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Precision Engineering of Targeted Nanocarriers

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Abstract

Since their introduction in 1980, the number of advanced targeted nanocarrier systems has grown considerably. Nanocarriers capable of targeting single receptors, multiple receptors, or multiple epitopes have all been used to enhance delivery efficiency and selectivity. Despite tremendous progress, preclinical studies and clinically translatable nanotechnology remain disconnected. The disconnect in targeting efficacy may stem from poorly-understood factors such as receptor clustering, spatial control of targeting ligands, ligand mobility, and ligand architecture. Further, the relationship between receptor distribution and ligand architecture remains elusive. Traditionally, targeted nanocarriers were engineered assuming a 'static' target. However, it is becoming increasingly clear that receptor expression patterns change in response to external stimuli and disease progression. Here, we discuss how cutting-edge technologies will enable a better characterization of the spatio-temporal distribution of membrane receptors and their clustering. We further describe how this will enable the design of new nanocarriers that selectively target the site of disease. Ultimately, we explore how the precision engineering of targeted nanocarriers that adapt to receptor dynamics will have the potential to drive nanotechnology to the forefront of therapy and make targeted nanomedicine a clinical reality.

Graphical Abstract

Keywords

Targeted nanocarriers; multi-valency; spatial control; epitopes; receptor clustering

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Introduction

Paul Ehrlich first postulated the ‘magic bullet’ theory of targeted drug delivery in 1908, proposing that cells had specific ‘side chains’ or receptors that could be used for specific targeting.¹ In 1980, the first reports emerged attaching targeting ligands to liposomes for active targeting.^{2, 3} Since then, there have been numerous attempts to use targeted nanocarriers to discriminate between diseased and healthy tissues. Although many targeted therapies such as monoclonal antibodies (*e.g.*, CD20 in lymphoma) are clinical success stories,⁴ the Food and Drug Administration (FDA) has yet to approve a targeted nanocarrier.⁵ Delivering drugs using both active targeting and nanocarriers could, however, significantly improve therapeutic efficacy and decrease toxic side effects.

Of the several thousand preclinical studies reporting the use of targeted nanocarriers, only a small number have progressed into clinical trials.⁶ Of the search results for “targeted nanoparticles” on clinicaltrials.gov, several trials investigate the previously approved, non-targeted paclitaxel albumin-stabilized nanoparticles nab-paclitaxel (Abraxane) combined with different chemotherapeutics and/or for different indications. Another study is examining the use of siRNA lipid nanoparticles decorated with vitamin A to treat fibrosis.⁷ Although the first liposomes delivering doxorubicin (Doxil®) were approved by the FDA in 1995,⁸ no targeted liposomal carrier has since been approved. Currently, a human epidermal growth factor receptor 2 (HER2)-targeting liposomal formulation delivering doxorubicin is in a phase 1 clinical trial (NCT01304797).⁹ Epidermal growth factor receptor (EGFR)-targeting minicells (bacteria-derived nanoparticles) demonstrated significant tumor growth inhibition upon miR-16 delivery [NCT02369198].¹⁰

Aside from therapeutics, targeted nanocarriers have also been developed for diagnostic purposes.¹¹ For instance, silica nanoparticles labeled with near infrared fluorophores and decorated with RGDY-targeting peptides are currently in clinical trials for melanoma and malignant brain tumor imaging [NCT02106598]. While harnessing the unique properties of nanoparticles for enhanced multivalent interactions is a promising strategy to increase target tissue selectivity, clinical translation of targeted nanocarriers seems to be challenging and has yet to produce a clinically viable product. Ligands ranging from aptamers, proteins, and small molecules have been used on nanoparticles to selectively target tissues of interest.¹² For overviews of specific targeting ligands used to engineer targeted nanocarriers, such as antibodies, antibody fragments, small molecules, peptides, and aptamers we refer readers to the review by Peer and colleagues.¹³

In this review, we discuss how receptor clustering and ligand architecture on nanoparticles can be used to increase nanocarrier accumulation and specificity.¹⁴ Receptors are commonly used as disease targets in diagnostics and therapeutics.^{15, 16} However, we currently do not have a good understanding about how receptor clustering and spatio-temporal changes affect nanoparticle binding, endocytosis, and downstream signaling. Although many carriers are designed to display multiple ligands and target multiple receptors, most lack spatial control over ligand display. Exploiting the spacing between targeting domains and/or receptors allows for controlled ligand display on spatially organized nanocarriers. This has the potential to engage targets more effectively than traditional approaches.^{17–19} Spatial control

is especially important for multiple epitope targeting because the binding of one ligand can direct and control other ligand interactions.²⁰ Ligand mobility in fluidic nanocarriers also offers a unique ability to adapt to spatio-temporal changes in receptor expression. Future research into receptor dynamics and receptor interactions is warranted to enable precision engineering of targeted nanocarriers.

Membrane proteins: the effect of receptor mobility and clustering on targeting

Membrane proteins are highly important therapeutic and diagnostic targets.²¹ Membrane proteins or receptors facilitate communication with the extracellular milieu and other cells *via* a range of ligands such as growth factors, cytokines, hormones, and small molecules. Under physiological and pathophysiological conditions, cells continuously change and often alter expression of specific membrane proteins, which can be used for targeted therapeutics or as diagnostic markers.²² For instance, malignant cells show specific membrane receptor expression patterns that produce unique “fingerprints.” Many receptors such as HER2, chemokine receptors (CXCR4, CCR2), and growth receptors are upregulated in cancer cells compared to healthy cells.^{23, 24} For example, in the case of the breast cancer oncogene HER2, clusters are different between healthy and cancerous cells and even distinguishable by density in different cancer cell lines.²⁵ HER2 is targeted clinically by trastuzumab and other agents and used as a companion diagnostic biomarker. Since HER2 expression is a known prognostic indicator, characterizing cluster density could further refine diagnosis. Cluster pattern and receptor distribution can change dynamically in diseases such as cancer and receptor distribution can also change over time as the disease progresses.²⁶ For example, in breast cancer with high HER2 expression at initiation, the expression and distribution sometimes decreases as the disease progresses.²⁷

Designing carriers that specifically target cancer cells while sparing healthy cells is key to successful, non-toxic therapy. While targeted therapeutics such as antibodies have improved treatment outcomes for many patients, they still produce off-target toxic effects or do not fully eradicate the tumor cells.²⁸ Developing more precise therapeutics could address these shortcomings, but developing a greater understanding of membrane receptors, how receptor density affects targeting, and the role of binding epitopes is important.

