on the surface of a target cell to form a heptamer, referred to as $[PA_{63}]_7$. The heptamer binds the toxic enzymes EF and LF with high-affinity, and transports them into the cytoplasm of a target cell. The design of molecules that recognize $[PA_{63}]_7$ represents a promising strategy for neutralizing anthrax toxin.

A combinatorial technique, phage display, was used to identify a peptide (HTSTYWWLDGAP) that binds to the region on $[PA_{63}]_7$ that interacts with the toxic enzymes LF and EF.[5] This peptide prevented the binding of LF_N (an N-terminal protective antigen-binding fragment of LF) to cell-bound $[PA_{63}]_7$ with a half-maximal inhibitory concentration or $IC_{50} \sim 150\ \mu M$. The weak inhibitory activity of this peptide precluded its use as an inhibitor of toxin action *in vivo*. In contrast, a polyvalent inhibitor — a derivative of polyacrylamide that had multiple copies of the peptide covalently attached to it — prevented the binding of LF_N to $[PA_{63}]_7$ with an IC_{50} of 20 nM, indicating that the attachment of the peptide to the polyacrylamide backbone increased the inhibitory activity by 7,500-fold (on a per-peptide basis).

The polyvalent inhibitor also neutralized anthrax toxin in a rat model. Rats were injected with 10 times the minimal lethal dose of PA and LF and monitored for the appearance of symptoms. The addition of 75 nmol of the polyvalent inhibitor prevented symptoms completely, whereas a mixture of monovalent peptide and polyacrylamide (75 nmol, uncoupled) had no effect on toxicity. This work provided the first demonstration of the *in vivo* efficacy of a polymeric polyvalent inhibitor. The work also demonstrates a general strategy for designing potent ligands, by combining combinatorial screening (by phage display in this case) and polyvalency. The significant enhancement in the efficacy of polyvalent ligands compared to their monovalent counterparts, and the demonstration of the efficacy of polyvalent molecules *in vivo* illustrate the promise of polyvalency as a strategy for designing potent pharmaceuticals.

Polyvalent Interactions: Insights from Polymer and Colloid Science

As described earlier, considerations of equilibrium thermodynamics suggest that polyvalency could provide significant enhancements in the affinity of interactions, and enhancements in inhibitory potency are indeed observed in experimental systems. However, as chemical engineers are well aware, it is important to consider both thermodynamics and kinetics, as thermodynamic equilibrium may not be attained on experimentally relevant timescales. In this context, studies of polymers and biopolymers at interfaces — a field that chemical engineers have made immense contributions to - provide important insights into polyvalent interactions.[6-14] The "nonspecific" adsorption of polymers onto surfaces is itself inherently polyvalent; while the strength of each interaction between segments of the polymer and the surface may only be of order kT, or a few multiples of kT, the sum of the adsorption free energy of the sequences can be large.[10] Furthermore, the probability that all segments will detach simultaneously from the surface is very low, rendering the adsorption process essentially irreversible.[10] Thus, even nonspecific polymer adsorption provides evidence of the enhancement of affinity that is the hallmark of polyvalency.

Studies of the adsorption and dynamics of polymers at interfaces in systems where the segment-surface interactions are strong and specific are particularly relevant for understanding the interaction of polyvalent ligands with their biological targets. The adsorption of polymer molecules on surfaces with strong and specific segment-surface interactions is kinetically controlled.[9] The energy hypersurfaces for such systems are characterized by several minima separated from each other by barriers greater than kT. Consequently, the chains adsorb in and can be "frozen" in nonequilibrium conformations, and the system is kinetically constrained from attaining equilibrium. Chakraborty et al. have also suggested that the relaxation behavior of the adsorbed polymer molecules in these systems is analogous to that observed for glass-forming liquids.[8] Muthukumar's studies of pattern recognition by polymers also illustrate the presence of rugged free energy landscapes, and the resulting delays in the approach to the global minimum of free energy.[14] Recent work suggests that these concepts are also important for polyvalent recognition in biological systems, as described later.

