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The Design of Polyvalent Therapeutics

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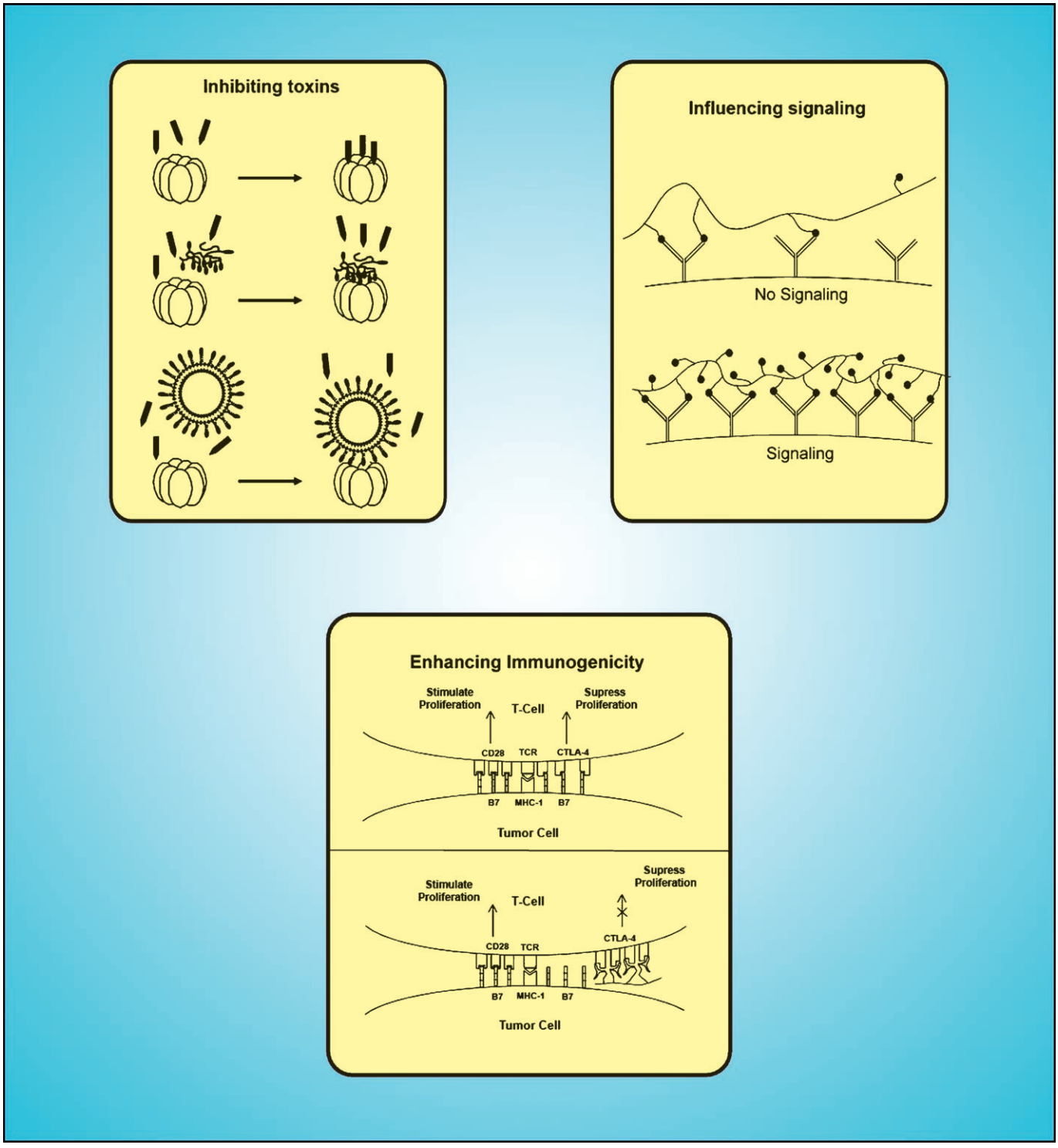

Abstract: This article reviews recent developments in the design of polyvalent ligands for in vivo applications. Topics discussed include the design of polyvalent inhibitors of toxins and viruses, the use of polyvalency for targeted drug delivery and imaging, and applications of polyvalency for enhancing or suppressing immune responses.

Keywords: drug delivery · imaging agents · immunology · inhibitors · polyvalency

Introduction

Polyvalency refers to the simultaneous attachment of multiple ligands on one entity to multiple complementary receptors on another entity and is a concept that nature makes extensive use of.[1,2] Examples of naturally occurring polyvalent interactions include antigen–antibody interactions and the binding of viruses or bacterial toxins to their cellular receptors.[1] Polyvalent interactions can not only be collectively much stronger than the corresponding monovalent interactions, but can also have properties that are different from those displayed by their constituents that interact monovalently.[1–4] Consequently, there has been an increasing interest in recent times in both the development of theoretical models to analyze polyvalent interactions and in the design of polyvalent ligands.[1,2,5–9] In particular, synthetic polyvalent ligands, consisting of scaffolds presenting multiple copies of suitable molecules (e.g., peptides or sugars), have numerous medical applications, ranging from the inhibition of toxins and pathogens to drug delivery, imaging, and influencing the function of the immune system. This review highlights recent reports in this area, with major emphasis on studies that have demonstrated efficacy in vivo.

Design of Polyvalent Inhibitors of Toxins and Viruses

The design of a polyvalent inhibitor involves the attachment of multiple copies of a suitable ligand to a scaffold such as a polymer or a liposome. Multiple simultaneous interactions between a polyvalent inhibitor and its polyvalent target can result in an interaction affinity that is orders of magnitude greater than that for the corresponding monovalent interaction. The discussion below focuses on inhibitors presenting multiple copies of a biospecific ligand (e.g., a peptide or a sugar that binds specifically to the target).

An important target that has been successfully neutralized by synthetic polyvalent ligands in vivo is anthrax toxin. Anthrax toxin, which is secreted by *Bacillus anthracis*, is responsible for the major symptoms and death in anthrax.[10,11] The toxin comprises a receptor-binding protein, protective antigen (PA) and the toxic enzymes lethal factor (LF) and edema factor (EF).[10] Following attachment to its receptor on the target cell, PA is cleaved by proteases into a 63 kDa fragment that oligomerizes on the cell surface to form a heptamer, $[PA_{63}]_7$, which binds LF and/or EF (Figure 1a) and transports them into the cytoplasm of the host cell.[10,12] The design of molecules that recognize $[PA_{63}]_7$ represents a promising strategy for neutralizing anthrax toxin. To that end, Mourez et al. used phage display to identify a peptide that binds to $[PA_{63}]_7$.[12] Attachment of multiple copies of this peptide to a polyacrylamide backbone resulted in a polyvalent inhibitor that was almost four orders of magnitude more potent than the corresponding monovalent peptide in vitro and neutralized the toxin in a rat model.[12] This work represented the first demonstration of the in vivo efficacy of a synthetic, polymeric, polyvalent toxin inhibitor.