Receptors are generally mobile and fluid in cellular membranes.²⁹ During signaling they can localize to lipid rafts, plasma membrane domains rich in cholesterol, glycosphingolipids, and saturated phospholipids.³⁰ Cholesterol and saturated lipids are the main non-fluid components of lipid rafts and facilitate tight packing and stability, thus allowing these microdomains to “float” and move in the relatively disordered and fluid-like unsaturated lipids of the remainder of the cell membrane. Proteins and receptors are specifically organized on lipid rafts by lipid-lipid, lipid-protein, and protein-protein interactions.³¹ Folate receptors, transferrin receptors, vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and many more receptors cluster upon ligand binding (Table 1). When ligands interact with membrane receptors *via* specific epitopes, they can exert specific biological functions and affect disease progression. Further, by binding to

specific sites, the ligands can cluster the receptors in cellular membranes. For instance, death receptors 4 and 5 (DR4 and DR5, respectively) are popular cancer targets due to their role in apoptosis.³² DR4 and DR5 cluster *via* a lipid raft-mediated mechanism, and clustering significantly increases the rate of apoptosis. In one study, a combination of tumor necrosis-related apoptosis inducing ligand (TRAIL) and epirubicin increased apoptosis by over ten-fold compared to TRAIL alone because of clustering and high local death receptor density.³³ To control for receptor clustering, nystatin, a cholesterol-sequestering agent, was used to limit receptor mobility in the bilayer. Nystatin partially reversed the increased apoptosis seen with the combination therapy. It is now known that several drugs that influence DR4/5 receptor clustering in lipid rafts including cryptocaryone,³⁴ cisplatin,³⁵ and oxaliplatin,³⁶ have similar positive outcomes in combination with TRAIL.

Similar to DR4 and DR5, EGFR clusters into lattices after ligand binding, speeding up internalization, and forcing sorting into lysosomes for degradation.³⁷ Receptor clusters form higher-order arrangements and are believed to play an intricate role in regulating signaling cascades.³⁸ HER2 also exhibits faster internalization and sorting into lysosomes after forming clustered lattices.^{39–41} The size of the aggregated lattice appears to be proportional to the receptor entry rate and lysosomal shuttling.³⁷ The relationship between lattice size and degradation rate is further supported by the inability of internalized EGFR clusters to return to the cell surface.⁴² Receptor clustering is also critical to phosphorylation, kinase activity, and downstream signaling.^{17, 43} Cluster-induced signaling leads to other biological activities such as cytoskeletal rearrangement, cellular migration, and extravasation.^{44–47} T-cell recognition is enhanced by major histocompatibility complex (MHC) class I clustering because it improves peptide presentation.⁴⁸ Thus, exploiting these principles in an orchestrated multivalent targeting strategy that uses receptor clustering is likely to control several cellular functions including processes such as apoptosis for therapeutic benefit.

Most receptors including the folate receptor, transferrin receptor, cell adhesion molecule (CAM) receptors, growth factor receptors, and chemokine receptors will homo-cluster, creating a high local receptor density and controlled activation for cell signaling (Figure 1).^{49–51} For instance, ligand binding to VEGFR causes a positive feedback loop between clustering and downstream signaling.⁵² The ligand effect on VEGFR clusters enhances the sensitivity compared to spatially homogenous VEGFR distributions. Thus, clustering facilitates activation and signaling by organizing receptors into ligand-accessible patterns on the cell surface.

Other receptors require co-clustering, typically in lipid rafts, to initiate an effect. Notable examples include VCAM-1 and ICAM-1 for leukocyte adhesion,⁴⁷ CD4 and the T cell receptor (TCR) for kinase recruitment,⁴³ lectin receptors for NF- κ B signaling,⁵³ and somatostatin receptors (SSTRs) to control cellular trafficking.⁵⁴ CD14 co-clusters are especially important in monocytes because they control several overlapping signaling pathways, including inflammatory responses, gene expression, growth regulation/apoptosis, nitric oxide (NO) production, cytoskeletal re-arrangement, and cellular migration.⁵⁵ Signaling is dependent on monocyte stimulation, and co-clustering with receptors such as CD47a, CD32, CD55, and CD64 (unstimulated) or CD36, CD16, CD81, and CD11b (stimulated) affects downstream signaling. However not all CDs required co-clustering for

signaling. For instance, binding of multivalent hyaluronic acid to CD44 homo-clusters mediates embryogenesis, hematopoiesis, lymphocyte activation, and inflammatory reactions.⁴⁴

Leukocyte recognition, adhesion, and rolling along the endothelial surface is a function of the spatial distribution of selectin targeting ligands and have become a focus for targeted drug therapies.⁸⁴ For example, lateral mobility of the ligand sialyl LewisX (sLex), enhances binding interactions with selectin clusters.⁶² Parameters such as ligand flexibility and linker length also influenced selectin binding and leukocyte rolling.⁸⁵ For instance, lipid biofilms decorated with 0.1 mol% sLex ligands connected through a short linker did not mediate cell adhesion and rolling. Increasing the linker length, however, mediated cell adhesion and rolling at concentrations as low as 0.001 mol%. When comparable linker lengths with differing flexibility were tested, no noticeable differences in cell adhesion and rolling were observed. The authors concluded that linker length and flexibility of ligand were important parameters to engage selectin clusters and mediate cell rolling. The results of these studies could be pertinent in designing cell-mimicking particles. These will be capable of migrating and rolling to the site of inflammation as effectively as leukocytes do.

Viruses such as human immunodeficiency virus type 1 (HIV-1) have long exploited receptor clustering, using the envelope glycoprotein gp-120 to induce clustering of CD4 and its co-receptors (CCR5 or CXCR4) for internalization.⁸⁶ For several decades, nanoparticles have been designed to mimic viruses to improve cellular uptake. Similar to viruses, nanoparticles can be designed for multivalent display, which has the potential to further improve receptor clustering compared to free ligands. For example, with the adhesion markers ICAM-1 and PECAM-1, monomeric antibodies were poorly internalized compared to multimeric immuno-beads, which were completely internalized after 30 minutes.⁵⁸ Multivalency also plays an important role in targeting ASGPR. It has been shown that the number of galactose residues displayed on a ligand significantly affects ASGPR binding: synthetic oligosaccharides displaying galactose-residues in tetra-antennary-like structures much more effectively inhibited the binding of a natural ligand to ASGPR than tri-antennary, bi-antennary, and mono-antennary structures.⁸⁷ In a different application, increased galactose valency translated into improved ASGPR targeting, recognition, and internalization.⁶⁷ Sixty-fold more liposomes were internalized with 7.5 mol% of galactose compared to formulations with less than 2.5 mol% galactose. Similar results were observed with N-acetylgalactosamine (GalNAc), a tri-antennary ASGPR ligand that demonstrated improved uptake and promising results in clinic trials for siRNA delivery.⁸⁸ Multivalent carbohydrates are commonly used in nature to form lectin clusters and improve specificity and cellular recognition.^{89, 90} Multivalent carbohydrates are superior in these cases, because binding interactions can be over 50,000-fold stronger than a monovalent ligand.