Arranz-Plaza et al.[15] have studied the polyvalent interactions between galactoside-functionalized polymers and the carbohydrate-binding protein XL35. As noted by these authors, the South African clawed frog, *Xenopus laevis*, provides a useful animal model to study phenomena associated with fertilization. In this organism, the polyvalent interaction of the protein XL35 with the jelly coat protein (JCP) enrobing the egg is considered to be critical for the prevention of polyspermy (the entry of more than one sperm into the egg during fertilization). Arranz-Plaza et al.[15] studied the interaction between synthetic galactoside-functionalized polymers and XL35, and found that the polyvalent interaction required a very long time to reach equilibrium, consistent with the results of the studies described earlier.[9,14]

Recent work also suggests that enhancements in potency in polyvalent systems are not governed solely by thermodynamics, and that kinetics plays a role.[16,17] Gujraty et al. and Joshi et

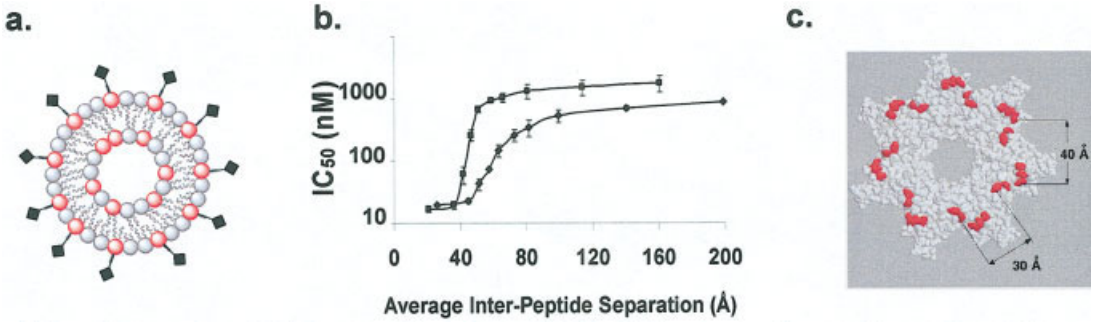

Figure 2. (a) Representation of a liposome-based polyvalent inhibitor composed of two different lipids; phosphatidyl choline head groups are labeled gray, thiol-reactive head groups are labeled red, and the diamonds represent the ligands (peptides); (b) influence of peptide density on the potency of liposome-based anthrax toxin inhibitors, A sharp transition is seen for gel-phase DSPC-based liposomes (■) (the transition is less sharp for fluid DOPC-based liposomes (♦)[4]); (c) structure of the lethal factor (LF)-binding face of the heptameric subunit of anthrax toxin, $[PA_{63}]_7$; residues 197, 200, 207, 210, and 214, which form part of the LF-binding site[40] are highlighted in red; approximate distances between residue 200 on adjacent monomers (30 Å) and residue 210 on adjacent monomers (40 Å) are indicated.

al. tested whether kinetics might limit the observed enhancement in potency of polyvalent inhibitors of anthrax toxin[16,17]—inhibitors that prevent the binding of LF to cell-bound $[PA_{63}]_7$. The authors hypothesized that equilibrium between the polyvalent inhibitor and $[PA_{63}]_7$ might not be reached on a biologically relevant time-scale - before the cell-bound $[PA_{63}]_7$ is internalized; in such a case, kinetics, that is, the relative binding rates of the polyvalent inhibitor and LF to $[PA_{63}]_7$ might limit the observed enhancement in potency. The polyvalent inhibitors were preincubated with $[PA_{63}]_7$ for different amounts of time before adding LF and exposing cells to this mixture. The potency of the inhibitors increased with increasing preincubation time, indicating that the potency of the polyvalent inhibitor was not governed solely by thermodynamics as is often assumed in biological systems, and that kinetics played a role on biologically relevant timescales. It would be interesting to test whether a similar phenomenon is observed in other polyvalent systems. Importantly, the slow approach to equilibrium and the relevance of kinetics should not limit practical applications of polyvalency, since the observed enhancements in inhibitory potency are still very substantial; an appreciation of the importance of kinetics would, however, be critical for the design of more potent polyvalent inhibitors.

