On the design of precision nanomedicines

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Teaser
We proposed the design of nanomedicines that target cells phenotypically enabling binding only when a precise receptor(s) composition is present.

Abstract
Tight control on the selectivity of nanoparticles' interaction with biological systems is paramount for the development of targeted therapies. However, the large number of synthetically tunable parameters makes it difficult to identify optimal design `sweet spots" without rational guiding principles. Here we address this problem combining super-selectivity theory (SST) with analytical models from soft matter and polymer physics into a unified theoretical framework. Starting from an archetypal polymersome functionalized with targeting ligands, we use our model to identify the most selective combination of parameters in terms of particle size, brush polymerization degree and grafting density, as well as tether length, binding affinity and ligands number. We further show how to combine multivalent interactions into multiplexed systems which act holistically as a function of the density of more than one receptor type, so as to achieve binding only when multiple receptors are expressed above a threshold density. We show that theory can be used to effectively fit experimental data and, hence confirming its suitability. We thus propose the design of “barcode” targeting approach that can be tailor-made to unique cell populations enabling personalized therapies.

Introduction
Possibly, the most defining feature of a drug is its ability to interact with its biological target as selectively as possible, and indeed most drug discovery tools are fined to identify those molecules that bind the strongest. Such a concept goes back to the 19th Century, when Nobel laureate Paul Ehrlich postulated the side-chain theory proposing the existence of receptors and ligands (1). Selective drugging, popularized as the "magic bullet", made the fortune of Ehrlich and indeed it is still the cornerstone of modern medicine. Today drug discovery is a highly rigorous process that spans across structural and cell biology, bioinformatics, computational and medicinal chemistry. It is now evolving and merging with -omic technologies to promise personalized therapies (2). Alongside drug development, we have also advanced our ability to deliver drugs combining molecular recognition with nanoscopic carriers equipped with the necessary attributes to navigate biological environments (3). Here selectivity is bestowed decorating the carrier with ligands that enable targeting and crossing biological barriers. Drug discovery is thus now extending to target biological macromolecules that are not accessible via simple passive diffusion such as the inside of cells (4) or the central nervous system (5). Today, our ability to create ligands is well advanced and can be extended to almost any biological unit. When the targeted receptor is exogenous to the host, such as the case of infections and poisoning, ligands can be made with selectivity close to 'Ehrlich's magic bullet'. However, in most diseases, with cancer being the most exemplary one, the malfunction is often associated with receptors that are endogenous and hence expressed by both healthy and diseased cells. Such a promiscuity is the major reason why most drugs come with side-effects and many failed to go through the clinical pipeline. Yet, such a promiscuous nature is managed with exquisite precision within a living system with molecules, proteins, nucleic acids, and cells interacting with one another with extremely high selectivity.

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Fig. 1 Examples of polymer brushes. Schemes of glycocalyx syndecan 4 LRP1 and SRB1 receptor. Proteins were reconstructed with atomic resolution using computational methods and minimized for a stretched brush conformation. Both insets show the details of the end part of the LRP1 next to the four heparan sulphate chains and the SRB1 size with respect to the syndecans (a). Scheme of a POEGMA-PDPA polymersomes decorated with angiopep peptides. The polymersomes were reconstructed using minimized atomistic model of the single blocks which in turn have been assembled into a 50nm vesicle. The inset detail show that the peptide is well embedded in the PEO brush.

Historically, the strength of interaction between a given ligand, $L$, and its receptor, $R$, is measured by its affinity and this is defined by the same thermodynamic principles that apply to a reversible reaction. The reaction association constant $K_A = \frac{k_{on}}{k_{off}}$ where $k_{on}$ and $k_{off}$ being the rates of binding and unbinding, respectively, is defined as the ligand affinity. The higher the ligand affinity, the lower the ligand concentration required to saturate its receptor. Affinity can be augmented by combining different ligands into multivalent scaffolds (6) and in such cases, the binding is defined by the term avidity which represents the total effect of the bound units collectively (7). Multivalent interactions are critical in most biological processes as they allow the translation of weak bonds into strong ones enabling clustering and signal transduction (8). Similarly, multivalent interaction is the bread and butter of supramolecular chemistry and often at the core of the design of nanoscale devices (9). From a theoretical standpoint, the probability of a single ligand to bind to a receptors-coated target can be expressed roughly as:
$$p_{\text{bind}} \approx \frac{\rho N_R K_A}{1 + \rho N_R K_A}$$