Thus, designing (a) nanocarriers that dynamically respond to spatiotemporal changes in receptor distribution on cellular membranes or (b) nanocarriers that can spatially capture the receptors on cellular membranes could further enhance selectivity and efficiency. These approaches are discussed in more detail below.

Nanocarriers – platforms for ligand architecture and spatial organization

Nanocarriers provide a highly promising and versatile platform for the display of multivalent and multifunctional ligands by providing ligand architecture and geometrically enhanced multivalent avidity. Ligand density, ligand ratio, and patterning are all parameters that can be controlled to varying degrees by different nanocarrier systems that influence targeting efficiency and specificity.⁹¹ It is well known that ligand density on nanoparticle surfaces can favorably affect binding, indicating that effective ligand engagement with receptors has a spatial component.¹⁸ Further, ligand density is thought to influence cellular binding and also the extent and rate of nanoparticle internalization.⁹² Further, nanomaterials can also function as scaffolds to provide spatial control of multiple ligands. These include, but are not limited to, DNA scaffolds,¹⁷ dendrimers,⁹³ viruses,⁹⁴ protein capsids and cages.^{95, 96}

Enhancing selectivity and specificity through multivalency

Multiple ligands on nanoparticles mediate polyvalent interactions (avidity) that are typically orders of magnitude stronger than free ligands.²⁰ This not only has the potential to enhance binding affinity to cellular targets but also maximizes selectivity for ubiquitously expressed targets. A target overexpressed in diseased tissue or a tissue of interest is often also present at low levels in other tissues throughout the body. Thus, superselective carriers that bind to target cells based on the binding site density are urgently needed.

In this regard, many nanocarriers have been engineered that display multiple surface ligands. These include polymeric, lipidic, and protein-based nanocarriers.⁹⁷ Selective approaches are urgently needed for chemotherapeutics and other biotherapeutics due to off-site toxicity issues caused by free ligand binding to other tissues with low-level target expression. Mathematical modeling and experimental results have recently supported the use of multivalent strategies for drug delivery.

Using a computational and experimental approach, Martinez-Veracoechea and Frenkel established that the number of receptor-target interactions increases non-linearly with receptor coverage in a multivalent system.⁹⁸ In their multivalent system, binding was saturated above a certain receptor threshold, whereas there was no binding with low receptor numbers. Superselective targeting was achieved with drug delivery carriers displaying the optimal number of targeting ligands. There was an inverse relationship between selectivity and binding strength: the stronger the binding affinity, the greater the decrease in selectivity, meaning that selectivity did not improve with increased binding affinity. Another worthwhile observation from their mathematical model was that little or no multivalent nanoparticles bound at low numbers of membrane receptors. The relationship between bound particles and the number of receptors was sigmoidal such that, as the number of receptors increased, the number of bound particles also increased significantly. The steepness of the slope between number of receptors and bound nanoparticles was similar to an on-off switch. Model performance was validated with Monte Carlo simulations, which suggested that the model accurately described “super” selective multivalent particle binding.

To confirm their predictions experimentally, the authors designed a model system in which receptors were attached to multivalent polymers and ligands were displayed on surfaces. Ligand density was systematically varied, and the effects of binding affinity, polymer valency, polymer linker and polymer concentration on super-selectivity were assessed.⁹⁹ Hyaluronic acid- β -cyclodextrin derivatives were capable of distinguishing between surfaces with different ligand densities. By adjusting either (a) the polymer linker, (b) the receptor/ligand affinity, or (c) the valency of the polymeric systems the authors could modulate the binding affinity by one order of magnitude.

In line with these observations, a minimum number of ligands (nine in this specific example) on PAMAM dendrimer-based nanoparticles were required for high-avidity interactions, and this threshold number was found to be important for slow target dissociation depending on the number of receptors.¹⁰⁰ The rate-determining factor for avidity in the multivalent system was determined to be the number of ligands per particle: below the ligand valency threshold, the avidity approached the affinity of a single ligand-target pair. Above this threshold, the avidity strength was robust to changes in ligand number.¹⁰¹ It is clear that multivalency contributes to achieving selective and specific targeted nanocarriers.

Multiple-receptor targeting and the effects of ligand ratios

Multi-receptor targeting has been used to target different cell types within the same tissue or the same cell type displaying heterogeneous membrane receptor expression patterns. By targeting receptors that are simultaneously expressed on cells and tissues of interest, delivery selectivity and specificity can be enhanced. Further, multi-receptor targeting could be useful in cases in which receptor expression changes with time as the disease progresses.¹⁰²

Several studies have demonstrated that dual-targeted nanoparticles show enhanced and selective targeting of cells of interest. For example, dual-targeting liposomes against folate receptors and EGFR displayed superior selectivity compared to single ligand controls by both reducing target cell viability and avoiding off-target cells.¹⁰³ Similarly, dual CD44 and folate receptor-targeting hyaluronic acid-ceramide (HACE) nanoparticles accumulated at approximately five-fold higher levels in a SKOV-3 ovarian cancer xenograft mouse model than mono-targeted HACE-based nanoparticles.¹⁰⁴ Dual CD44 and folate receptor targeting nanoparticles also increased cellular uptake into SKOV-3 cells compared to mono-targeted nanoparticles.

Using a combination of *in silico* predictions and experimental studies, Adefeso and colleagues not only showed that dual-targeting particles equipped with ligands binding to sialyl Lewis A (sLe) and anti-intercellular adhesion molecule-1 (ICAM-1) displayed approximately three- to seven-fold better attachment to the endothelium but also that the ligand ratio was important.¹⁰² They found that binding of particles with different ligand ratios was affected by receptor expression and that the ligand targeting the lower expressed receptor needed to be present at a higher ratio. Specifically, since ICAM-1 showed greater endothelial expression than sLe, particles with a sLe/ICAM ratio of 75:25 had the highest binding. Conversely, when cells had low ICAM-1 expression but high sLe expression, particles with a sLe/ICAM ratio of 25/75 had the highest binding. Ligand ratios are clearly

important for the efficacy of targeted carriers. Further, as diseases progress, cellular status, gene expression, and receptor expression may change.²² Thus, one size may not fit all, and ligand ratios may need to be adjusted throughout the natural course of the disease.

