



Synthetic Multivalency for Biological Applications

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Review

Synthetic Multivalency for Biological Applications

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Current directions and emerging possibilities under investigation for the integration of synthetic and semi-synthetic multivalent architectures with biology are discussed. Attention is focussed around multivalent interactions, their fundamental role in biology, and current and potential approaches in emulating them in terms of structure and functionality using synthetic architectures.

Introduction

Interfacing synthetic multivalent materials with biological systems in a controlled manner is an ongoing area of investigation, with pronounced potential in the generation of novel therapeutics and diagnostics. However, designing and synthesising materials at the nanoscale to engage biological processes in a functional way remains an extremely intricate task, justifiable given the complexity present for "native" interactions which have evolved at the molecular scale in an integrated and cooperative way.

Regarding synthetic assemblies, rational design approaches, involving the conjugation of biomolecules to nanoscale materials have shown functional behaviour at the level of ligand-receptor binding through various biophysical methods and in vitro assay. However, using multivalent synthetic structures for modulating biological outcomes, through peptide or glycan interactions, remains a complex handle to manipulate in a predictive way, given the finely tuned interactions specificities, avidities and dynamics which regulate biological interaction networks. As the discovery of novel synthetic approaches for multivalent biofunctional nanostructures continues, future work will likely see synthetic material biology integrated with the philosophy of the systems biology paradigm to enable broader understanding. Designed biological intervention by synthetic materials would thus focus on controlled modulation of whole biological process networks as understood from a systems biology perspective, applying holistic consideration to sequential interaction behaviours and whole system response. Biological interactions are, after all, cooperative, reciprocal and cascading, responding not only to epitope interaction but also structural dynamics¹.

Increasingly effective interactions, through the employment of

synthetic multivalent scaffolds, remains a highly promising avenue to novel therapeutics, given the growing understanding in how nature uses this model as a means of dynamic interaction control; Multivalent balance has evolved at multiple biological scales from sub-nanometre to micron scale, involving ionic metal interactions in metalloproteins, supramolecular interaction^{2, 3}, amino acids⁴, macromolecular assemblies⁵ (e.g. viral capsid) and cell-cell communication⁶. It is thus evident that it is a mechanism fundamental in homeostasis, regulating recognition, adhesion, and signalling processes.

An essential feature of multivalency is augmentation of selectivity for interactions in complex mixtures. Thus, in addition to enabling finer control in avidities, it is understood that such spatial expansion of interaction nodes can lead to exquisite selectivity at composite recognition interfaces. Such interaction selectivity is apparent for the evolution of the human glycome⁷ in the presence of sugar binding pathogens, through combinations of low affinity glycan interactions.

Biomimetic Multivalency

Multivalent display is involved in numerous interactions and plays a number of functions in biology involving fundamental structural elements, nucleic acids, amino acids and sugars⁵. With advancement in synthetic capabilities at the nanoscale many current approaches look to structurally emulate biology giving rise to biological functionality. Multiple nanomaterial types can now be reliably synthesised in a size range which spans from that of proteins to viruses, and thus a rational approach would be to examine structure/function relationships for these biological structures and aim to mimic their structure through rational synthetic approaches. However, while many synthetic systems have been developed which deliver specific epitope binding, nanoscale display of corresponding functional moieties with controlled orientation and correct spacing remains a challenging task, without yet considering those dynamic features present for proteins and fundamental to biological interaction sequences including

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internal molecular mobility and characteristic association/dissociation rates.

An additional consideration is the population heterogeneity typical of many nano-synthetic preparations when contrasted with the one-by-one programmed biological synthesis of proteins. For proteins multivalent display control is regulated at the genetic level through structural language of peptide assembly in combination with post-translational modification processes (contrast glycoprotein glycan presentation imparted by peptide folding prior to post-translational glycosylation with a glycoNP). By comparison, while potentially superior in yield, nanoparticle synthetic methods are crude, with comparable display control difficult to envisage. For proposed *in vivo* diagnostic and therapeutic solutions, such surface heterogeneity for synthetic multivalent display systems and the impact of population outliers, remains an issue, given the recognition sensitivity present in biology, where altered protein functionality can arise from single amino acid sequence mutations⁸.

Hence, to move beyond a role as synthetic mimics aiding in the understating of multivalency, and to progress further as therapeutic and diagnostic solutions, there remains a need for novel methods in the synthesis of multivalent nanoscale architectures which provides requisite surface control and population homogeneity.