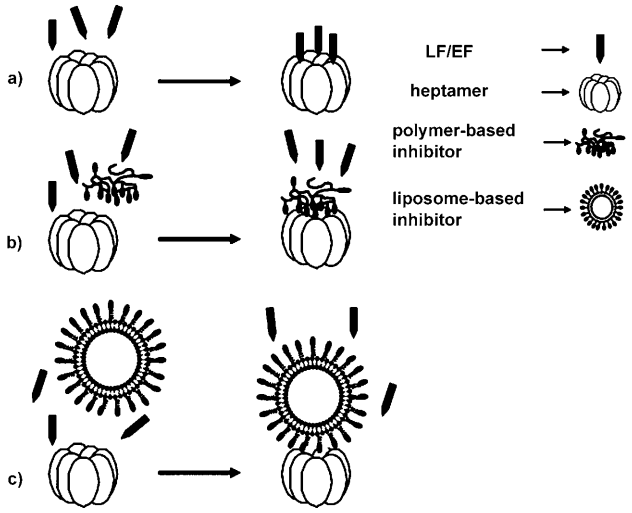

Figure 1. Illustration of the binding of the toxic enzymes (EF or LF) to the heptameric subunit of anthrax toxin, $[PA_{63}]_7$ in the absence of any inhibitor (a), in the presence of a polymer-based polyvalent inhibitor (b), and in the presence of a liposome-based polyvalent inhibitor (c).

More recently, we have synthesized potent polyvalent inhibitors of anthrax toxin based on a variety of scaffolds[13–17] (Figure 1b–c) and elucidated structure–activity relationships. Studies with liposome-based inhibitors have revealed the influence of peptide density, membrane fluidity,[16] and membrane heterogeneity[17] on the inhibitory potency. We functionalized liposomes with the $[PA_{63}]_7$-binding peptide at different densities and observed a distinct transition in half-

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maximal inhibitory concentrations (IC_{50} values) from $\approx 10^{-6}$ M to lower, more potent values ($\approx 10^{-8}$ M) over a narrow range of peptide densities. Moreover, the average inter-peptide distance at this transition (35–50 Å) matched the average separation between peptide-binding sites on the heptamer.[12] Previous theoretical studies had found that random heteropolymers recognize surfaces presenting multiple binding sites when the statistics characterizing the distribution of heteropolymer ligands along the polymer backbone and the density of surface binding sites were similar or "matched".[18] Our results suggest that the recognition of target proteins (e.g., $[PA_{63}]_7$) by peptide-functionalized liposomes is also facilitated by statistical pattern matching, that is, by statistically matching the pattern of ligands on the polyvalent liposomes to that of a polyvalent target. "Pattern-matched" polyvalent liposomes inhibited anthrax toxin in vitro at concentrations four orders of magnitude lower than the corresponding monovalent peptide and neutralized the toxin in vivo.[16] We also recently demonstrated that phase separation, that is, the clustering of inhibitory peptides into membrane domains, can significantly enhance the potency of liposome-based polyvalent inhibitors.[17]

We have also recently synthesized liposome-based polyvalent inhibitors that target the cellular receptors for anthrax toxin.[13] By using phage display to identify a peptide that binds the anthrax toxin receptors and attaching multiple copies of this peptide to liposomes, we designed inhibitors that neutralized anthrax toxin both in vitro and in vivo. This work was the first demonstration of a receptor-directed anthrax antitoxin and also represents a promising strategy to combat a variety of viral and bacterial diseases. In particular, blocking conserved host receptors used by pathogens represents a powerful strategy to overcome the problem of pathogen resistance to antimicrobial therapeutics, because extensive alterations to the pathogen may be required to enable it to switch to a new receptor that can still support pathogenesis.[13]

Several groups have designed potent polyvalent inhibitors of other toxins such as the heat-labile enterotoxin (LT) from *E. coli*, the Shiga-like toxin (Stx) from enterohemorrhagic *E. coli* (EHEC) strain O157:H7 and the cholera toxin.[1,19–25] Guided by the crystal structure of the Shiga-like toxin I (Stx-I) from *E. coli* in complex with an analogue of its carbohydrate receptor, Kitov and co-workers designed oligovalent inhibitors consisting of receptor analogues tethered to a glucose molecule as the central core. The inhibitor, termed as "Starfish" (Figure 2), protected Vero cells against both Stx-I and II.[20] "Starfish" could also inhibit Stx-I in vivo but not Stx-II; however, a derivative of this molecule termed "Daisy" (Figure 2) protected mice against both Stx-I and II.[22] In another study, Neri et al. synthesized potent inhibitors of Stx by conjugating multiple copies of a derivative of globotriaosyl ceramide (Gb_3), which is a natural receptor of Stx, to a polyacrylamide backbone. The resultant inhibitor was found to inhibit both Stx-I and II in vivo.[24] Similar inhibition of toxicity in mice was observed by Nishikawa and co-workers, when they administered a carbosilane-based-dendrimer presenting multiple copies of a Gb_3 derivative.[21] In another study, by screening a polyvalent peptide library, Nishikawa et al. identified a tetravalent peptide that exhibited a high affinity for the Stx-II and was able to protect mice from a lethal dose of an EHEC serotype.[23]

The principle of polyvalency has also been used to synthesize potent inhibitors of influenza viruses that are effective in vivo. These inhibitors target the viral proteins neuraminidase or hemagglutinin. Honda and co-workers have developed polyvalent inhibitors by attaching multiple copies of the monovalent neuraminidase inhibitor Zanamivir to polyglutamic acid backbones. These inhibitors were more potent in vivo than the monovalent inhibitor.[26,27] Matrosovich and co-workers have synthesized polyacrylamide-based inhibi-

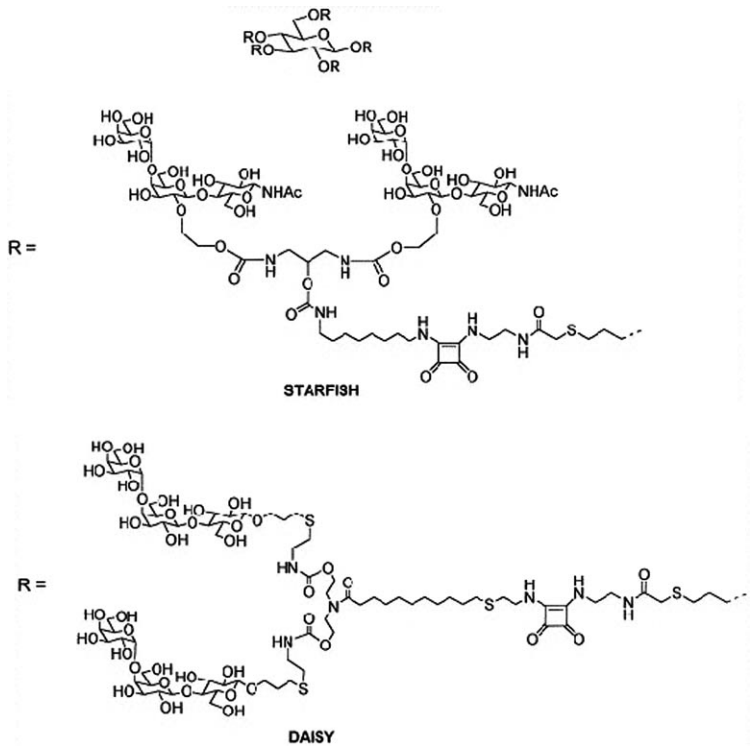

Figure 2. Chemical structure of "Starfish", which inhibits Stx-I in mice and "Daisy" which protects mice from both Stx-I and II. Reproduced with permission from reference [22]. Copyright 2003, The University of Chicago Press.

tors of the influenza virus, presenting multiple copies of a modified sialic acid monomer. The inhibitor was found to increase the survival of mice infected with a mouse-adapted variant of the H3N2 strain of the influenza virus.[28] Researchers have also used dendrimers for the synthesis of inhibitors of the influenza virus. Polyvalent sialic acid conjugated polyamidoamine dendrimers were effective in preventing infection by a H3N2 influenza subtype.[29]

Polyvalency in Targeted Drug Delivery and Imaging

The attachment of multiple copies of ligands to nanoscale scaffolds for targeted drug delivery or imaging is well established. This section highlights recent innovation in polyvalent targeting, the design of targeting ligands, and the engineering of nanoscale scaffolds.