In a study targeting the VCAM-1 and E-selectin adhesion molecules, the authors assessed the uptake of VCAM-1 and E-selectin-targeting liposome uptake into endothelial cells in response to spatiotemporal receptor expression alterations. Endothelial VCAM-1 and E-selectin expression was quantified by flow cytometry before and after TNF- α and IL-1 α activation. VCAM-1 and E-selectin expression was dynamic and changed over 24 h, with VCAM-1 showing the highest expression at 24 h and E-selectin peaking at 6 h. The authors tested different ratios of VCAM and E-selectin antibodies attached to liposomes and found that a 1:1 ratio resulted in a 2.5-fold increase in binding over the other ratios tested (1:0, 1:4, 1:8, and 0:1) at both 6 h and 24 h after activation. While with the 1:1 ratio was determined as optimal, it would also be interesting to assess if the binding affinities of the antibodies affects the optimal ratio.¹⁰⁵ These studies showed that by modulating ligands and ligand ratios it is possible to harness differences in spatial organization of target molecules to improve binding and specificity.

Effect of spatial control on targeting

Spatial control of targeting ligands has emerged as an approach to enhance selectivity. A spatially informed ligand design on nanocarriers could allow for more effective target engagement. Also in the case of multiple epitope targeting, the binding of the first ligand to the target will favor the second binding interaction because of spatial proximity. Designing multivalent systems with ligands in a spatially controlled space has tremendous benefits for selectivity and activity.

DNA scaffolds provide precise spatial control over ligand display

Using DNA as a scaffold for ligand attachment and spatially controlled display is appealing because of unmatched precision for site-specific attachment. Over the last three decades, “DNA origami” has evolved as a useful tool for spatial arrangement of targeting ligands or nanoparticles. In one example, DNA origami was used to anchor viral capsids with high spatial accuracy by attaching DNA strands to the exterior of viral capsids *via* unnatural amino acids.¹⁰⁶ The complementary single-strand DNA positioned on DNA origami tiles anchored the capsids at precise locations 100 nm apart. Rinker *et al.* explored another application of DNA tiles and attached aptamers targeting two external sites of thrombin to DNA tiles for ligand capture.¹⁰⁷ The distance between aptamers was varied and precisely controlled by anchoring them to different DNA helix bundles tiles, creating bivalent binding motifs. Compared with bivalent modified scaffolds with aptamers at distances greater/shorter than that of thrombin, the bivalent motif with aptamer separation of 5.3 nm (the width of one thrombin molecule) bound the most thrombin (>40%) compared to ~25% for the shorter and longer spaced constructs (Figure 2 A & B).¹⁰⁷ The bivalent system also outperformed the monovalent-modified scaffold, capturing about 12-fold more protein than a monovalent DNA origami tile. This was one of the first examples of using DNA origami engineered for spatial control of ligand display, and it emphasized the importance of *a priori*

knowledge of the distance between target domains to optimize the targeting system. This spatially organized design strategy could be extrapolated to many other applications from biosensors to drug delivery.

EphA2 is activated by the membrane-tethered ligand ephrin A1. EphA2 acts as a cell adhesion molecule and as a tumor suppressor by inhibiting invasion and metastasis in breast cancer.¹⁷ Ephrin A1 monomers in solution were unable to activate EphA2 unless they were displayed in a pattern on an artificial membrane scaffold. Ligand clustering therefore appears to be a prerequisite for EphA2 activation. Shaw *et al.* took advantage of DNA origami to precisely present ephrin A1 and successfully targeted and activated EphA2.¹⁷ They also prepared monovalent or bivalent scaffolds with separation distances of 14.3, 42.9, or 100 nm. The relative binding signal per cell was highest and the relative invasion was decreased by about four-fold when bivalent ephrin A1 was separated by 42.9 nm (compared to empty control), indicating that spatial control of ephrin A1 is a pertinent factor in EphA2 binding and activity. Spatial organization using the DNA origami scaffold approach demonstrated its utility and potential for use as a therapeutic agent against metastasis.

Ke *et al.* synthesized a DNA origami scaffold that controlled ligand distance by taking advantage of mechanical forces transmitted by the DNA helices and the flexibility of single-stranded DNA hinges.¹⁰⁸ By altering the length of DNA staples, the size and conformation of the nanoactuator could be precisely controlled. By modulating the geometry and shape, the DNA origami scaffold was devised to sense changes in buffer composition, respond to enzymatic activation, and to selectively detect microRNAs. Distance-sensing DNA origami could be crucial for studying ligand-receptor interaction mechanisms and has potential for customizing therapeutics such as for drug targeting and signaling pathway interference.

Ligand geometry has also been shown to be relevant for single ligand applications. Lee *et al.* used oligonucleotide nanoparticles (ONPs) shaped as tetrahedrons and decorated with folate to show that ligand orientation and geometry influenced gene silencing (in this case of green fluorescent protein, GFP).¹⁰⁹ DNA base pairing of the ONPs allowed precise positioning of targeting ligands on either the face, vertex, or along the side of the ONPs to investigate the impact of inter-ligand distance and geometry. Orientating the folate molecules on either the face or the vertex of the tetrahedron particle decreased GFP expression by 50%, while there was no knockdown when folate molecules were placed along the side. The sophisticated control of spatial organization and geometry offered by the tetrahedron ONPs was vital for targeting and gene silencing.

DNA origami has demonstrated its utility in a new field of multivalent ligand display where spatial control is required. The exciting ability to control scaffolds, ligands, and particles at nanoscale resolution *via* DNA origami is very appealing for applications in bio-sensing, imaging, cell-cell communications, and receptor targeting.

Spatial control using a small molecule scaffold

Three small molecule trigonal scaffolds based on phloroglucinol, tripropargylamine, and 1,4,7-triazacyclononane were used to control ligand distance and geometry when targeting the melanocortin receptor.¹⁹ MC4R melanocortin receptor forms dimers or asymmetric

trimers on the cell surface and thus was an ideal choice for a spatially controlled multivalent targeting strategy. Divalent display with these scaffold resulted in 10- to 30-fold inhibition of signaling compared to monovalent display, and a trivalent display resulted in an additional two-fold inhibition. In each case, an increase in valency resulted in a significant improvement in the levels of inhibition. Furthermore, a distance of 17 to 23 Å between ligands was found to be optimal for inducing multimerization and binding.

Triarylpyridines (TAPs) scaffolds have three rotatable bonds that were used to target secondary structures of guanine-rich domains in DNA helixes called G-quadruplexes.¹¹⁰ By changing the ligands at the end of the three rotatable bonds, the authors were able to selectively target quadruplex DNA rather than duplex DNA. The spatial arrangement and flexibility of the three ligands resulted in a high binding affinity ($K_d = 180$ nM). Small molecules offer great advantages as a scaffold because of their essentially limitless structures and three-dimensionality. Libraries of small molecules with unique three-dimensionality have been shown to be synthesized in a facile method.¹¹¹ The emergence of spatial control in drug targeting and the ability to control small molecule scaffold with exquisite spatial control has the potential for selective drug targeting in biological systems.