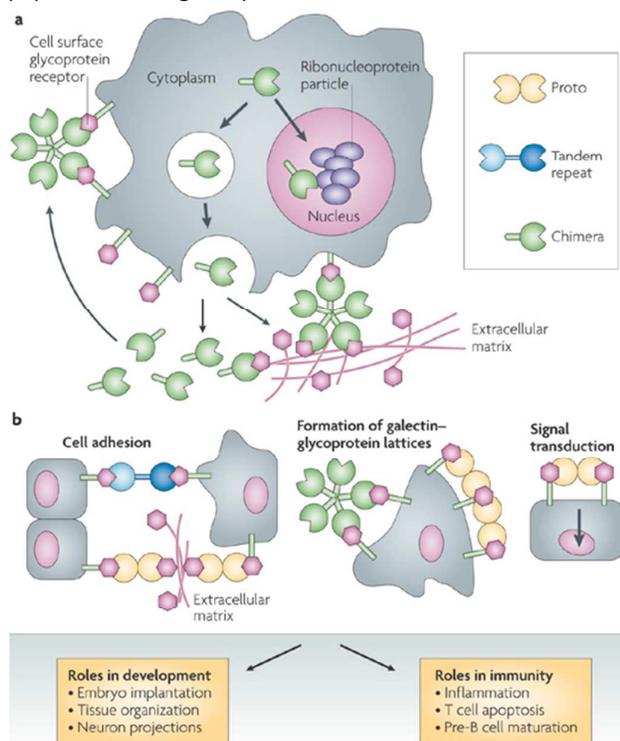


Figure 1. Multivalency in lectin interactions as proposed for galectin family depicting roles in cell-cell, cell-ECM and cell signalling pathways. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology⁹, copyright (2009).

Mimicking the Immune system

Progress in understanding human immunobiology at the molecular level can be taken advantage in prompting advances

in synthetic multivalency, particularly in terms of controlling the balance between selectivity and avidity. Immuno-biological recognition processes have evolved through multivalent structural displays to facilitate high specificity through increasing number of atomic scale interactions, while preserving avidity in the appropriate reversible range, adapted for the concert of interaction time-scales present. As presented further below, through selected examples, the role of multivalency in immune response extends widely across both innate and adaptive immune recognition events.

Innate Immunity

For the innate immune response, following initial exposure to “non-self”, multivalency plays a critical role in its capacity for outcome selection. A prime example is the discrimination of non-self by the lectin associated complement pathway⁹⁻¹¹. Multivalency in protein-carbohydrate recognition has been previously well described¹², and is recognized as a fundamental feature of the innate immune response as well as in the regulation of (glyco)protein interactomes, “fine” tuning surface structure and function^{12, 13} (see Figure 1). Lectin proteins display CRD (carbohydrate recognition domains) which generally bind terminal monosaccharide moieties with high specificity and low affinity with sufficient avidities gained through multiple interactions^{14, 15}, so exhibiting glycan density dependence. To illustrate, mannose binding lectin (MBL), a serum protein, utilizes multivalent pattern recognition to recognize, in a threshold dependant manner, mannosylated surfaces, typical of many pathogens, and as a result activate the complement system¹⁶. This is an example of multivalent modulation of a biological interaction through molecular pattern recognition, and evidences the ligand density and threshold effects present in biological multivalency. Thus threshold avidities are achieved in a highly selective way through multivalent recognition of glycosylation patterns¹⁵. This hints at approaches, discussed also further on, in achieving biospecific recognition and yet controlled primary immune evasion through the tuning of synthetic glycosylated nanosystems in a combinatorial way.

At a larger interaction scale, multivalent “recognition of self” or “detection of missing self” plays a primary role in innate immune surveillance by Natural Killer (NK) cells. The interaction of “self”-ligands with inhibitory receptors expressed on NK cells suppresses their cytotoxic activity, whereas the absence of self-ligands frees the killer function¹⁰. This is an example of multivalent modulation at the multi-receptor level, whereby, absence of multivalent recognition of a combination of self-ligands will lead to internal suppression signals falling below a threshold level thus liberating killer function.