Polyvalent targeting: One key principle of targeted nanoparticle (NP) drug delivery is that attaching multiple targeting molecules to the surface of NPs will improve their ability to bind to target cells and deliver the drug.[30] Holl et al. demonstrated that polyvalency improves the specificity of targeting of NPs to tumor cells that overexpress the folate receptor.[31] They tested the binding characteristics of a series of dendrimers with the number of presented folic acid molecules ranging from zero to 15. The experiments showed that dendrimers bound more tightly to the cells—by as much as 170000-fold—as the number of folic acid molecules increased. Calculations suggested that 5–6 folate receptors can clump together on the surface of a cell to bind to a single dendrimer presenting multiple folic acid moieties.

While strategies for delivering cytotoxic drugs to tumor cells are based on targeting cell-surface receptors overexpressed on tumor cells, these receptors are often also present on healthy cells, which can therefore also receive the drug. To improve the selectivity of drug delivery to target cells, Saul et al. took advantage of the fact that tumors usually overexpress multiple cell-surface receptors.[32] Liposomes loaded with a chemotherapeutic agent, doxorubicin, and functionalized with either folic acid, a monoclonal antibody against the epidermal growth factor receptor (EGFR), or both ligands, were used to deliver doxorubicin to healthy cells and to tumor cells overexpressing both the folate receptor (FR) and EGFR.[32] The singly targeted NPs were less specific compared to the dual-labeled liposomes. The optimal formulation contained three monoclonal antibodies and an average of 200 folic acid molecules per liposome. In the future it would be very interesting to explore the use of this dual-ligand approach for targeted delivery in vivo.

Identification of ligands: Ruoslahti and co-workers screened random peptide libraries displayed on phage for their ability to mediate the homing of phage to tumors in vivo.[33] Selection of phage from the libraries yielded three peptide motifs capable of homing to tumor vasculature: an RGD (arginine-glycine-aspartic acid) motif embedded in a double-cyclic peptide (termed RGD-4C), an NGR (asparagine-glycine-arginine) motif, and a GSL (glycine-serine-leucine) motif.[33] Conjugating an anticancer drug or a proapoptotic peptide to the RGD-4C peptide yielded compounds with increased efficacies against tumors and lowered toxicity to normal tissues in mice.[33]

Weissleder et al. described the parallel synthesis of a library consisting of magnetofluorescent NPs functionalized polyvalently with 146 different synthetic small molecules in an array format.[34] Screening this library against different cell lines led to the discovery of a series of NPs with specificity for endothelial cells, activated human macrophages, or pancreatic cancer cells. The polyvalent attachment of small molecules to nanoparticles also contributed to an increase in their specific binding affinity.

DNA and RNA aptamers represent another promising class of ligands for targeting. Langer and co-workers have shown that aptamer-conjugated NPs enable the specific delivery of a cytotoxic drug, docetaxel, both in vitro and in vivo.[35] They used an aptamer that binds to the extracellular domain of the prostate-specific membrane antigen (PSMA) expressed on the surface of prostate cancer cells.[36] In experiments with mice bearing human prostate tumors, the tumors shrank drastically, and all treated mice survived. In contrast, only 57% of the animals treated with an untargeted NP survived and only 14% of the animals treated with docetaxel alone survived.

Novel polyvalent scaffolds: There are several different classes of NPs currently in development for targeted delivery and imaging. Lewis et al. have designed polyvalent fluorescently labeled NPs, based on a plant virus called the cowpea mosaic virus (CPMV), for non-invasive imaging and targeting of the mammalian cardiovascular system.[37] CPMV can be labeled with fluorophores at high densities without quenching the fluorescence, resulting in an extremely bright, nontoxic material that is an outstanding tool for imaging vasculature in live animals(Figure 3).[37] CPMVs have been used to effectively image the vasculature in the embryos of several species, and were reported to be superior to other imaging particles such as lectins, fluorescent dextrans, or polystyrene microspheres.[37] Destito et al. have functionalized CPMV with tumor ligands such as folic acid.[38] These novel targeted particles had a high degree of specificity for the receptors and for uptake by tumor cells.

The unique optical properties of quantum dots (QDs) make them ideal scaffolds for in vivo optical imaging. Akerman et al. explored the use of peptide-functionalized and polyethylene glycol (PEG) coated QDs for targeting and imaging.[39] Ex vivo histology revealed that these QDs were directed to the tumor vasculature and organ-specific targets by the peptides. Peptide-functionalized QDs were injected into the tail vein of nude mice with breast carcinoma xenograft tumors. The blood vessels were visualized by co-injecting fluorescently-labeled tomato lectin. QD fluorescence co-localized with blood vessel staining in the tumor tissue due

to specific targeting. Gao et al. used triblock copolymer-coated QDs for prostate cancer targeting and imaging in vivo by conjugating a prostate specific membrane antigen (PSMA)-specific monoclonal antibody to the QDs.[40] Multicolor capability of QD imaging in live animals was also demonstrated by using QD-tagged cancer cells. Cai et al. developed RGD peptide-modified QDs that exhibited high affinity integrin $\alpha_v\beta_3$-specific binding as demonstrated by in vitro cell labeling, ex vivo tumor tissue staining, and in vivo tumor vasculature imaging.[41]

Rao and co-workers have developed a QD–protein conjugate for in vivo imaging applications, that generates its own light based on the principle of bioluminescence resonance energy transfer (BRET).[42] They covalently conjugated multiple molecules of *Renilla reniformis* luciferase (Luc8) to a single fluorescent QD. The Luc8-catalyzed oxidation of its substrate coelenterazine results in the emission of broad-spectrum blue light peaking at 480 nm. Due to the complete overlap of the luciferase emission and QD absorption spectra, QDs conjugated to Luc8 were efficiently excited in the absence of external light (Figure 4a,b).[42] In vivo imaging showed greatly enhanced signal-to-background ratio after injection of cells labeled with the Luc8–QD probe into the blood stream (Figure 4c).[42] This bioluminescent quantum dot technology has the potential to greatly improve near-infrared fluorescence detection in living tissue.

Bhatia et al. have developed a new small interfering RNA (siRNA) delivery platform using PEG-coated, peptide-functionalized QDs that also double as imaging agents.

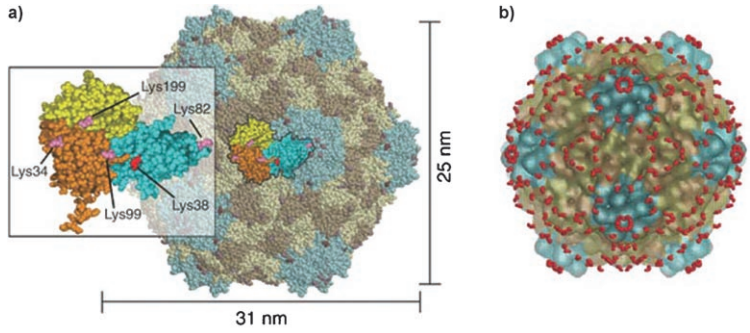

Figure 3. a) Subunit organization of CPMV: domain A (blue) represents the small subunit, domains B (orange) and C (yellow) represent the two domains of the large subunit. The maximum (31 nm) and minimum (25 nm) particle diameters according to the refined crystal structure are indicated. b) Surface model of CPMV particle showing predicted arrangement of conjugated fluorochromes. Reproduced with permission from reference [37]. Copyright 2006, Nature Publishing Group.