Potential applications of protein cages as platforms for spatially controlled ligand display

Self-assembled protein structures also have potential as platforms for spatially controlled ligand display. Protein subunits or monomers can assemble into nanoscale cage-like structures *via* electrostatic, hydrophobic, and van der Waals attractive forces.¹¹² Due to their highly geometric spatial organization ligand distance can be finely controlled. Further, protein cages can be modified at their exterior surface, interior surface, and their interfaces. Ligands can be attached to the exterior surface by epitope recognition, chemical modifications, or genetic fusion.^{113, 114} Thus, the exterior surface is ideal for anchoring ligands for cell targeting.

Using molecular epitope recognition, we recently demonstrated the utility of clathrin triskelions, the building block for clathrin cages, as carriers for spatially-controlled multi-protein display.⁹⁵ Fluorescent proteins genetically fused to a clathrin binding peptide (CBP) were attached to clathrin in a site-specific and spatially organized design *via* molecular epitope binding. The local proximity of the simultaneously attached fluorescent proteins was confirmed by Förster resonance energy transfer (FRET). The ability to display two proteins within a biologically relevant distance (~ 10 Å) provides the ability to target multiple epitopes, or receptors or function as a biosensor. Clathrin triskelions and clathrin cages are ideal scaffolds for controlling spatial organization by molecular epitope recognition, and their higher-order architecture also provides future scope for increasing loading capacity, improving specificity, and exquisitely controlling ligand placement.

Ferritin is composed of 24 subunits that self-assemble into 12 nm cages. The size of the cages can be increased to 16 nm (48 subunits) or decreased to 10 nm (16 subunits) by genetic manipulation.^{115, 116} These different sizes may be favorable depending on the intended application. Ferritin cages have been used as polyvalent nano-platforms to display monosaccharides, attached at defined sites by thiol-maleimide Michael-type addition for cell targeting.¹¹⁷ A mannose or a galactose derivative was used to target cell surface lectins on

HepG2 cells. Further, the ability of ferritin monomers to reassemble at physiological pH has been exploited to create hybrid cages with customizable ratios of a genetically fused arginylglycylaspartic acid (RGD) peptide and a chemically conjugated dye (Cy5.5).¹¹⁸ Of note, the authors also explored the ability of heavy metal cations, in this case ⁶⁴Cu, to bind at well-conserved metal binding sites on the interior of the ferritin cage. The result was a multifunctional delivery vehicle that could target tumor vasculature and act as an imaging agent for positron emission tomography (PET) and near-infrared fluorescence (NIRF) imaging. It would be interesting to display two targeting ligands simultaneously and with spatial control in an effort to enhance selectivity for the target tissue.

Viral capsids are another promising class of protein cages suitable for spatially defined multivalent display due to their ability to self-assemble and form geometric shapes (Figure 2 C & D).^{119, 120} Capsid size and shape can be controlled by changing buffer properties such as ionic strength and pH. For instance, assembling a permutant TMV capsid at acidic pH promotes rod-like assembly, whereas a neutral pH favors a disk conformation.^{121–123} The ability to control capsid shape and geometry is quite promising for multivalent presentation.

Genetic fusion, specific protein-protein interactions, and click chemistry have all been used for organized ligand display on virus-like particles (VLPs). Strable *et al.* demonstrated site-specific incorporation of unnatural amino acids on the hepatitis B virus (HBV).¹²⁴ Copper-catalyzed [3 + 2] cycloaddition was used to attach 120 triazole-linked small molecules in a spatially organized manner on the protein cage without affecting intrinsic particle stability. Expanding on this technology, Patel *et al.* used a global replacement scheme on VLPs to introduce methionine analogues (azidohomoalanine and homopropargylglycine) that contain azide and alkyne side chains to the VLP for direct attachment of ligands *via* click chemistry. The VLPs simultaneously displayed three ligands: an antibody fragment fusion (50 molecules per VLP), CpG DNA oligonucleotides (20 molecules per VLP), and granulocyte-macrophage colony stimulating factor (GM-CSF; 6 molecules per VLP).¹²⁵ This demonstrated the potential of VLPs for spatially controlled multifunctional display of three distinct ligand classes: proteins, small molecules, and nucleic acids. Another way to control ligand ratio on VLPs is to mix the subunit proteins from a cowpea chlorotic mottle virus (CCMV) capsid with different modifications (either biotin or digoxigenin) and then combine the differently modified subunits into one batch to form capsids with the distinct modifications.¹²⁶ By modulating subunit stoichiometry, this method achieved controlled density to a certain degree. However, the drawback of this approach was that the subunits incorporated randomly so spatial control between ligands was not achievable.

Dendrimers

Dendrimer generations and branching offer controllable attachment points to explore spatial control. In one example, polyphenylene branching was controlled to create dendrimers with hydrophobic and hydrophilic surface group patterns.¹²⁷ The hydrophilic and hydrophobic regions made for unique and tunable particle solubility, which the authors speculated would directly impact cell interactions and toxicity. The ability to control the spatial patterns offered by dendrimer branching lays the foundation for multivalent display of targeting ligands. In another example, GM1 ligands attached to dendrimers in a spatially organized

pattern were significantly more effective at inhibiting cholera toxin binding than free GM1 ligands (5- to 15-fold lower concentrations were required).⁹³ This was due to the fact that the display of GM1 on branched dendrimers mirrored the clustered arrangement of GM1 on cellular membranes more effectively than free ligands.¹²⁸

DNA dendrimer particles are of particular interest in spatial ligand control because of their tunable design. An acetylcholine bio-sensing probe was designed using evenly spaced enzymes and fluorescent probes on the dendrimer branches.¹²⁹ The local proximity of the enzyme and probe was utilized to create a local pH drop to activate a pH-sensitive fluorescent indicator in the presence of acetylcholine. Although in this application targeting was not investigated, this technology can be translated into a delivery strategy.

Effect of membrane fluidity in receptor/ligand interactions

While the movement of the majority of targeting ligands on nanocarrier surfaces is mostly restrained, liposomes have lipid bilayer mobility that mimics receptor mobility on cellular membranes. Liposomes were one of the first nanocarriers to be surface decorated with ligands to target specific cells.^{2, 3} The unique ability of lipids to diffuse and rearrange can be exploited to overcome asynchronous binding and other shortcomings in targeting observed with solid nanoparticles. The impact of bilayer fluidity, whether at the cell membrane or the surface of a liposome, is discussed in this section in relation to how ligand and target fluidity may favorably improve multiple epitope targeting.