Furthermore, in contemplating synthetic approaches to targeting biology and evading innate immunity, we can also interrogate mechanisms which pathogens have evolved in response. Indeed, the promise of controlled synthetic glycosylation is further demonstrated upon studying pathogens. Some have evolved their glycomes in parallel with their hosts, utilizing host lectin to direct their survival⁹. They have evolved to mimic host glycosylation patterns as both a

means of immune evasion as well as ensuring successful attachment or invasion through host lectin, a strategy similarly applicable to synthetic glyco-architectures.

Viruses meanwhile, mainly utilize host machinery to glycosylate their capsid proteins and it is evident from virus evolution that adaptation to host glycosylation machinery has produced interfacial displays selected for infectivity and immune evasion^{17, 18}. Using host glycosylation machinery to develop synthetic glycosylation patterns is an area under increasing investigation. Methods using glycosyltransferases and glycosidases, have recently been applied to synthetic architectures¹⁹⁻²¹.

In Adaptive Immunity

“Natural” multivalent macromolecular assemblies IgM, are circulating natural antibodies expressed by naive B cells. Individual IgM display low antigen affinity but when assembled into a pentameric construct are efficient in opsonizing (coating) antigens for destruction. This initial response is facilitated by multivalent recognition. Individual Ig components can bind a broad range of antigenic sequences but with ineffective low affinity. When assembled however, multimeric recognition facilitates sufficient binding avidity for antigenic surface displays, connecting with the complement pathway^{22, 23}. In other words, the low affinity, polyreactive nature of the individual IgM, by assembly, provides an effective broad pathogenic surface specificity through multivalency^{24, 25}. In a related synthetic biology approach, heterologated polyreactive antibodies to HIV²⁶, thus multivalently displayed low affinity polyreactive ligands, showed enhanced binding avidity.

The case of IgM thus demonstrates that multivalent display of low affinity ligands can give rise to highly selective surface type recognition, behaviour also under study for synthetic systems^{27, 28}. This leads on to the general design principle for multivalent ligand displays—that the binding interaction should be constructed of low affinity individual interaction to deliver selectivity, yet arrive at a suitable avidity with suitable dissociation half-life. Effective biological interactions normally fall within a K_d window, thus facilitating dissociation half-lives in a suitable range e.g. Affinity constants for Abs are rarely higher than 10^{10} M^{-1} because this would require that the dissociation half-life of the Ab must be longer than 1 h^{29, 30}.

Regarding these and other aspects of immune response not expanded upon here, such as antigen density dependant T-Cell activation, we can surmise that optimal selectivity and avidity for concerted functional recognition in a complex surrounding milieu of similar peptide and glycan arrangements, has evolved using multivalency at various scales. Thus, expansion of recognition modes beyond molecular to supramolecular and multi-macromolecular enables higher selectivities amid balanced avidities. These observations hint at the therapeutic intervention potential of synthetic architectures which show controlled ligand densities, spatial distributions and combinatorial recognition properties.

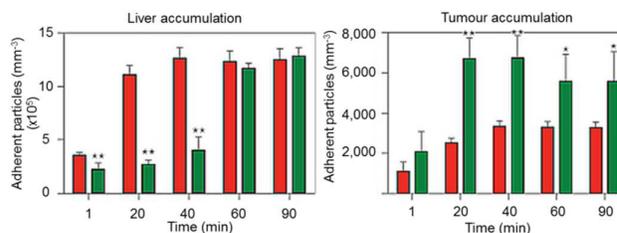


Figure 2. Human leucocyte membrane coated 3.2 μm NPs show decreased liver accumulation and increased tumor accumulation in mice. Red bars: nanoparticle, Green bars: LeukoLike Vectors i.e. membrane coated nanoparticles. Adapted by permission from Macmillan Publishers Ltd: Nature Nanotechnology³¹, copyright (2012).

Synthetic Multivalency

Synthetic multivalent systems encompass a vast array of nanoarchitectures including multimers, polymers, dendrimers, nanoparticles, micellar and other supramolecular assemblies which the following discussion aims to treat in a broad sense.