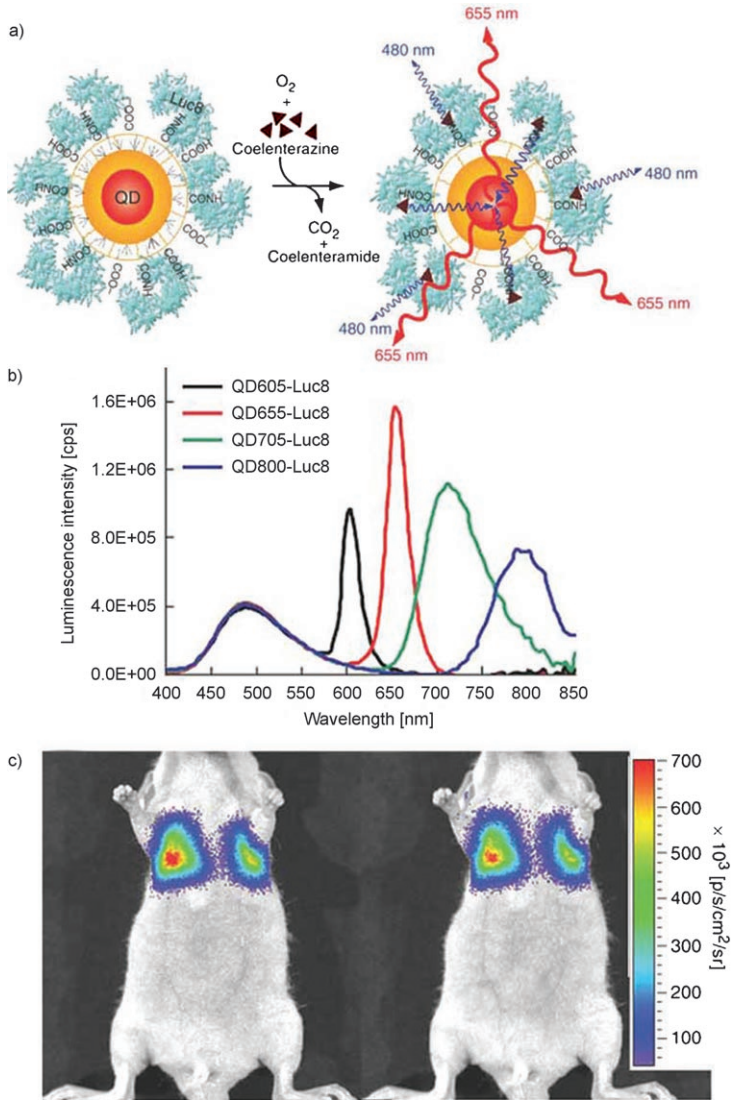

Figure 4. a) A schematic of a quantum dot that is covalently coupled to a BRET donor, Luc8. The bioluminescence energy of Luc8-catalyzed oxidation of coelenterazine is transferred to the quantum dots, resulting in quantum dot emission. b) Bioluminescence emission spectra of indicated QD-Luc8 conjugates, each QD has fluorescence emission at indicated wavelength. c) Representative bioluminescence images of a nude mouse injected via tail vein with C6 glioma cells labeled with QD655-Luc8-R9, acquired with a filter (575–650 nm) (left) and without any filter (right). Reproduced with permission from reference [42]. Copyright 2006, Nature Publishing Group.

Their studies determined that a ratio of 20 homing peptides to one siRNA produced the optimal level of protein suppression.[43] Using a similar strategy, Moore et al. have designed a multifunctional delivery vehicle using NPs that are not only capable of transporting antitumor siRNA into cells but also imaging them using magnetic resonance imaging (MRI) and fluorescence optical imaging (FOI).[44] They attached an average of three dye molecules, four membrane-penetrating molecules, and five siRNA molecules to the iron oxide NPs. They were able to track NP uptake by the tumors in vivo using both MRI and FOI and showed that large numbers of the NPs accumulated in tumors. Using fluorescence microscopy they showed that the siRNA agent was taken up along with the NPs and was able to silence a known cancer gene in the tumors.

Ruoslahti and co-workers have designed dextran-coated superparamagnetic iron oxide (SPIO) NPs that act like a developing clot in order to target tumors.[45] In vivo phage display yielded a peptide, that is, cysteine-arginine-glutamic acid-lysine-alanine (CREKA), that homed to the vasculature inside breast cancer tumors growing in mice. Using a mouse strain that lacks fibrinogen and a control group of normal mice they showed that the CREKA peptide recognizes clotted blood, which is present in the lining of tumor vessels but not in vessels of normal tissues. Multiple copies of fluorescein-labeled CREKA peptide were coupled to the SPIO particles. The peptide–NP conjugates that accumulated in the tumor enhanced blood clotting in tumor vessels and thus created additional binding sites for the NPs. This "self-amplification" of the tumor-homing property of the NPs greatly enhanced the ability to image the tumors and contributed to blocking about 20% of the blood vessels in the tumor.

Metal nanoshells and carbon nanotubes—nanomaterials that absorb near-infrared (NIR) radiation—constitute another promising class of polyvalent scaffolds. The generation of heat by the NIR irradiation of these nanomaterials has been used to kill tumor cells. NIR light holds particular promise for these applications because of the relative transparency of biological systems at these wavelengths. Hirsch et al. used silica-gold nanoshells as scaffolds for the photothermal treatment of tumors both in vitro and in vivo.[46] Magnetic resonance temperature imaging (MRTI) demonstrated that tumors reached temperatures which caused irreversible tumor damage within a matter of minutes. Dai and co-workers targeted single-walled carbon nanotubes (SWNTs) to tumor cells in vitro by functionalizing them with multiple copies of folic acid; the NIR irradiation of the nanotubes resulted in cell death.[47] They also functionalized SWNTs with PEG chains bearing multiple copies of an RGD peptide and a macrocyclic chelating agent called DOTA, which in turn was used to conjugate a positron emitting radionuclide ^{64}Cu. RGD binds to integrins, which are over-expressed on tumor cells; consequently, the researchers were able to target the polyvalent SWNTs to cancer cells and image their biodistribution in mice by using in vivo positron emission tomography.[48] Recently our group has demonstrated the ability to remotely deactivate proteins adsorbed onto carbon nanotubes by near-infrared and visible irradiation.[49] The mechanism of protein deactivation was found to be photochemical—mediated by the photoinduced generation of reactive oxygen species. We also designed polyvalent peptide-functionalized nanotubes that could selectively recognize and destroy a target protein (anthrax toxin) from a mixture of proteins.[49]

Development of Molecules That Can Modulate Immune Responses

Polyvalency plays an important role in the functioning of the immune system. Synthetic polyvalent ligands can be designed to both enhance and suppress immune responses as summarized below.

Enhancing immunogenicity: In recent years, engineered viruses have been used as novel scaffolds for the polyvalent display of antigens,[50–54] enabling the generation of both T-cell and antibody based immune responses without an adjuvant. Kaltgrad et al. arrayed tetra- and hexasaccharides on the surface of cowpea mosaic virus.[53] Chickens were then inoculated with these viral assemblies, raising avian IgY antibodies (the equivalent of human IgG) that were shown to have high avidity and specificity when analyzed on a printed glycan array. Liu et al. conjugated a synthetic version of the Cap tetrasaccharide, which is displayed on the lipophosphoglycan of leishmania parasites, to phospholipids and to the influenza virus coat protein hemagglutinin.[54] These conjugates were incorporated into the membrane of influenza virus particles, which then elicited IgM and IgG anti-glycan antibodies in mice. Moreover, these antibodies cross-reacted with the natural carbohydrate antigens expressed by leishmania cells.