To elucidate the impact of membrane fluidity and nanoparticle fluidity in targeting, Gunawan *et al.* prepared two different liposome formulations using 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), an unsaturated lipid with a phase transition temperature of $T_m = -17^\circ\text{C}$, and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), a saturated lipid with a phase transition temperature of $T_m = 41^\circ\text{C}$, to investigate how these two formulations would impact targeting of ICAM and E-selectin on endothelial cells.¹³⁰ Notably, in the presence of lipid rafts, the more fluid DOPC formulation increased liposome binding three-fold, most likely as a result of lateral mobility of the antibody within the bilayer.¹³⁰ Upon inhibition of lipid raft formation in cellular membranes by chelating membrane cholesterol with methyl- β -cyclodextrin,¹³¹ both the DOPC and DPPC liposomes bound to the same extent. The change in binding of the liposomes to the cell membrane before and after lipid raft depletion indicates a pivotal role for these microdomains in cellular targeting. It is important to also consider that receptor complexes exhibit decreased diffusion in biological membranes compared to single receptors and that receptor density is a regulator of diffusion rates in cellular membranes.¹³²

Fluidic particles with attached targeting ligands have been studied using nanoparticles called protocells. Protocells are lipid bilayers supported by a porous silica core, and they are versatile and customizable for many delivery strategies.¹³³ One attribute of protocells is their high membrane fluidity. The proposed enhanced membrane fluidity is advantageous for multi-domain targeting because of the kinetically favorable ligand movements within the fluidic bilayer, which allow adequate ligand-target interactions *via* ligand clustering. A schematic of ligand mobility is shown in Figure 3, in which targeting ligand 1 and ligand 2

can diffuse from their 'starting' position towards a region where they interact with receptor A and receptor B, respectively. Enhanced lateral diffusion of targeting ligands has also been observed in liposomes. A gel-phase liposome composed of DSPC and fluid-phase liposomes composed of DOPC were compared, and binding of peptide-functionalized liposomes to anthrax toxin receptor was markedly better for the DOPC-containing liposomes.¹³⁴ The authors reasoned that enhanced lateral diffusion offered by the DOPC liposomes provided a ligand arrangement complementary to that of the binding sites of the target receptor and caused a decrease in the IC₅₀ by greater than 100-fold.¹³⁴

Receptor dimerization is a pivotal step in cellular signaling, making the disruption and/or induction of dimerization an appealing drug target.^{135, 136} Both experimental and computational models have shown that lipid rafts play a role in dimerization and signaling.¹³⁷ Inspired by the ability of lipid rafts to influence receptor clustering and signaling, Rai *et al.* designed liposomes with gel- and fluid-phase lipid ratios mimicking microdomains on cellular membranes.¹³⁸ The IC₅₀ was reduced over 50-fold by using gel- and fluid-phase lipids in the liposome compared to solely gel-phase liposomes. The designed liposomes formed microdomains due to phase separation (confirmed by FRET) that mimicked lipid rafts. Multivalency has also been suggested to be a driver of receptor clustering in some scenarios. A multicomponent system with a 1,3,5-benzenetricarboxamide derivative (BTA) polymer scaffold, BTA monomers, and multivalent single-stranded DNA, when mixed in solution, defined the spatiotemporal distribution of the target monomer along the polymer by exploiting multivalent clustering.¹³⁹ Since target proteins are mobile in the lipid bilayer, this study suggested that multivalency can be used to recruit and cluster receptors. Targeting strategies that control the spatial arrangement of ligands may have the ability to target and/or prevent receptor dimerization by exploiting the colocalization of receptors in lipid rafts. As more studies explore multiple epitope targeting and spatial control, application of these novel strategies to modify receptor clustering could be of great benefit in pre-clinical and clinical studies.^{127, 129}

Several studies have attributed enhanced activity and/or selectivity to pattern matching.^{105, 130, 133, 134, 138, 140–143} Liposomes are very similar to cells, so they provide a promising platform to implement ligand-target pattern matching.^{105, 141} In addition to experimental data, pattern matching has also been described by several theoretical and mathematical models.^{144–146} Further, simulations have shown that pattern matching is superior to an "unmatched" pattern for biomimetic recognition.¹⁴⁷ The models and simulations tend to agree that the free energy is minimized for multiple interactions between a protein pattern and the target surface (by overcoming the entropic penalty for binding), binding affinity is highly dependent on the target arrangement on a surface, and the binding interaction between ligand and target attempt to maximize the number of contact points. These factors are satisfied in conditions where the ligand-target are matched, giving merit to strategies that aim to organize ligands in specific patterns and orientations. The techniques discussed in this review such as modulating membrane fluidity and applying spatial organization using nanocarrier scaffolds are innovative approaches to ligand-target matching. Further studies on fluidic particles and geometric scaffolds may provide the opportunity to mimic nature's recognition system and create highly specific biotherapeutic delivery strategies.

Quantifying receptor density and spatial distribution of receptors and epitopes

As well as studying and developing design guidelines for multivalent particles, it is also important to accurately quantify receptor density and characterize the spatial distribution of receptors and epitopes on cell membranes. Methods and approaches to accurately and precisely quantify the distance between receptors on cell membranes exist but are not widely utilized. The spatiotemporal changes in cell membranes associated with cellular function and homeostasis further complicate accurate quantification and must be addressed for successful implementation of multi-epitope and spatially controlled targeting strategies.

Using dual-color photoactivated localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM), Davis and colleagues determined the spatial distribution of CD4 in relation to TCR and the active form of Src kinase p56lck (Lck) before and after T cell activation.⁴³ Through statistical analyses, dual-color imaging allowed spatial determination of CD4 to within 20–50 nm. The authors found that, in non-activated states, CD4, TCR, and Lck localized in isolated clusters without much interaction. However, after T cell activation, the TCR and CD4 rearranged, formed microclusters, and assembled into supramolecular activation clusters. Receptor distribution on cellular membranes can therefore be highly dynamic depending on the cell's state. Another notable example is EGFR clusters responding to cell polarity (also visualized with dSTORM).⁷⁵ Spectral precision distance/position determination microscopy demonstrated that three different cell lines varied significantly in the amount and mean diameter of HER2 clusters.²⁵ Thus, an ideal nanocarrier should be able to dynamically respond to changes in cell membrane receptors.

Other methods to determine the spatial distribution of membrane receptors and their respective subunits involve atomic force microscopy (AFM). To characterize the glycine receptor (GlyR) in terms of ligand-binding sites and its stoichiometry, Yang *et al.* labeled the $\alpha 1$ and β subunits with FLAG and His-tag epitopes for detection with antibodies by AFM imaging.¹⁵⁰ Using this technique, they identified the subunit stoichiometry as $2\alpha 1:3\beta$. This information will be very useful in the analysis and development of drugs designed to target the subunits.