where $N_R$ is the number of receptors on the target and $\rho$ is the number concentration of ligands and can be written as $\rho = [L] N_A$ where $[L]$ is the molar concentration of the ligands and $N_A$ is the Avogadro number (see supporting information annex 1 for derivation). Thus, probability saturates to 1 for either large number of receptors $N_R$ or high binding strength. For this reason, high affinity means that a large proportion of ligands will bind to any cells that express the targeted receptors, not just those over-expressing them. This inevitably leads to unwanted interactions, which in the case of anticancer therapy, where the final aim is often to kill the abnormal cells, can lead to reactions that outweigh the clinical benefits. For multivalent systems the binding affinity has a strong contribution from combinatorial entropy (7), which can be exploited for targeting. In 2007, Carston et al. showed that multivalent targeting was more selective when multivalent low affinity ligands were used(10). Noting such a peculiar feature of multivalency, in 2011 Martinez-Veracoechea and Frenkel came up with a very interesting approach proposing, based on a statistical mechanical description, the super-selectivity theory (SST) (11). They show that, in contrast to what happens with high affinity ligands, the combination of multiple low-affinity ligands creates on-off association profiles where the multivalent scaffold saturates the receptors only above a given onset receptor density, whilst does not bind at all at lower densities. Such a scenario is indeed what required to target cancer cells which often over-express receptors otherwise present in several healthy tissues. SST was proven experimentally in model systems such as supramolecular polymers (12) and multivalent polymers (13, 14). However, a major limitation to the applicability of SST is that the affinity required to create super-selective profiles is rather low, corresponding to binding energies of order of few $k_B T$s, where $k_B$ is the Boltzmann’s constant and $T$ is the absolute temperature. Indeed, the receptor and its ligand interact via supramolecular forces emerging such as Coulombic forces, hydrogen bonds, aromatic interaction, hydrophilic and hydrophobic effects and van der Waals interactions. Although, these are usually weak forces, the range of realistic binding energy is much higher than that required by SST with lowest limit being the water hydrogen bond of about $8k_B T$ (15) to the strongest biological supramolecular bond known so far, the avidin/biotin complex, with association energy of c.a. $30k_B T$ (16). In addition to this, as recently demonstrated by Angioletti-Uberti, multivalent systems are strongly affected by unspecific interactions induced by the presence of ligands other than the targeted ones and this is exacerbated by the use of low-affinity ligands (17). In the following, we show how these problems can be solved by combining the general concept of SST theory with principles from soft matter and polymer physics that allow to concurrently modulate the bond-mediated specific interaction and avoid non-specific ones. In doing that, we also show how to achieve multiplexed targeting based on multiple receptor types.
Fig. 2 Repulsive steric potentials. Schematics of the binding of a multivalent POEGMA-PDPA polymersome decorated with angiopep peptide and targeting LRP1 (a) with PMPC chains and targeting SRB1 receptors (b) and with both ligands and targeting both receptors (c). The detail of the interaction between angiopep and LRP1 (d) and PMPC and SRB1 (e) modulated by both the PEO and glyocalyx brushes. The corresponding repulsive steric potentials exerted on the LRP1 insertion in the PEO brush (f) and the polymersome inserting in the glyocalyx brush (g). These are calculated as a function of the polymersome radius, $R$, and insertion parameter for the PEO chains, $\delta_P$, and for the glyocalyx heparan sulphate chains, $\delta_G$, respectively.

Theory

Rules of engagement in a biological environment. From blood plasma to interstitial fluid to the cell cytosol, biological liquids are crowded aqueous over-saturated solutions with high molecular diversity. Typical protein volume fractions range from 10% in the blood plasma to up to 40% in cellular organelles. Assuming hexagonal packing, the ratio between the protein radius $r_P$, and the inter-protein distance, $r_{ij}$ is a function of the volume fraction, $\phi_P \frac{r_P}{r_{ij}} = \sqrt{\frac{\phi_P \sqrt{3}}{2\pi}}$. Hence each protein is packed with average inter-particle distance ranging from 6 to 0.3 times its own radius. Macromolecular crowding means that proteins diffusion is considerably
decreased (18, 19) while metabolites and ions diffusing through the protein network exhibit enhanced percolation (18). The same water that bathes the proteins is confined in a thin interface and hence exhibits properties different from bulk behavior (20). In addition to the constitutive bonds that characterizes each unit, biological molecules and macromolecules interact via weaker supramolecular forces. These interactions are either electrostatic such as ionic, hydrogen bonding, π-π, metal complexation, and van der Waals or emerging from the interaction with the solvent (i.e. the water) including attractive hydrophobic and repulsive hydrophilic interaction. Supramolecular forces combine into isotropic non-specific potentials that, for the single unit to be stable, ought to be repulsive and stronger than the thermal fluctuations at very short distances (i.e. $U(r \rightarrow 0) > k_B T$). At larger distances, the word fluid already indicates that these forces are weaker and similar magnitude to thermal fluctuations. Any attractive net potential stronger than the thermal fluctuations will lead to association and aggregation. A good example of such a scenario is when an exogenous element placed within a biological fluid quickly attracts all the proteins around. Such a fouling process, known as opsonization, is a critical step of immunological surveillance and correlates with fast riddance (21). Many biological structures are also capable of creating unique chemical combinations that make supramolecular forces between two complementary molecular arrangements very specific, directional and stronger than thermal fluctuations. Molecular recognition processes such as ligand/receptor binding are critical to control interaction as well as to serve as template for drug design. It is also important to notice that in complex multicellular organisms opsonization acts together with preprogrammed proteins that recognize non-self or abnormal self-species and hence bestowed with the ability to detect chemical signatures classified by the adaptive immune system as non gratae (22).