There has also been a fundamental interest in understanding how the display of haptens on polymeric scaffolds influences their immunogenicity. In 1976, Dintzis et al. described the synthesis of a series of linear polyacrylamides functionalized with haptens at various densities. Polymers with hapten substitutions of less than 12–16 groups were not immunogenic in mice, whereas all polymers with a higher degree of substitution triggered an immune response. Based on this result, the authors concluded that a minimum number of antigens was required to cluster together to initiate a cellular immune response.[55] Recently, Puffer et al. used ring-opening metathesis polymerization (ROMP) to synthesize a series of polymers functionalized with defined valencies of the 2,4-dinitrophenyl (DNP) group, which can be recognized and internalized by specific B-cells.[56] These polymers were injected into mice to activate B-cell signaling and elicit an antibody response in vivo. Despite the fact that no differences in BCR internalization were observed, only the ligands of high valencies were able to promote an antibody response. By clustering the BCRs on the surface of the B-cell into membrane microdomains, the ligands with high

valencies were able to initiate a signaling response that led to the release of Ca^{2+} from the endoplasmic reticulum. Ca^{2+} release in turn induced other signaling events that eventually led to antibody production (Figure 5).

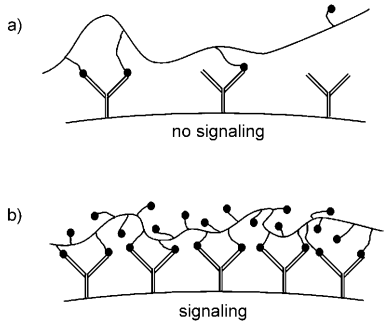

Figure 5. Polymers presenting varying ligand densities are used to cluster B-cell receptors on the surface of B-cells to initiate signaling and elicit an antibody response in vivo. a) Polymers with low ligand density cannot effectively cluster cellular receptors, and do not lead to signaling. b) High ligand density enables clustering of cellular receptors and leads to signaling.

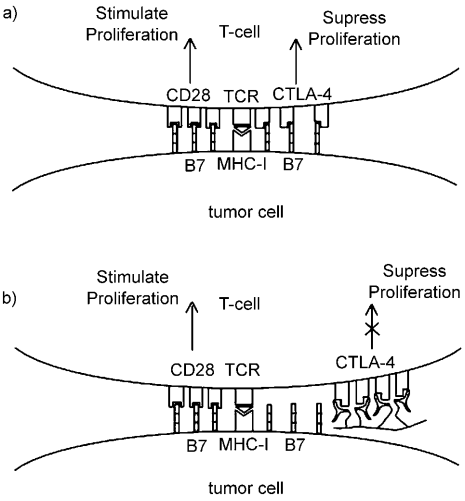

Figure 6. a) B7 receptors presented on a tumor cell can bind to either CD28, causing proliferation of anti-tumor T-cells, or to CTLA-4, causing inhibition of T-cell proliferation. b) A polyvalent aptamer binds to and blocks CTLA-4 and disrupts the down-regulation signal, thereby leading to enhanced proliferation of anti-tumor T-cells.

Immunogenicity can also be enhanced by designing polyvalent ligands that interfere with a natural signal that suppresses the immune response. When a T-cell recognizes a specific major histocompatibility complex (MHC)–peptide complex on an antigen presenting cell, a co-stimulatory signal, delivered by the B7 family of receptors to CD28, is required for T-cell proliferation. CTLA-4 is a receptor on T-cells that competes with CD28 for binding to B7 receptors, keeping proliferation in check. Unfortunately, this negative signal also interferes with the cytotoxic T-lymphocyte-(CTL)-dependent clearance of tumor cells. To combat this problem, Santulli-Marotto et al. used a screening process to identify RNA aptamers that bound to CTLA-4 with high affinity and specificity.[57] The aptamers inhibited CTLA-4 function in vitro, and when they were assembled into tetramers, their ability to enhance the proliferation of anti-tumor T-cells greatly increased both in vitro and in vivo (Figure 6). This enhanced proliferation resulted in a significant decrease in tumor growth rate in vivo.

Much effort has been put into developing cancer vaccines that would direct the immune system against the antigens that are upregulated on the surface of tumor cells. Polyvalent formulations that contain many of these tumor-associated antigens have been synthesized and tested. However, these approaches have been reviewed previously[58] and will not be discussed in detail here.

Immunosupression: While the previous section focused on enhancing immune responses, it can be equally important to suppress undesirable immune system processes. For instance, inflammation may need to be reduced in the event of allergic reactions. Suppression of immune system function may be required in the context of autoimmune disease. Successful organ transplantation also requires prevention of the rejection of transplanted organs by the immune system. The design of synthetic polyvalent molecules provides a promising approach to immunosuppression.

Anti-inflammation: One way to reduce inflammation is to stop immune cells from entering the site of injury or allergy. Since these cells arrive at their site of action by rolling along the arterial walls, inhibiting this rolling of leukocytes is one strategy that has been pursued.[59–61] Recent work has shown the utility of this approach in vivo. Ali et al. polyvalently presented Sialyl-Lewis(x) and related ligands on a polylysine backbone.[62] The polymers, when conjugated at high enough densities, inhibited E-selectin-dependent leukocyte rolling. The degree of inhibition and the time scale over which inhibition was seen was influenced by the identity of the ligand, the density of ligand on the backbone, and the concentration of polyvalent ligand injected. Kretzschmar et al. synthesized trivalent Sialyl-Lewis(x) ligands, and showed inhibition of E-selectin and P-selectin mediated cell adhesion both in vitro and in vivo.[63]

Autoimmunity: The immune system employs many processes to ensure that self-reactive B-cells and T-cells are eliminated. Failure of these processes leads to autoimmunity, and combating self-reactive immune cells can be challenging. Attempts to block the activity of these cells have met with some success. Piaggio et al. have evaluated the therapeutic efficacy of multimerized epitopes at the effector phase of a type 1 diabetes model and assessed their mechanism of action.[64] Diabetes was induced in transgenic mice expressing the influenza hemagglutinin (HA) in pancreatic β-cells by adoptive transfer of T helper 1 cells that were specific to a peptide derived from HA. A multimerized self-peptide, consisting of four covalently linked linear peptides derived

from HA, was seen to protect the mice from a challenge with diabetes-inducing T-cells specific for HA. The tetramer was shown to bind to and cluster MHC class II molecules on the surface of antigen presenting cells (APCs). This clustering promoted activation-induced death of the diabetogenic T-cells, possibly by co-localizing these cells with $CD4^+$ $CD25^+$ regulatory T-cells of the same specificity. These mice were also resistant to a second challenge with the diabetogenic T-cells.[64]

Multiple sclerosis is an autoimmune disease in which the immune system is directed against myelin, the protein sheath that surrounds nerve fibers to protect neurons. $CD4^+$ T-cells that recognize complexes of MHC II with a peptide fragment (amino acid residues 85–99) derived from the myelin basic protein (MBP) play a key role in the development of autoimmunity. A polymer therapeutic composed of a random sequence of four amino acids (tyrosine, glutamic acid, alanine, and lysine, termed YEAK) in a specific ratio has been approved by the FDA as the therapeutic Copaxone. This copolymer is broken down by antigen presenting cells and fits into the MHC II used by the MBP-derived fragment, blocking its association with the fragment.[65] It has been reported that Copaxone only reduces MS relapse rate by ≈30% in patients.[66] Stern et al.[66,67] and Illes et al.[68] modified the composition of the copolymer, substituting different amino acids based on the size of the binding pocket in the MHC class II protein. Two modified random copolymers showed greater activity against experimental autoimmune encephalomyelitis in a mouse model. One copolymer, dubbed VWAK, promoted anergy of T-cells displaying the specific disease related MHC II molecule, while the other, FYAK, induced the production of anti-inflammatory T helper 2 cytokines IL-4 and IL-10.