Biological Environment

Presenting multivalent recognition interfaces in biology requires consideration for interactions with the complex surrounding environment. Surrounding biomolecules will rapidly equilibrate with the synthetic surface through composite supramolecular interactions, thereby altering behaviour³². For instance, this biomolecular “corona” can lead to macrophage engulfment due to adsorbed opsonin or scavenger recognition of non-opsonin “de-natured” proteins³³. Many reports have concluded opsonizing pathways for nanoparticles³⁴, with serum protein adsorption activating immune responses and complement pathways leading to rapid Reticuloendothelial System (RES) removal of nanoparticles from *in vivo* systems³². Surface chemistry dependant, such clearance is also in evidence for non-adapted viral carriers^{35, 36}. The result is rapid clearance from circulation largely at the liver, making for ineffective biomedical agents. Indeed this general property does suggest nanosynthetics as ideal candidate macrophage imaging agents³⁷, also for macrophage inhibition³⁸ and in vaccine preparations. However, a more general aim is prolonged circulation, increasing target tissue residence time and specific receptor recognition opportunity. “Self” cells such as lymphocytes and red blood cells show circulation half-lives of 70-80 days³⁹ and 120 days respectively, comparable synthetic surface configurations are yet to be discovered. Biomimetic approaches have shown some success in stealthy NPs by coating with such blood cell membranes^{31, 40} (figure 2), reducing phagocytosis and liver accumulation, thus enhancing circulation and increasing passive accumulation in tumor.

That synthetic surface chemistry can modulate immune cell response to nanoscale presentations^{41, 42} is now well known, and understanding of the relationship between synthetic surface chemical spatial distributions, surrounding biological environmental interactions, and macrophage behaviour is ever increasing. PEGylated surfaces greatly suppress protein adsorption resulting in inhibition of opsonisation pathways. The application of the promising “PRINT” fabrication approach,

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for example, has shown that nanoparticles can avoid induction of an innate immune response through highly controlled PEGylation, yet monocyte phagocytosis still occurs, albeit more slowly⁴³. Other factors including ligand displacement and population surface heterogeneities connecting to serum adsorption, can be forwarded to explain the macrophage accumulation evident for many *in vivo* studies⁴⁴. It is also proposed that such removal mechanisms, where opsonisation is considered avoided, are mediated through pattern recognition receptor (PRR). PRR are low specificity proteins which can recognize a broad range of pathogen associated chemical functionalities including glycan and their lipido and peptido conjugates, and polyanions, and so are expected to recognize many “non-self” repetitive synthetic surfaces.

An additional consideration for synthetic multivalent architectures is adaptive immunity. Antibodies have been described in the human population for PEGylated surfaces⁴⁵⁻⁴⁷, and could thus be expected to arise for other multivalent surface types, limiting applicability.

Immune avoidance through multivalent “self”-recognition may provide an alternative to the “stealth” approach. Recently it has been shown that self-peptides, designed to bind CD 172a and so impede phagocytosis, when attached to NP surfaces, have the potential to inhibit clearance⁴⁸. Additionally glycosylated surfaces appear promising as “mimics of self”, and can be encoded for specific recognition through transaction with native lectins. Controlled combinatorial displays could thus be envisioned to enable controlled immune interaction, simultaneously presenting targeting patterns.

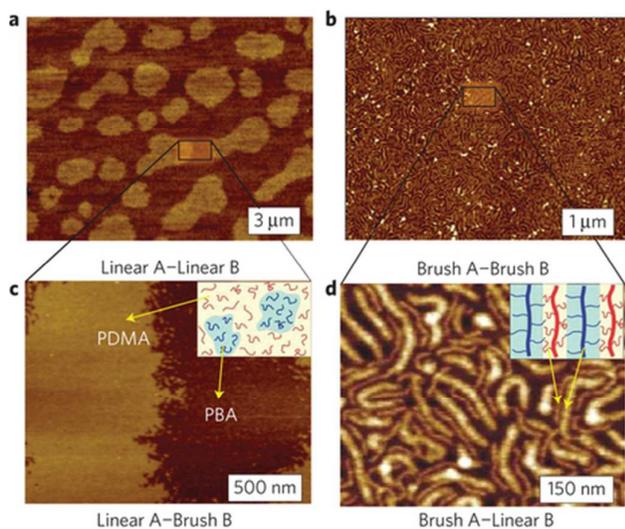


Figure 3. Controlled surface microdomain formation linear-linear polymer (left) and linear-branched polymer mixtures (right). PBA: poly(*n*-butyl acrylate), PDMA :poly(dimethyl acrylamide) Reprinted by permission from Macmillan Publishers Ltd: Nature Materials⁴⁹, copyright (2013).