Proteins control their repulsive potential via their surface charge and structure. For large objects such as cells and viruses, their surfaces are often coated with long polysaccharides also known as glycans (23), either chemically linked to proteins forming glycoproteins (e.g. syndecan, mucins, etc) or bound to dedicated receptors (e.g. CD44/Hyaluronan) (24, 25). Glycans are often packed densely and this in combination with their strong affinity with water drives the chain to stretch forming brush-like profiles. Such an arrangement creates a very strong repulsive steric repulsive potential that prevents non-specific interaction (26, 27).

Synthetic hydrophilic polymer brushes are the most common strategy in biomaterial design to prevent unspecific interaction and protein fouling so as to ensure long-term compatibility (28-30). Experimental observations have shown that unspecific interaction are best controlled using non immunogenic moieties that have either neutral polar or zwitterionic functional groups (30-32). These are known to interact with water orienting it in their close proximity creating repulsive potentials relatively insensitive to other species (30, 32). The most common polymer that fulfil such requirements is the poly(ethylene oxide) (PEO) also known as poly(ethylene glycol) (PEG) (28-30). This is one of the very few synthetic polymers that is generally recognized as safe for most medical applications and it is used routinely in the clinic as adjuvant/coating for several devices and drugs (33). Alternatives to the PEO include poly(vinyl pyrrolidone) (PVP), poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC), poly(glycerol)ls, poly(amino acid)s, polysarcosine, poly(2-oxazoline)s, and poly(N-(2-hydroxypropyl)methacrylamide) (28, 29).

The case study. To help the discussion and facilitate theory derivation and calculations, while at the same time studying a relevant system, we selected brain endothelial cells (BEC) as biological target. BECs are the most important actors in the brain-blood barrier (BBB) and hence the most critical target to devise brain delivery strategies (5). BECs are very much like any other endothelial cells in our body and their major function is forming the vessels that carry blood supply. Yet every organ, and brain in particular, conditions endothelial cells to control the crossing of metabolites and immune cells according to their specific needs. BECs are programmed to be very ‘permeable’ to glucose and several other small metabolites but almost impermeable to most drugs and to immune cells (5). This has created a considerable hurdle to any neurological pharmaceutical development (5). We demonstrated that BECs can be targeted and crossed using multivalent polymersomes bearing ligands for the Low density lipoprotein receptor-related protein 1 (LRP1) (34, 35). We also demonstrated the poly[2-(methacryloyloxy)ethyl phosphorylcholine] (PMPC) polymer chains target the scavenger receptor B1 (SRB1) (36), also expressed by brain endothelial cells (34). We know from the literature (37, 38) that endothelial cells (39, 40), and BECs (38) in particular, express high level of the glycocalyx syndecan 4. Based on these information, we thus reconstructed a possible arrangement of LRP1, SRB1 and syndecan 4 proteins on a stereotypical BEC membrane. The resulting scheme is shown in fig.1a, where all the molecules are drawn to scale. The structure of the LRP1 was reconstructed.
using iterative threading assembly refinement (I-TASSER) (see supporting information) while the glycocalyx structure was used as reported by Cruz-Chu et al (41). For the SRB1 we used the PDB deposited crystal structure (4F7B) as reported by Neculai et al.(42). The syndecan 4 is decorated with 4 heparan sulphate (HS) chains with polymerization degree of 100 and placed in the membrane with density estimated by experimental reconstructions (37, 38). The resulting arrangement clearly shows that anything interacting with either LRP1 or SRB1 will be also affected by the steric hindrance of the HS chains and suggests that only analyzing the holistic interaction we can disclose the most effective targeting strategy.