Hyperacute rejection: In the event of organ failure, finding a donor is usually very difficult; the number of recipients in need is always greater than the number of available donors. Xenotransplantation from pigs is a way to overcome this problem. However, most non-primate mammals have oligosaccharides on their cells termed αGal, the three main epitopes being Galα1–3Galβ1–4Glcβ-R, Galα1–3Galβ1–4GlcNAcβ-R, and Galα1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ-R.[69] Unfortunately, humans have natural polyclonal anti-αGal antibodies, including IgM, IgG, and IgA isotypes. After a xenograft, hyperacute rejection (HAR) occurs when the anti-αGal IgG binds to the αGal epitopes on the cells of the transplanted organ, leading to antibody-dependent cell-mediated cytotoxicity. The anti-αGal IgM also bind to the αGal epitopes, causing complement-dependent cell lysis.

A few strategies have been pursued to overcome this difficulty, including immune suppression and the use of affinity columns that remove circulating anti-αGal antibodies from the recipient's serum. Though both methods have had some success, immune suppression can leave a patient vulnerable to disease, and antibody removal by columns can be incomplete (80–100% removal).[70] Soluble monomeric αGal cannot be used as an anti-αGal blocker, since the monovalent interaction is too weak. Liu and Roy have developed methods of presenting αGal epitopes polyvalently, making di-, tri-, tetra-, and hexameric disaccharide clusters.[71,72] These constructs have been successfully tested in vitro for their ability to protect αGal presenting cells from anti-αGal antibodies. Katopodis et al. have developed a glycoconjugate of αGal epitopes on a poly-L-lysine backbone.[73] This anti-αGal binding polymer had been tested in numerous in vivo studies of baboons receiving pig organ transplants, and has shown to be effective at preventing hyperacute rejection of the xenotransplants.[73–78] Diamond et al. have also developed a polyvalent display of Galα1–3Gal conjugated polyethylene glycol polymers that can bind to anti-αGal antibodies in non-human primates.[79]

Conclusions and Future Directions

The examples presented in this review clearly illustrate the versatility of polyvalent interactions, and the numerous therapeutic applications of this phenomenon. Promising directions for the future range from improvements in the fundamental understanding of polyvalent recognition, to the design of novel ligands and polyvalent scaffolds, and the design of polyvalent ligands for novel therapeutic targets.

Fundamental studies that relate the structure and composition of polyvalent ligands to their activity, and that provide a better mechanistic understanding of polyvalent recognition are critical to enable the rational design of potent polyvalent ligands. As discussed in this review, several polyvalent ligands have shown efficacy in animal models, and further pre-clinical and clinical studies with these lead compounds would be of tremendous interest. It would also be important to design effective polyvalent therapeutics for other disease targets; receptor-directed inhibitors would be of particular interest, given the growing resistance to available antimicrobial agents. Another promising approach involves the design of polyvalent inhibitors based on self-assembly. Individual molecules comprising of a ligand and a self-assembling moiety consisting of novel dendrimers have been shown to form noncovalent nanoparticles which function as polyvalent ligands. These self-assembled nanoparticles, but not the individual molecules, were shown to efficiently inhibit polyvalent interactions such as IgM binding to the αGal epitope.[80] Using this design principle, it is conceivable that a polyvalent receptor can be utilized as a template to optimize its own polyvalent inhibitor (Figure 7).[80] Another important area involves the development of novel scaffolds for the polyvalent display of ligands, e.g., those based on proteins[81–84] or multifunctional nanoparticles which would carry therapeutic agents, be amenable to imaging, and have the ability to be delivered to a specific site by surpassing permeation barriers (Figure 8).[85]

Thus, with the rapid expansion of this field, the identification of novel ligands and scaffolds, and the development of novel targeting principles, polyvalency can be expected to find even more exciting applications in the future.

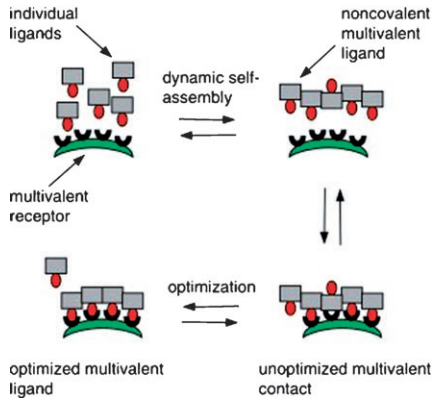

Figure 7. Monomer units, composed of a ligand and self-assembling moiety, can non-covalently assemble and bind polyvalently to cellular receptors. If self-assembly is dynamic, the polyvalent receptor can be utilized as a template to optimize its own polyvalent inhibitor.

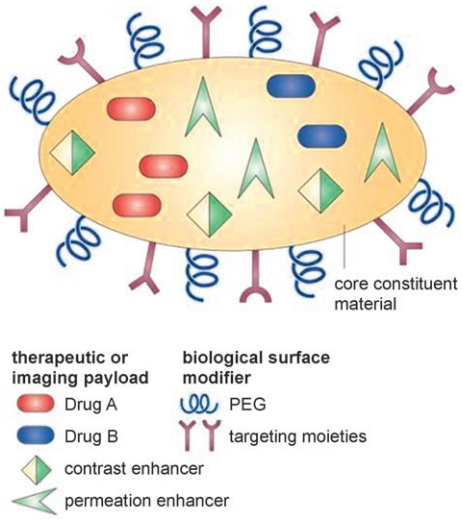

Figure 8. The following features are illustrated: the ability to carry one or more therapeutic agents; biomolecular targeting through polyvalent conjugation with one or more recognition ligands; imaging signal amplification, by way of co-encapsulated contrast agents; and biobarrier avoidance by a permeation enhancer, and by polyethylene glycol (PEG) for the avoidance of macrophage uptake. Reproduced with permission from reference [85]. Copyright 2005, Nature Publishing Group.