Surface Display

The chemical synthetic approach to nanoscale therapeutics remains attractive, as there are large yield and economic advantages when compared to the synthetic biology route.

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Interaction super selectivity^{27, 28} has been predicted for synthetic architectures which display combinations of low affinity ligands, where ligand combination, density, and specificities are determining characteristics for *in vivo* behaviour⁵⁰ and biological response⁵¹. To achieve such biological accordance requires exquisite control of surface presentation. Providing core scaffolds toward this goal, many synthetic systems have been investigated, based mainly around covalent conjugation or supramolecular assembly, dendrimers⁵², virus-like particles⁵³⁻⁵⁶ and proteins⁵⁷⁻⁵⁹.

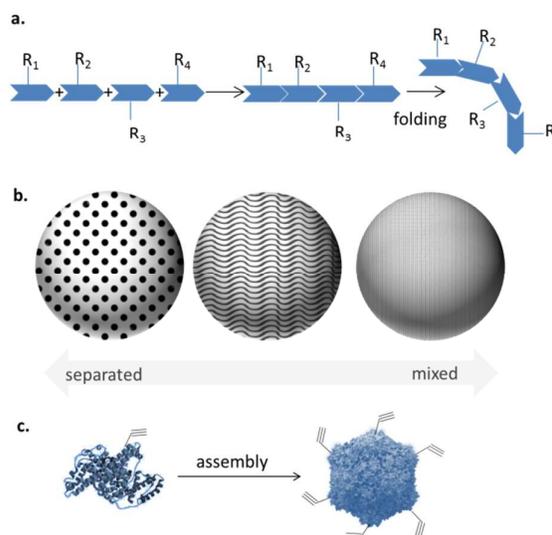


Figure 4. Recent Approaches to interfacial ligand display control: a) molecule by molecule polymeric conjugation approach followed by controlled folding (see example⁶⁰). b) Self-organization into supramolecular domains domain as in examples using intermolecular interactions^{61,72,73} or entropic forces⁴⁹. c) Structurally directed assembly of addressable reaction points for example using protein-capsid assembly in combination with gene engineering⁶².

Ligand density and spatial control

While general biologically suitable conjugation chemistry has been extensively reviewed^{32, 63}, here we focus on methods applied in controlling nanoscale interfacial display. In the case of nanoparticles, typical approaches involve ligand mixtures applied in surface functionalization to control ligand surface density. A commonly employed alternative is ligand replacement post-stabilisation⁶⁴, with a general assumption of homogenous final distribution for both. Such stochastically controlled conjugation approaches have traditionally been applied to nanoparticles⁶⁵, dendrimers^{52, 66}, liposomes⁶⁷ and result however in population functionality distributions⁶⁸⁻⁷⁰. In figure 4 recent approaches to addressing the issue of how to control in terms of ligand density and spatial distribution are presented and further discussed in the following text. Approaches to gaining greater control over density usually involve surface programming through supramolecular complementarity. Through the self-assembly approach, thermodynamic supramolecular control can be incorporated for guiding interfacial display numbers as well as spatial distribution and orientation. This is well illustrated by

examples which use biomimetic approaches such as the viral capsid mimics (protein-protein interactions)^{71, 72} or cell membrane lipid-protein nanodomains⁷³ (hydrophobic interactions, lipid rafts, protein-protein etc), where lateral supramolecular interactions are used to define equilibrium display. Correspondingly, micellar assemblies provide a means to monomer programmed induction of interfacial order through self-assembly. They may also be harnessed as organic templates for other “fixed” materials such as silica, generally applied in related material science approaches to material transcription of chirality⁷⁴. Indeed, it has been demonstrated using block copolymer, that through hierarchical micellar self-assembly, increased population and nano-domain homogeneity^{75, 76} can be attained. Additionally, small molecule micellar assemblies, such as those based on short peptides, have recently shown promise as condition responsive uniform targeting architectures⁷⁷ and may be further developed in terms of interfacial targeting architecture, thus addressing nanoscale heterogeneity in multivalent targeting.

Another approach to greater display definition is given by the controlled molecule-by-molecule conjugation as applied in solid phase peptide synthesis. Stepwise conjugation allows for fully defined chemical sequence thus enabling controlled ligand placement. This approach has been duly exploited through incorporation of “click” able functions to generate defined heterogeneous glyco-display, exerting control over number, spacing, position, and type of sugar ligand displayed⁶⁰.