Fluorophore localization imaging with photobleaching has been applied to quantify EGFR dimerization events. This strategy has a resolving power of 10–50 nm and requires low-level receptor expression, limiting its practicality.¹⁵¹ Nevertheless, the approach was able to measure EGFR dimers (8 nm) and EGFR pentamers (59 nm).

Recent advances in energy transfer techniques have resulted in the development of nanometal surface energy transfer (NSET), which uses similar dipole-dipole interactions as FRET. However, NSET uses a luminescent donor molecule and a metallic nanoparticle surface as an acceptor to increase energy transfer efficiency.¹⁵² Since the distance determined by NSET is inversely proportional to the 4th power of the separation distance, and FRET is inversely proportional to the 6th power of the separation distance, reports of distances in the order of 40 nm have been suggested using NSET.¹⁵³

Similar to analyzing spatial distributions of receptors on cell membranes, it is also important to determine the distance between epitopes of membrane receptors for the purpose of multi-epitope targeting. PTK7 is a membrane-bound receptor with two binding sites in the extracellular domain, and it is highly expressed in colon cancer, making it an ideal target for multiple epitope targeting.¹⁵⁴ However, constructing a nanocarrier directed against these domains has proven difficult without information about proximity. Chen *et al.* used an aptamer and an antibody directed against the respective domains to measure the distance between these two sites using NSET. Using an Alexa-488-anti-PTK7 monoclonal antibody and aptamer-functionalized gold nanoparticles, the authors demonstrated that the two sites are 13.4 nm apart.^{154, 155} NSET is useful for the accurate quantification of two binding sites on live cells, which is tremendously beneficial for informing targeting strategies for countless biological systems. Applications of these methods to characterize and determine the distance between membrane receptors would be highly useful for the rational design of carriers with improved targeting and binding specificity.

Summary and Perspective

Although nanocarriers have undergone tremendous developments in recent decades there is currently no approved targeted nanotherapeutic agent. The challenges in clinical implementation can in part be attributed to deficient knowledge about the fundamental and underlying mechanisms governing nanocarrier-target interactions such as spatial proximity, ligand mobility, targeted epitopes, and ligand density. A more thorough understanding of the spatial organization of ligands will aid the development of nanocarriers that are less toxic and more efficacious. Therefore, we believe the following developments will be necessary to develop clinically viable, targeted nanotherapeutics:

- Techniques are in place to study the spatial distribution of receptors and their respective epitopes on cellular membranes. However, their use is still limited because they require specific expertise and equipment. Expansion of studies that characterize the spatial distribution of receptors and epitopes and their spatio-temporal expression as a function of triggers could vastly improve our understanding of drug targeting and aid in the design of targeted carriers.
- Receptors can cluster with the same or different receptors in cell membranes. Homo- and hetero-clustering can affect endocytosis and downstream signaling. To design specific and effective targeted nanocarriers, the phenomenon of receptor clustering between the same and different receptors must be fully characterized.
- The binding domains targeted should be precisely defined and well characterized. Although binding domains can consist of several epitopes with distinct functions, they are often not well distinguished. Any ambiguities with regard to targeting receptors should be eliminated.
- For multiple epitope or multiple receptor targeting to be successful, precise spatial control of ligands is necessary to guarantee the most effective engagement between the nanocarrier and receptors or proteins in general.

- While spatial control of targeting ligands has been shown to be important for effective targeting, receptor density on cell membrane can change due to disease progression or membrane clustering upon triggering cues. In those cases, having nanocarriers that can adapt to the changes in the spatial distribution of receptors would be highly valuable.
- Mathematical models to explore the advantages of spatial control with multiple receptor and multiple epitope targeting could aid in the development of targeted nanocarriers.
- The engineering of targeted nanocarriers can lead to heterogeneous particle populations with varying ligand densities. Thus, some subpopulations may be more effective than others. It is important to synthesize nanocarriers in such a way that ensures well-characterized nanoparticles in terms of ligand density.

In conclusion, precisely engineered nanotherapeutics have the potential to decrease off-site target effects by increasing delivery efficiency and selectivity.

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Figure 1.
Nanoparticle-mediated receptor clustering affects endocytosis and downstream signaling. Clustering can be mediated between the same receptors (homo-clustering) or different receptors (hetero-clustering).

Figure 2.

(A,B) DNA scaffold displaying two thrombin binding aptamers at varying distances. (B) Optimal thrombin binding is achieved when the distance between both aptamers is 5.3 nm. Image adapted from Rinker and colleagues.¹⁰⁷ (C,D) Spatially controlled display of targeting ligands on virus-like capsids. The distance between the targeting ligands can be controlled through epitope targeting or genetic fusion and affects binding to cell membrane receptors. PDB code for virus particle: 3J40.

Figure 3. Membrane fluidity allows clustering of targeting ligand 1 and 2 on fluid particles to respond to their respective receptor targets on cellular membranes. This can result in higher affinity and enhanced interactions with membrane receptors.

Table 1

Effects of homo- and hetero-clustering on endocytosis, phosphorylation, kinase activity, signaling, and other downstream effects.