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[1] M. Mammen, S. K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908–2953.
[2] L. L. Kiessling, L. E. Strong, J. E. Gestwicki, *Annu. Rep. Med. Chem.* **2000**, *35*, 321–330.
[3] J. Rao, J. Lahiri, L. Isaacs, R. M. Weis, G. M. Whitesides, *Science* **1998**, *280*, 708–711.
[4] E. J. Gordon, W. J. Sanders, L. L. Kiessling, *Nature* **1998**, *392*, 30–31.
[5] M. Mammen, E. I. Shakhnovich, G. M. Whitesides, *J. Org. Chem.* **1998**, *63*, 3168–3175.
[6] J. M. Gargano, T. Ngo, J. Y. Kim, D. W. Acheson, W. J. Lees, *J. Am. Chem. Soc.* **2001**, *123*, 12909–12910.
[7] P. I. Kitov, D. R. Bundle, *J. Am. Chem. Soc.* **2003**, *125*, 16271–16284.
[8] A. Mulder, J. Huskens, D. N. Reinhoudt, *Org. Biomol. Chem.* **2004**, *2*, 3409–3424.
[9] R. S. Kane, *AIChE J.* **2006**, *52*, 3638–3644.
[10] R. J. Collier, J. A. Young, *Annu. Rev. Cell Dev. Biol.* **2003**, *19*, 45–70.
[11] G. J. Rainey, J. A. Young, *Nat. Rev. Microbiol.* **2004**, *2*, 721–726.
[12] M. Mourez, R. S. Kane, J. Mogridge, S. Metallo, P. Deschatelets, B. R. Sellman, G. M. Whitesides, R. J. Collier, *Nat. Biotechnol.* **2001**, *19*, 958–961.
[13] S. Basha, P. Rai, V. Poon, A. Saraph, K. Gujraty, M. Y. Go, S. Sadacharan, M. Frost, J. Mogridge, R. S. Kane, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13509–13513.
[14] K. V. Gujraty, A. Joshi, A. Saraph, V. Poon, J. Mogridge, R. S. Kane, *Biomacromolecules* **2006**, *7*, 2082–2085.
[15] A. Joshi, A. Saraph, V. Poon, J. Mogridge, R. S. Kane, *Bioconjugate Chem.* **2006**, *17*, 1265–1269.
[16] P. Rai, C. Padala, V. Poon, A. Saraph, S. Basha, S. Kate, K. Tao, J. Mogridge, R. S. Kane, *Nat. Biotechnol.* **2006**, *24*, 582–586.
[17] P. R. Rai, A. Saraph, R. Ashton, V. Poon, J. Mogridge, R. S. Kane, *Angew. Chem.* **2007**, *119*, 2257–2259; *Angew. Chem. Int. Ed.* **2007**, *46*, 2207–2209.
[18] A. J. Golumbfskie, V. S. Pande, A. K. Chakraborty, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11707–11712.
[19] E. K. Fan, Z. S. Zhang, W. E. Minke, Z. Hou, C. Verlinde, W. G. J. Hol, *J. Am. Chem. Soc.* **2000**, *122*, 2663–2664.
[20] P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read, D. R. Bundle, *Nature* **2000**, *403*, 669–672.
[21] K. Nishikawa, K. Matsuoka, E. Kita, N. Okabe, M. Mizuguchi, K. Hino, S. Miyazawa, C. Yamasaki, J. Aoki, S. Takashima, Y. Yamakawa, M. Nishijima, D. Terunuma, H. Kuzuhara, Y. Natori, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 7669–7674.
[22] G. L. Mulvey, P. Marcato, P. I. Kitov, J. Sadowska, D. R. Bundle, G. D. Armstrong, *J. Infect. Dis.* **2003**, *187*, 640–649.
[23] K. Nishikawa, M. Watanabe, E. Kita, K. Igai, K. Omata, M. B. Yaffe, Y. Natori, *FASEB J.* **2006**, *20*, 2597–2599.
[24] P. Neri, S. I. Nagano, S. Yokoyama, H. Dohi, K. Kobayashi, T. Miura, T. Inazu, T. Sugiyama, Y. Nishida, H. Mori, *Microbiol. Immunol.* **2007**, *51*, 581–592.
[25] B. D. Polizzotti, R. Maheshwari, J. Vinkenborg, K. L. Kiick, *Macromolecules* **2007**, *40*, 7103–7110.
[26] T. Honda, S. Yoshida, M. Arai, T. Masuda, M. Yamashita, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1929–1932.
[27] T. Masuda, S. Shibuya, M. Arai, S. Yoshida, T. Tomozawa, A. Ohno, M. Yamashita, T. Honda, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 669–673.
[28] A. S. Gambaryan, A. B. Tuzikov, A. A. Chinarev, L. R. Juneja, N. V. Bovin, M. N. Matrosovich, *Antiviral Res.* **2002**, *55*, 201–205.
[29] J. J. Landers, Z. Cao, I. Lee, L. T. Piehler, P. P. Myc, A. Myc, T. Hamouda, A. T. Galecki, J. R. Baker, Jr., *J. Infect. Dis.* **2002**, *186*, 1222–1230.
[30] C. B. Carlson, P. Mowery, R. M. Owen, E. C. Dykhuizen, L. L. Kiessling, *ACS Chem. Biol.* **2007**, *2*, 119–127.
[31] S. Hong, P. R. Leroueil, I. J. Majoros, B. G. Orr, J. R. Baker, M. M. B. Holl, *Chem. Biol.* **2007**, *14*, 105–113.
[32] J. M. Saul, A. V. Annapragada, R. V. Bellamkonda, *J. Controlled Release* **2006**, *114*, 277–287.
[33] W. Arap, R. Pasqualini, E. Ruoslahti, *Science* **1998**, *279*, 377–380.
[34] R. Weissleder, K. Kelly, E. Y. Sun, T. Shtatland, L. Josephson, *Nat. Biotechnol.* **2005**, *23*, 1418–1423.
[35] O. C. Farokhzad, J. J. Cheng, B. A. Teply, I. Sherifi, S. Jon, P. W. Kantoff, J. P. Richie, R. Langer, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 6315–6320.
[36] S. E. Lupold, B. J. Hicke, Y. Lin, D. S. Coffey, *Cancer Res.* **2002**, *62*, 4029–4033.
[37] J. D. Lewis, G. Destito, A. Zijlstra, M. J. Gonzalez, J. P. Quigley, M. Manchester, H. Stuhlmann, *Nat. Med.* **2006**, *12*, 354–360.