As has been discussed with reference to the immune system, and has been demonstrated for synthetic systems⁷⁸, ligand grouping and nanoscale surface pattern are fundamental determinants of multivalent recognition behaviour. Viewing proteins as biological nanoparticles, as noted earlier, the biological one-by-one synthesis of proteins, and directed folding process endows exquisite surface function distribution control, enabling highly specific cooperative interactions and behaviour. Controlling surface display comparably through the typical surface functionalization methods applied to synthetic nanoparticles presents a considerable task. Besides the inherent population variability, in terms of size and surface distributions, produced in many nanoparticle syntheses, the task of controlling ligand density and spacing requires advancement. Rational design approaches to controlling spatial distribution of ligands, facilitating optimal recognition capacities, have been attempted previously through various means; Methods have been developed for single ligand attachment points, on gold NPs for example⁷⁹ and also for polymeric nanoparticles with single addressable reaction points⁸⁰. However, exacting control on combinatorial surface displays are generally attempted through stochastic functionalization methods. Such an approach may yet prove profitable, where selection/fractionation methods can also be applied to select for avidity and selectivity ranges using chromatography or precipitation selection methods, ideally using target biology.

Mentioned above in relation to surface density, the supramolecular self-assembled multivalency approach

promises programmable control of architecture distributions. Self-assembled systems can be chemically encoded for display control and so enable the presentation of defined ligand numbers in defined spatial distributions^{81, 82}. The majority of current research in this area involves amphiphilic self-assembling systems based on peptides^{83, 84} and glycopeptides^{85, 86}, with other prominent examples based on RNA assemblies⁸⁷. Increasing scales of multivalency can also likely be accessed through programmed nanoparticle and macromolecular assembly⁸⁸.

For nanoparticle scaffolds, further innovation has been reported recently for nanoscale display control driven by supramolecular organisation at dynamic surfaces. Entropic repulsion between linear and branched polymer has been demonstrated as a route to controlled addressable domain formation on nanoscale surfaces (figure 3)⁴⁹. Also surface curvature⁸⁹ has been shown to influence ligand display. “Attractive” supramolecular interactions can also be used to drive domain formation within dynamic layers, organising ligands at the recognition interface⁶¹. Biological fluid 3-D interfaces, phospholipid bilayers, provide a medium for controlled multivalent ligand presentation. Combined with supramolecular intra-layer control of ligand display they provide a route to adaptive interfaces reminiscent of cooperative recognition present at biological membranes⁹⁰. An alternative approach meanwhile uses surface assembly of proteins⁶² as spacing elements for controlled chemical function display.

As detailed above, binding, recognition and signalling in many biological processes, such as cell-cell and cell-extracellular vesicle, are dynamic and cooperative processes involving multiple interactions. Such dynamic binding modes can be mimicked for synthetic assemblies tuning the lability present in many supramolecular and dynamic covalent systems^{91, 92}. Dynamic assemblies may ultimately provide scaffolds allowing the programming of sequential adaptation into nanoarchitectures, reminiscent of biological systems.

Innovative Synthetic Approaches:

Numerous methods have been applied in the synthesis of multivalent structures typically involving conjugation of biological functions such as sugars^{35, 93-95} at nanoscale surfaces. Self-assembly of synthetic biological mimics⁹³⁻⁹⁵ is another approach to demonstrate enhanced avidity, to further relate some of our previous investigations^{32, 96-98}. Similar approaches are widely applied using protein/peptide and DNA. In the following section some emerging and exciting approaches for synthetic and semi-synthetic multivalent nano-systems are conveyed, focussing on biologically inspired methods.

Biologically Derived Multivalency

Some emerging approaches rearrange or reconstitute biological components in different forms as a means of acquiring the structural complexity required to relate effectively with biology. Biological membranes, fundamental structure directing and display components in biology, can be taken and reformed as nanoscale display systems whilst

maintaining desirable properties such as immune compatibility and optimised functional display.

A recent example used leucocyte cell membrane to coat microparticles showing promise in inhibiting particle opsonization and phagocytosis³¹. This approach has the potential to confer the originating cell-like behaviour to a synthetic particle. Another interesting approach uses engineered *E. coli* to display a functional protein in the outer membrane, before budding functional targeting outer membrane vesicles^{99, 100} (OMVs). In general, synthetic exosomes and microvesicles are an area of intensive research, and can be generated by multiple methods¹⁰¹ and combined with nanoscaffolds¹⁰².