Finally, we note that steric stabilization[7,10] — a mechanism for stabilizing colloids in solution by adsorbing polymers onto the surfaces of the particles — may also contribute to the polyvalent inhibition of the attachment of viruses to target cells.[1] When polymer-coated colloids approach each other (or an uncoated surface), the adsorbed polymer is compressed; the resulting repulsive force due to entropic and osmotic contributions prevents colloidal aggregation. By analogy, the adsorption of a polyvalent molecule on the surface of a virus may sterically inhibit the attachment of the "polymer-coated" virus to receptors on a target cell. Steric stabilization may enable the design of polyvalent inhibitors that bind to any molecule on the surface of a pathogen, regardless of whether this molecule itself binds to receptors on the target cell.[1]

Statistical Pattern Matching Facilitates the Polyvalent Inhibition of Anthrax and Cholera Toxins

Rai et al. have recently synthesized liposome-based inhibitors of anthrax toxin assembly.[4] Liposomes are assemblies composed of a phospholipid bilayer and an aqueous core, and represent excellent systems for fundamental studies of polyvalent inhibition. Recent work using such systems has not only highlighted the strength of polyvalency as a paradigm in drug design, but has also provided considerable insight into the biophysics of recognition by polyvalent inhibitors. In particular, these studies have illustrated the role of pattern matching and membrane fluidity in determining the potency of liposome-based inhibitors.[4]

Rai et al. synthesized liposomes composed of two different lipids (Figure 2a) — gel phase lipids 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), and the thiol-reactive lipid (PDP-DPPE) in initial experiments — in a molar ratio of 9:1. The liposomes were functionalized with a peptide that binds $[PA_{63}]_7$ and inhibits the binding of LF. Peptide-functionalized liposomes inhibited the toxicity of a mixture of PA and LF in cells with an IC_{50} of 20 nM *on a per-peptide basis* (data not shown). In contrast, the monovalent peptide did not inhibit cytotoxicity at concentrations as high as 250 μM. Polyvalency therefore provided an enhancement in inhibitory potency of at least four orders of magnitude.

Rai et al. next investigated the effect of altering the density of pendant peptides on the potency of the functionalized liposomes in a cytotoxicity assay (the molar fraction of peptide-modified lipids was varied between 0 and 10%). The IC_{50} values of these liposomes on a per-peptide basis were plotted as a function of the average interpeptide separation on the functionalized liposomes (Figure 2b). A distinct transition in IC_{50} (from $\sim 10^{-6}$ M to $\sim 10^{-8}$ M) was observed over a narrow range of average inter-peptide distances of 35–50 Å, suggest-

ing the onset of recognition of $[PA_{63}]_7$ by the polyvalent liposomes due to pattern matching. Recent theoretical studies of the recognition between random heteropolymers — one of the simplest models of proteins — and surfaces that bear multiple "binding sites"[12,13,18] suggest that recognition occurs when the distributions and densities characterizing the heteropolymer ligands and the surface binding sites are similar or "matched". In other words, statistically matching a polyvalent ligand with a polyvalent target facilitates recognition.

To test whether the location of the transition (Fig. 2b) was consistent with statistical pattern matching, the average interpeptide separation at the transition was compared to the average separation between peptide-binding sites on the polyvalent target, $[PA_{63}]_7$. Although the exact position of the peptide-binding site on $[PA_{63}]_7$ is not known, Mourez et al.[5] suggested that the 12-mer peptide binds PA_{63} at or near the EF/LF sites (Figure 2c), which would indicate that the average separation between adjacent peptide binding sites on $[PA_{63}]_7$ is $\sim 30-40$ Å. The results indicated, therefore, that recognition of anthrax toxin by peptide-functionalized liposomes was facilitated by statistical pattern matching — a significant enhancement in potency occured when the average separation between the peptides matched the average separation between the binding sites on the target protein. Furthermore, this phenomenon was not unique to anthrax toxin; statistical pattern matching also facilitated the inhibition of cholera toxin by galactose-functionalized liposomes.[4]