[38] G. Destito, R. Yeh, C. S. Rae, M. G. Finn, M. Manchester, *Chem. Biol.* **2007**, *14*, 1152–1162.
[39] M. E. Akerman, W. C. W. Chan, P. Laakkonen, S. N. Bhatia, E. Ruoslahti, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12617–12621.
[40] X. Gao, Y. Cui, R. M. Levenson, L. W. Chung, S. Nie, *Nat. Biotechnol.* **2004**, *22*, 969–976.
[41] W. Cai, D. W. Shin, K. Chen, O. Gheysens, Q. Cao, S. X. Wang, S. S. Gambhir, X. Chen, *Nano Lett.* **2006**, *6*, 669–676.
[42] M. K. So, C. Xu, A. M. Loening, S. S. Gambhir, J. Rao, *Nat. Biotechnol.* **2006**, *24*, 339–343.
[43] A. M. Derfus, A. A. Chen, D. H. Min, E. Ruoslahti, S. N. Bhatia, *Bioconjugate Chem.* **2007**, *18*, 1391–1396.
[44] Z. Medarova, W. Pham, C. Farrar, V. Petkova, A. Moore, *Nat. Med.* **2007**, *13*, 372–377.
[45] D. Simberg, T. Duza, J. H. Park, M. Essler, J. Pilch, L. L. Zhang, A. M. Derfus, M. Yang, R. M. Hoffman, S. Bhatia, M. J. Sailor, E. Ruoslahti, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 932–936.
[46] L. R. Hirsch, R. J. Stafford, J. A. Bankson, S. R. Sershen, B. Rivera, R. E. Price, J. D. Hazle, N. J. Halas, J. L. West, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13549–13554.
[47] N. W. S. Kam, M. O'Connell, J. A. Wisdom, H. J. Dai, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 11600–11605.
[48] Z. Liu, W. B. Cai, L. N. He, N. Nakayama, K. Chen, X. M. Sun, X. Y. Chen, H. J. Dai, *Nat. Nanotechnol.* **2007**, *2*, 47–52.
[49] A. Joshi, S. Punyani, S. Bale, H. Yang, T. Borca-Tasciuc, R. S. Kane, *Nat. Nanotechnol.* **2008**, *3*, 41–45.
[50] A. A. McCormick, T. A. Corbo, S. Wykoff-Clary, K. E. Palmer, G. P. Pogue, *Bioconjugate Chem.* **2006**, *17*, 1330–1338.
[51] H. Kwak, W. Mustafa, K. Speirs, A. J. Abdool, Y. Paterson, S. N. Isaacs, *Virology* **2004**, *322*, 337–348.
[52] T. M. Kundig, G. Senti, G. Schnetzler, C. Wolf, B. M. Prinz Vavricka, A. Fulurija, F. Hennecke, K. Sladko, G. T. Jennings, M. F. Bachmann, *J. Allergy Clin. Immunol.* **2006**, *117*, 1470–1476.
[53] E. Kaltgrad, S. Sen Gupta, S. Punna, C. Y. Huang, A. Chang, C. H. Wong, M. G. Finn, O. Blixt, *ChemBioChem* **2007**, *8*, 1455–1462.
[54] X. Liu, S. Siegrist, M. Amacker, R. Zurbriggen, G. Pluschke, P. H. Seeberger, *ACS Chem. Biol.* **2006**, *1*, 161–164.
[55] H. M. Dintzis, R. Z. Dintzis, B. Vogelstein, *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 3671–3675.
[56] E. B. Puffer, J. K. Pontrello, J. J. Hollenbeck, J. A. Kink, L. L. Kiessling, *ACS Chem. Biol.* **2007**, *2*, 252–262.
[57] S. Santulli-Marotto, S. K. Nair, C. Rusconi, B. Sullenger, E. Gilboa, *Cancer Res.* **2003**, *63*, 7483–7489.
[58] S. F. Slovin, S. J. Keding, G. Ragupathi, *Immunol. Cell Biol.* **2005**, *83*, 418–428.
[59] P. Mowery, Z. Q. Yang, E. J. Gordon, O. Dwir, A. G. Spencer, R. Alon, L. L. Kiessling, *Chem. Biol.* **2004**, *11*, 725–732.
[60] W. J. Sanders, E. J. Gordon, O. Dwir, P. J. Beck, R. Alon, L. L. Kiessling, *J. Biol. Chem.* **1999**, *274*, 5271–5278.
[61] H. Maaheimo, R. Renkonen, J. P. Turunen, L. Penttila, O. Renkonen, *Eur. J. Biochem.* **1995**, *234*, 616–625.
[62] M. Ali, A. E. Hicks, P. G. Hellewell, G. Thoma, K. E. Norman, *FASEB J.* **2004**, *18*, 152–154.
[63] G. Kretzschmar, U. Sprengard, H. Kunz, E. Bartnik, W. Schmidt, A. Toepfer, B. Horsch, M. Krause, D. Seiffge, *Tetrahedron* **1995**, *51*, 13015–13030.
[64] E. Piaggio, L. T. Mars, C. Cassan, J. Cabarrocas, M. Hofstatter, S. Desbois, E. Bergereau, O. Rotzschke, K. Falk, R. S. Liblau, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 9393–9398.
[65] D. Teitelbaum, A. Meshorer, T. Hirshfeld, R. Arnon, M. Sela, *Eur. J. Immunol.* **1971**, *2*, 242–248.
[66] J. N. Stern, Z. Illes, J. Reddy, D. B. Keskin, E. Sheu, M. Fridkis-Hareli, H. Nishimura, C. F. Brosnan, L. Santambrogio, V. K. Kuchroo, J. L. Strominger, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11743–11748.
[67] J. N. Stern, Z. Illes, J. Reddy, D. B. Keskin, M. Fridkis-Hareli, V. K. Kuchroo, J. L. Strominger, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 1620–1625.
[68] Z. Illes, J. N. Stern, J. Reddy, H. Waldner, M. P. Mycko, C. F. Brosnan, S. Ellmerich, D. M. Altmann, L. Santambrogio, J. L. Strominger, V. K. Kuchroo, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11749–11754.
[69] Y. Wang, Q. Yan, J. Wu, L.-H. Zhang, X.-S. Ye, *Tetrahedron* **2005**, *61*, 4313–4321.
[70] X. Chen, P. R. Andreana, P. G. Wang, *Curr. Opin. Chem. Biol.* **1999**, *3*, 650–658.
[71] B. Liu, R. Roy, *J. Chem. Soc. Perkin Trans. 1* **2001**, 773–779.
[72] B. Liu, R. Roy, *Chem. Commun.* **2002**, 594–595.
[73] A. G. Katopodis, R. G. Warner, R. O. Duthaler, M. B. Streiff, A. Bruelisauer, O. Kretz, B. Dorobek, E. Persohn, H. Andres, A. Schweitzer, G. Thoma, W. Kinzy, V. F. Quesniaux, E. Cozzi, H. F. Davies, R. Manez, D. White, *J. Clin. Invest.* **2002**, *110*, 1869–1877.
[74] U. Brandl, M. Erhardt, S. Michel, H. Jockle, L. Burdorf, I. Bittmann, M. Rossle, V. Mordstein, P. Brenner, C. Hammer, B. Reichart, M. Schmoeckel, *Xenotransplantation* **2007**, *14*, 323–332.
[75] U. Brandl, S. Michel, M. Erhardt, P. Brenner, I. Bittmann, M. Rossle, H. Baschnegger, A. Bauer, C. Hammer, M. Schmoeckel, B. Reichart, *Xenotransplantation* **2005**, *12*, 134–141.
[76] T. T. Lam, B. Hausen, K. Boeke-Purkis, R. Paniagua, M. Lau, L. Hook, G. Berry, J. Higgins, R. O. Duthaler, A. G. Katopodis, R. Robbins, B. Reitz, D. Borie, H. J. Schuurman, R. E. Morris, *Xenotransplantation* **2004**, *11*, 517–524.
[77] K. Wiebe, M. Oezkur, J. Poling, A. Haverich, *Transplantation* **2006**, *82*, 681–688.
[78] R. Zhong, Y. Luo, H. Yang, B. Garcia, A. Ghanekar, P. Luke, S. Chakrabarti, G. Lajoie, M. J. Phillips, A. G. Katopodis, R. O. Duthaler, M. Cattral, W. Wall, A. Jevnikar, M. Bailey, G. A. Levy, D. R. Grant, *Transplantation* **2003**, *75*, 10–19.
[79] L. E. Diamond, G. W. Byrne, A. Schwarz, T. A. Davis, D. H. Adams, J. S. Logan, *Transplantation* **2002**, *73*, 1780–1787.
[80] G. Thoma, A. G. Katopodis, N. Voelcker, R. O. Duthaler, M. B. Streiff, *Angew. Chem.* **2002**, *114*, 3327–3330; *Angew. Chem. Int. Ed.* **2002**, *41*, 3195–3198.
[81] A. V. Terskikh, J. M. Le Doussal, R. Crameri, I. Fisch, J. P. Mach, A. V. Kajava, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 1663–1668.
[82] J. Silverman, Q. Liu, A. Bakker, W. To, A. Duguay, B. M. Alba, R. Smith, A. Rivas, P. Li, H. Le, E. Whitehorn, K. W. Moore, C. Swimmer, V. Perlroth, M. Vogt, J. Kolkman, W. P. Stemmer, *Nat. Biotechnol.* **2005**, *23*, 1556–1561.
[83] Y. Wang, K. L. Kiick, *J. Am. Chem. Soc.* **2005**, *127*, 16392–16393.
[84] H. K. Binz, P. Amstutz, A. Pluckthun, *Nat. Biotechnol.* **2005**, *23*, 1257–1268.
[85] M. Ferrari, *Nat. Rev. Cancer* **2005**, *5*, 161–171.

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