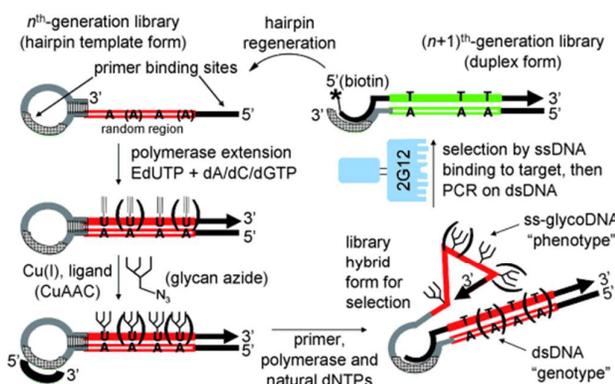


Figure 5. Directed evolution of Multivalency. Optimum Man_9 glycan presentation was accomplished through the SELMA (SElection with Modified Aptamers) technique, Reprinted by permission John Wiley and Sons Ltd.: Temme et al.¹⁰³, copyright (2013).

Outside of restructuring of biological membranes, synthetic biology provides powerful routes to controlled chemical function presentation and highly defined nanoparticle populations through gene engineering. Proteins can be used as display architectures themselves or assembled in virus-like particles to develop larger scales of controlled multivalency. Such approaches enable the scaling of multivalency from 1 nm through to 100 nm scale. The virus-like particle approach to displaying addressable conjugation points has been shown to deliver potent viral inhibition through competitive binding¹⁰⁴. By this approach nanoparticles of advanced biofunctionality for intracellular imaging^{105, 106} have also been prepared. The main limitations in such biological approaches, when compared with that of potential synthetic chemical replacements are material yield and economy.

Directed Evolution and Combinatorial Multivalency

Directed evolution uses selection and amplification to evolve constituents toward a user-defined functionality. Phage display technology is a prime example of an evolution driven approach to peptide discovery which uses diversified populations of bacteriophage to display proteins, encoded for by the corresponding gene inside^{107, 108}. Rounds of selection (e.g. through binding) and amplification can thus be used to identify functional genotypes. Besides macromolecule discovery, directed evolution may be applied to generate biological multivalent displays such as virus phenotypes with

defined properties¹⁰⁹. Incorporation of *In vitro* selection concepts into synthetic multivalent displays would typically extend only to prior peptidic ligand discovery, with subsequent conjugation to a synthetic scaffold. The aptamer approach^{110, 111}, which involves target specific RNA being selected through rounds of bind, release and amplification, has been successfully applied¹¹². In this case, aptamers were selected for specific cell internalizing ability before subsequent conjugation to NP surfaces. Evolution of polymeric glycocluster presentations for effective synthetic vaccines was also addressed using the aptamer approach (see Figure 5). Here *in vitro* selection and amplification of multivalent glycosyl ligand display enabled the evolution of DNA aptamers bearing large covalent oligosaccharide modifications^{103, 113}.

For fully synthetic multivalent display, although lacking the encoding and amplification mechanisms which enable directed evolution, related combinatorial approaches using diversification and selection to identify optimal architectures have raised some interest. Some recent examples describe the development of optimised nanoassemblies for *in vivo* siRNA delivery^{114, 115} and tumor targeting^{116, 117}, and can also be combined with innovative selection methods¹¹⁸. Additionally biosynthetic diversification approaches such as "glycorandomisation" through covalent chemical¹¹⁹ or enzymatic approaches¹²⁰⁻¹²² can be imported for fully synthetic displays, thus integrating further discovery power.

Modification of amphiphile molecular structure, prior to supramolecular structural translation upon micellar assembly, provides a control for features of multivalent presentation¹²³. Headgroup valency, shifting assembly properties, has been shown to impact micellar size and thus surface structure; Decreased size with increased fractional charge has been shown with increasing headgroup number for cationic amphiphiles^{124, 125}. Such a multi-headgroup approach has ultimately been shown to alter biological properties in gene transfection studies using liposomal assemblies¹²⁶. Building further upon combinatorial approaches in multivalent display, the principles of constitutionally adaptive chemistry¹²⁷ may enable enhanced modes of combinatorial discovery as constitutionally adaptive nanoscale surfaces can, in principle, access great diversity. Such approaches to optimal display discovery are only beginning to develop¹²⁸.