Receptor	Cluster Formation	Endocytosis Effect	Other Effects
Folate receptor	Homo-clustering	No enhanced endocytosis upon receptor clustering with multivalent folate conjugates versus monovalent ones ⁵⁶	N/A
Transferrin receptor	Homo-clustering	1.45-fold ↑ ^{49, 50}	Promotes clathrin-coated pit formation ⁵⁰
VCAM-1	Homo-clustering Co-clustering with ICAM-1	Cross-linked VCAM-1 molecules internalize with a half-life of 14.5 mins ⁵⁷	Adhesion and extravasation of leukocytes ↑ ⁴⁷
ICAM-1	Homo-clustering Co-clustering with VCAM-1	Clustering required for internalization ⁵⁸	Adhesion and extravasation of leukocytes ↑ ^{18, 58}
PECAM-1	Homo-clustering	Clustering required for internalization ⁵⁸	N/A
Death Receptor (DR)	Hetero-clustering of DR4 and DR5.	80% of ligand mediated DR4/5 clusters are internalized by 30 mins ^{59, 60}	Rate of apoptosis ↑ ³³
E-Selectin	Homo-clustering	Clustering occurs in lipid rafts prior to uptake. Disrupting lipid rafts prevents clustering ⁶¹	Clustering in lipid rafts and clathrin-coated pits enhances recognition, adhesion, and rolling of leukocytes ↑ ^{61, 62}
CD4	Homo-clustering Co-clustering with TCR	N/A	Recruitment of TCR in microclusters after T-cell activation ⁴³
CD14	Co-clustering with CD32, CD47, CD55, CD64, and others	N/A	Receptor cluster composition dictates cellular response ⁵⁵
CD44	Homo-clustering	Clustering promotes uptake of hyaluronic acid and phagocytosis of apoptotic cells ^{63–65}	Actin cytoskeleton rearrangement ⁴⁴
LRP1	Homo-clustering	Internalization does not occur without clustering ⁶⁶ (*delete Ghosh; 67)	N/A
ASGPR	Homo-clustering	~60-fold ↑ in uptake of liposomes with optimal targeting ligand density (7.5%) ⁶⁷	N/A
Integrins	Hetero-clustering of α and β subunits. Co-clustering with ligands, talin, PI(4,5)P ₂ , and JAM-A	Different hetero-clusters have distinct internalization kinetics (i.e. α6β1 and α6β4 internalizes 3-fold faster than α3β1) ⁶⁸	Cell motility ↑ ^{69–71}
EphA2 receptor	Homo-clustering	N/A	Phosphorylation and kinase activity ↑ ^{17, 72}
VEGFR	Homo-clustering Co-clustering with Neuropilin-1	Ligand binding induces dimerization, internalization, and subsequent downregulation of VEGFR ⁷³	Phosphorylation and kinase activity ↑ ⁵²
EGFR	Homo-clustering Co-clustering with HER2	Internalized homo-clusters unable to recycle back to cell surface ⁴²	EGFR activation and downstream signaling ↑ ^{74, 75}
HER2	Co-clustering with EGFR	~60-fold ↑ when large mAb-receptor complexes were formed ³⁷	N/A
SSTR	Homo-clustering Co-clustering of SSTR2 and SSTR5	SSTR2 homo-clusters must dissociate before internalization ⁷⁶ SSTR5 reduces internalization of SSTR2 by 35% ⁵⁴	Receptor activation ↑ ⁷⁷ Co-clustering of SSTR2/5 modulates trafficking ⁵⁴

Receptor	Cluster Formation	Endocytosis Effect	Other Effects
CEA	Homo-clustering Co-clustering with integrins	A bivalent mAb targeting multiple epitopes seem to cross-link and form larger clusters of CEA resulting in a 3-fold faster internalization ^{78, 79}	N/A
CCR2	Homo-clustering	N/A	Signaling and cell migration ^{↑46, 80}
CXCR4	Homo-clustering Co-clustering with CCR5, CD4, and CXCR7	Clusters distribute on the cell surface but upon ligand binding localize to lipid rafts to mediate activity and internalization ^{45, 81–83}	N/A

PECAM-1: Platelet endothelial cell adhesion molecule 1; LRP1: low density lipoprotein receptor-related protein; ASGPR: Asialoglycoprotein receptor; PI(4,5)P2: phosphatidylinositol 4,5-bisphosphate; JAM-A: junctional adhesion molecule A; EphA2: ephrin type-A receptor 2; CEA: carcinoembryonic antigen.

Table 2

Targeted nanocarriers and the effect of spatial control over ligand display on binding affinity and specificity and other biological applications

Nanocarrier/ Scaffold	Ligands	Spatial Control	Application	
Fluidic Particles	Liposomes	I-CAM mAb/E-selectin mAb	Free mobility of ligands on lipid membrane	Receptor targeting Binding $\uparrow^{130}$
		Galactose	Free mobility of ligands on lipid membrane	Receptor targeting Selectivity by 15-fold $\uparrow^{67}$
	Protocells	Targeting peptide (SP94)/fusogenic peptide	Free mobility of ligands on lipid membrane	Receptor targeting Non-specific binding $\downarrow$ Specific binding $\uparrow$ IC ₅₀ by 100-fold $\downarrow^{133}$
Protein Cages	Clathrin triskelion	eGFP/mCherry	Molecular epitope recognition	Multifunctional presentation Half-life and AUC $\uparrow^{95}$
	VLPs	Triazole-linked amino acid	Equidistant spacing <i>via</i> click chemistry	Polyvalent display ¹²⁴
		Tumor idiotype scFv/CpG DNA/GM-CSF	Ligands attached <i>via</i> click chemistry to azide and alkyne containing unnatural amino acids Spatial control <i>via</i> position of unnatural amino acids	Tumor vaccination ¹²⁵
		Digoxigenin/biotin	Mixed reassembly, no spatial control over ligand display	Multivalent display ¹²⁶
		CD40 ligand	Spatial control <i>via</i> molecular epitope recognition	Targeting Affinity for B lymphocytes $\uparrow^{94}$
	Bacteriophage	EGFR/HER2 scFv/ PEG2000/Alexa fluor 488	PEG and Alexa fluor 488 attached to M13 filamentous phage <i>via</i> oxime formation at the N-terminal amines scFv genetically fused to the p3 coat protein	Imaging agent for EGFR and HER2 cells ¹⁴⁸
		Transferrin	Polyvalent display on icosahedron scaffold <i>via</i> click chemistry. Spacing partially controlled by modulating ligand density	Receptor targeting Uptake kinetics $\uparrow^{149}$
	Ferritin cages	Monosaccharides	One ligand attached per single cysteine residue on ferritin subunit Uniform spacing of 24 ligands on cage	Receptor targeting Binding to cells $\uparrow^{117}$
		RGD peptide/Cy5.5	Genetic fusion of RGD peptide and amine conjugation of Cy5.5 Mixed reassembly, no spatial control over ligand display	Multimodality imaging probe ¹¹⁸
DNA Origami	DNA scaffold	Ephrin A1 monomers	Spaced 42.9 nm apart <i>via</i> DNA base pairing	Receptor targeting Binding $\sim$ 2-fold

Nanocarrier/ Scaffold	Ligands	Spatial Control	Application	
				↑ Invasion ~4-fold ↓ ¹⁷
	DNA “tiles” (rectangular and triangular)	VLPs	Spaced 100 nm apart <i>via</i> DNA base pairing	Spatially organized display of VLPs ¹⁰⁶
	DNA “tiles” (rectangular)	Two distinct anti-thrombin aptamers	spaced 5.3 nm apart <i>via</i> DNA base pairing	Targeting and capture Binding ~ 12-fold ↑ ¹⁰⁷
	DNA nanoactuator	Split eGFP	“Mirror” distance on driver arm of nanoactuator	Bio-sensor Sensitivity ~1.4–1.7-fold ↑ ¹⁰⁸
Small molecule	Trigonal scaffold	MSH(4) ligand	Optimal inhibition observed with a ligand distance of 17 to 23 Å	Receptor targeting Inhibition ~20–40 fold ↑ ¹⁹

eGFP, enhanced green fluorescent protein; AUC, area under the curve; GM-CSF, granulocyte macrophage-colony stimulating factor; scFv, single-chain variable fragment; PEG, polyethylene glycol; MSH, melanocyte-stimulating hormone.