To gain further insight into the recognition properties of these peptide-functionalized liposomes, Rai et al. investigated the influence of membrane fluidity on pattern recognition.[4] In addition to the gel-phase liposomes described earlier, they synthesized a series of fluid-phase peptide-functionalized liposomes from a mixture of the lipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and a thiol-reactive pyridyldithiopropionate derivative of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PDP-DOPE) in a molar ratio of 9:1. The peptide-functionalized fluid-phase liposomes were more potent than the gel-phase liposomes of corresponding average interpeptide separations (Figure 2b). These results suggested that lateral diffusion of peptide-functionalized lipids in the fluid-phase liposomes might allow them to adopt a pattern complementary to that of the binding sites on $[PA_{63}]_7$ after the initial binding event has occurred, but before the liposomes dissociate. These findings are also consistent with a previous study of cell adhesion to membranes, in which the lateral diffusion of free ligands into the contact area after the initial binding event was found to result in an increase in adhesive strength.[19]

The transition in potency was also less sharp in the series of fluid-phase liposomes than in the series of gel-phase liposomes (Figure 2b). Studies suggest that the sharpness of the transition is governed by a competition between the diffusion of free peptide in the liposome and the dissociation of bound peptide. The sharpness of the transition can be increased by either decreasing the fluidity of the liposomes or by decreasing the affinity of the ligand for its target (i.e., by increasing the rate of dissociation of bound peptide); near the transition, weakly bound ligands, or ligands in gel-phase liposomes would be more likely to dissociate before free ligands could diffuse and adopt a complementary pattern to strengthen the interaction. These results provide an excellent illustration of the role of fundamental chemical engineering concepts — kinetics and transport— in polyvalent recognition.

Pattern-matched liposomes could also neutralize anthrax toxin *in vivo*. The pattern-matching-based recognition strategy applied for the neutralization of anthrax toxin should be broadly applicable for the detection and neutralization of bacterial and viral pathogens and toxins, many of which possess repetitive surface structures.

Emerging Areas

Novel therapeutics

The design of therapeutics will continue to be an important area of research in polyvalency. It will be especially important to apply this powerful concept to diseases where alternative treatments are sorely needed, including diseases, such as influenza, AIDS, and malaria. While polyvalent inhibitors have been shown to be effective in a number of systems *in vitro*, the recent demonstrations of efficacy *in vivo*[4,5,16,17,20,21] are particularly encouraging. Current examples of polyvalent inhibitors of toxins and pathogens, however, have primarily involved the design of molecules that bind directly to the pathogen or toxin to inhibit their action. Basha et al. have recently developed a polyvalent inhibitor of anthrax toxin that targets the cellular receptor for the toxin.[21] Blocking "conserved" host receptors used by pathogens represents a powerful strategy to overcome the widespread problem of pathogen resistance to antimicrobial therapeutics, because extensive alterations to the pathogen may be required to enable it to switch to a new host receptor that can still support pathogenesis. The design of polyvalent inhibitors that bind host proteins represents a promising strategy to combat a variety of viral and bacterial diseases.

Stem Cells

Another exciting application of polyvalent molecules involves their use as cellular effectors — molecules that actively promote or activate biological processes and cellular responses.[22] The dimerization and oligomerization of cellular receptors has been recognized to be a general biological control mechanism contributing to the activation of several intracellular signal transduction pathways. The clustering of cell-surface receptors by *synthetic* polyvalent molecules, or effectors, can also activate biological systems and elicit cellular responses. Polyvalent effectors have been used to control bacterial chemotactic responses,[23] to elicit or down-regulate immune responses,[24] and to influence cell spreading and cell motility.[25] Literature reports also implicate the size of receptor clusters in regulating cellular responses. For instance, Engelmann et al. found that antibodies that aggregate tumor necrosis factor (TNF)-binding proteins activate the TNFα receptor, and that the resulting cytotoxicity was directly correlated to the extent of receptor aggregation.[26]

An important future application of polyvalency will involve the design of synthetic effectors that control stem cell fate and function. A stem cell is defined as a clonal precursor of both more identical stem cells, as well as specialized or differentiated progeny cells of one or more defined types.[27] Understanding the mechanisms that control stem cell fate is important for both basic biology as well as for therapeutic applications of stem cells. Important unresolved questions include: What pathways can polyvalent effectors activate in stem cells? How does the activation depend on the type of stem cell (e.g., adult vs. embryonic; precursor of blood cells vs. neurons)? Does the extent or rate of