Further examples are presented in more recent approaches to artificial gene delivery. Many effective non-viral vectors have been rationally designed in recent decades¹²⁶. Despite this progress, there remain issues related to effective and direct application, derived from the structural variations in cells as well as targeted DNA. A limitation in addressing this diversity through rational design, is the typically low number of primary components. Within this context, the dynamic combinatorial and constitutional screening approach appears an attractive strategy for the rapid identification of active delivery vectors from dynamically exchanging libraries of complex mixtures of components. The potential of such an approach was demonstrated by the Matile group, using reversible exchange between hydrophobic tails and hydrophilic heads. In dynamic screening methods, the fittest transfactor, adapting

simultaneously to the DNA target and the cell membrane barrier, was selected¹²⁹.

Dynamic adaptation has also been observed for the nanometric dynamic (dynamic polymeric) systems¹³⁰⁻¹³². These systems display the property of dynamic reversible component rearrangement at the molecular level, so adapting surface through supramolecular interaction with the target DNA and the cell membrane barrier.

For example, polyspermine imidazole-4,5-imine dynamers have been obtained by condensing bis-formaldehyde imidazole with spermine, through pH-responsive reversible covalent bonds. This dynamer was used to condense siRNAs for delivery into cells and enable release from endosomes. Cellular and *in vivo* assays indicated that the dynamic carrier is effective in silencing target genes, with compatible cytotoxicity¹³⁰.

Related studies on dynamic polymers composed of hybrid polyacrylhydrazone-monomers that combine bis-cationic heads with ethylene oxide containing segments show effective binding of dsDNA at N/P ratios comparable to polyethyleneimines. Dynamic covalent polymers, via the incorporation of reversible covalent bonds, is therefore a promising strategy for generating effective vectors accommodating dsDNA through multivalent interactions, while also tuneable for condition dependant release in biological contexts¹³¹.

Furthermore, 3D Dynamic Constitutional Frameworks (DCFs)¹³², generated from reversibly interacting constituents, show promise for novel biomedical agents, given that biotargets like DNA, contain diverse molecular members. In our recent example, multifunctional PEG core macromonomers and cationic heads have been used to generate DCFs for DNA binding. The optimal spatial distribution of multivalent biointeracting headgroups within the 3D scaffold of the DCFs together with environmental adaptation, leads to discovery of adapted active delivery systems. In other words, DNA supramolecular interactions, in combination with environmental interactions, are used to drive self-selection and self-construction of the optimal vector for its transfection. The simplicity of the synthetic constitutional strategy using simple building blocks to generate DCFs which display synergistic DNA and cell membrane affinities, thus presents a valuable approach to the systematic discovery of active delivery systems.

Conclusions

The current and emerging research in engaging biology with synthetic multivalent architectures involves many biosynthetic, chemical synthetic and combination approaches, which show respective advantages and drawbacks.

While an array of biological interactions are governed through multivalent interaction at different scales, it remains a challenge to effectively synthesise multivalent structures which can effectively engage, taking into account also expanded features beyond ligand-receptor binding including

spacing, density, complex environmental interactions, and dynamic effects.

To date, the most proven synthetic approaches to specific interaction *in vivo*, remain biological, using protein scaffolds with specificity achieved through directed evolution, regularly applied in novel mAb therapeutics and increasingly promoted for immunotherapeutic solutions.

For fully synthetic nanosystems, beyond the anthropological limitations of rational design in appreciating biological complexity, are the issues of the synthetic processes which produce variable population distributions in size and ligand display, and would likely require physical selection procedures for population optimisation, with such issues improved upon using the programmable supramolecular self-assembly approach to multivalency. Another intriguing prospect remains the controlled self-assembly of native constituents. Thus "simpler" chemical displays may also be adapted for *in vivo* self-organising of multivalent targeting assemblies where "native" ligands are recruited and assembled from the surrounding media through multivalent surface chemistries. Some recent nanoparticle based examples have shown the potential of this instructed translation of chemical to biological interfaces^{133, 134}.

In discussing these aspects of synthetic multivalency for biological applications it is clear that there are many exciting routes and solutions yet to be explored.

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