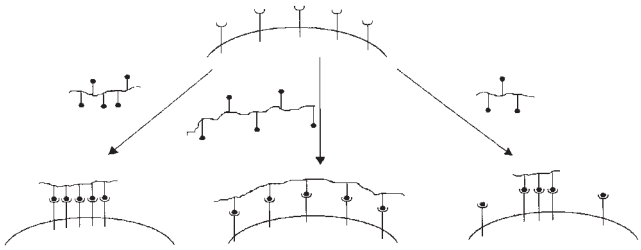

Figure 3. Polyvalent cellular effectors: tuning the molecular properties (e.g., valency and interligand spacing) of the polyvalent effectors enables control over the clustering of cellular receptors.

activation depend on the extent of receptor clustering (Figure 3)? How does the molecular structure and composition of the polyvalent effector (e.g., the dimensions, fluidity, and density of pendant ligands on a polyvalent liposome) influence signal transduction and the stem cell response? How do polyvalent effectors influence the rates of internalization of target receptors? Do polyvalent effectors provide control over stem cell proliferation and differentiation? Finding the answers to these questions will be of both fundamental and practical significance.

Opportunities for Chemical Engineers

Molecular and multiscale modeling

Modeling across multiple length and timescales[28] — another field of research where chemical engineers have had a significant impact — is well-suited for both understanding polyvalent recognition, and for designing polyvalent ligands. At the smallest length scales, molecular simulations may provide insight into the molecular basis for ligand-receptor affinity and the conformation and dynamics of an individual ligand (e.g., peptide) when bound to its complementary receptor, and help identify the binding site for a ligand on its receptor. Simulations may provide insight into the influence of the molecular structure and properties (e.g., the flexibility and architecture of the scaffold and the ligand density) on polyvalent recognition.[7,12,13,18] At larger timescales, simulations may provide insight into the formation of a polyvalent complex and the approach to thermodynamic equilibrium.

Ligand identification and design

A critical step in the design of a polyvalent molecule involves the identification of a suitable monovalent ligand that is specific for a particular biological target. As mentioned earlier, combinatorial strategies, such as phage display are well-suited for polyvalency; it is relatively easy to identify a weakly binding ligand by phage display, whose potency can be then enhanced significantly through polyvalency. Chemical engineers have played a leading role in the fields of molecular bioengineering and directed evolution,[29-31] and have developed powerful techniques including bacterial surface display[31] and yeast surface display,[29] that are well-suited for identifying ligands for polyvalent display for applications ranging from polyvalent inhibition to targeted drug delivery. The use of these techniques in conjunction with molecular simulations may enable further optimization of ligand potency and even the rational design of novel ligands.

Molecular engineering of polyvalent scaffolds

Another critical aspect of the design of polyvalent molecules involves the design of scaffolds, e.g., polymers, liposomes, nanoparticles, and even proteins or viruses. The expertise of chemical engineers in self-assembly, nanofabrication, polymer synthesis, and the design of proteins, including those incorporating unnatural amino acids could be creatively applied for the engineering of novel polyvalent scaffolds.[32]

Delivery of polyvalent molecules

Chemical engineers have played a major role in the design of strategies for the controlled delivery of drugs — both small molecules and proteins.[33,34] The development of new strategies for the effective delivery of polyvalent molecules — a new class of drugs — would certainly benefit from chemical engineering contributions.

Cellular Interactions

Chemical engineers could contribute in various ways ranging from the molecular engineering of novel polyvalent cellular effectors to the quantitative analysis of the cellular response and signal transduction, and the formulation of tissue-level models.[35]

Applications of polyvalency are only limited by one's imagination, and also include fields ranging from targeted drug delivery to imaging, immunology, and nanofabrication.[36] As discussed earlier, chemical engineers have the tools and skill set required to make important contributions to this field.

Conclusions

The chemical engineering profession has acquired an increasing molecular focus in recent times.[28, 37, 38] As discussed earlier, this molecular focus and the powerful chemical engineering tool-set are well-suited for understanding polyvalent recognition and applying it to solve problems that have a practical significance. To paraphrase a quote attributed to Jay Bailey[39], "chemical engineering is what chemical engineers do". Chemical engineering's molecular focus, and its traditional strengths in thermodynamics, kinetics, and transport position its practitioners ideally to make fundamental contributions to polyvalency, and, thus, to have a major impact in areas ranging from the design of novel therapeutics to tissue engineering, nanofabrication, and drug delivery.

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