As targeting unit we propose synthetic vesicles, known as polymersome, made of the self-assembly in water of amphiphilic poly[oligo(ethylene glycol) methyl methacrylate]-poly(2-(diisopropylamino)ethyl methacrylate) (P[(OEG)_{10}MA]_{20}-PDPA_{100}), which we will refer as EP for simplicity. Their structure is shown in fig.1b, where some of the POEGMA chains are functionalized with a peptide ligand. We have studied this particular system in large detail and demonstrated its use for the in vivo targeting of the blood brain barrier (34, 35) and peritoneal metastasis (43, 44). As shown in fig.1b, the oligo(ethylene glycol) chains cluster at high density on the surface creating a brush-like layer that protect polymersomes from unspecific interaction and modulate the ligand binding as it will be discussed in the following section.

**Steric potentials as interference effect.** Now that we have established the 'rules of engagement’ and the model study the quest is to define how either glycans or anti-fouling polymers contort the ligand/receptor interaction. We thus define three possible scenarios: in **Fig.2a**, the EP polymersome is decorated with angiopep-2 peptides, known to target the LRP1 receptor (45); in **Fig.2b**, the EP polymersome is decorated with poly[2-(methacryloyloxy)ethyl phosphorylcholine] (PMPC) polymer chains to target the SRB1 receptor (36); finally in **Fig.2c**, the two ligands are combined together (**Fig.2e**). In all three scenarios the EP polymersomes interact with LRP1 (**Fig.2d**) and/or SRB1 receptors dispersed in a matrix of syndecan 4. This
particular glycocalyx is known to be over-expressed by endothelial cells (39, 40) and the brain endothelial in particular (38) and its role is to control both the blood fluid dynamics as well as the transport across (37, 46). We use structural data available from the literature and inferred that each receptor is associated with at least two syndecan 4 (39, 40). In each scenario, the receptor/ligand interaction drives the association, and this is opposed by two steric potentials: one arising from the glycocalyx brush and one arising from the polymer brush that coats the nanoparticle. The magnitude of both depends on how accessible ligands and receptors are. This in turn depends on: (i) the relative height of the receptor with respect to the HS chains which we define as \( \delta_G h_G \) where \( h_G \) is the HS length and (ii) the tether length of the ligands which we define here as \( \delta_P h_P \) where \( h_P \) is the PEO chains length. Both, \( \delta_G \) and \( \delta_P \) are defined as the interference parameters, with \( \delta_P, \delta_G \in [0,1] \). For simplicity, we neglect the protein component of the syndecan and consider only the HS chains. For receptors shorter than the HS chains, binding requires that the nanoparticle inserts into the HS brush and hence feels a steric potential arising from both the osmotic pressure due to its volume being depleted and an elastic component due to the compressions of the chains. According to Halperin (47), the

Fig. 3 Scaling principles in super-selectivity. Heat maps showing the fraction of bound particle \( \theta \) as a function of the numbers of receptors \( \langle N_R \rangle \) and number of ligands \( N_L \). (a) the additive inverse Ligand affinity \(-\beta \Delta G^L_{ij}\) (b) and particle radius, \( R \) (c). Each map was analysed to calculate the selectivity \( \alpha_{\text{max}} \) and the corresponding \( \langle N_R \rangle_{\text{onset}} \) and the graph of these as function of the varying parameter are reported alongside.
two components depend on the relative ratio between the object entering the brush (herein the nanoparticle) and the brush height. If we assume that the nanoparticle radius is smaller than the brush height, i.e. \( R < h_G \), then, as in (47), the compression component can be neglected. Considering that HS chains are as long as 100nm (39, 40) we believe this condition applies to most nanomedicines whose optimal radius is always around 50nm. We can thus calculate the potential as a function of the glycol interference parameter, \( \delta_G \), considering only the osmotic pressure component as:

\[
\beta U_G = \frac{4\pi R^3 (1 - \delta_G^2) \frac{\varrho}{(\sigma_{HS})^2}}{3} \tag{2}
\]

where \( \beta = (k_B T)^{-1} \), \( R \) is the particle radius, \( \sigma_{HS} \) is the area per HS chain which can be derived as \( \sigma_{HS} = \frac{\pi}{24} d_s^2 \), with \( d_s \) being the interchain distance between two syndecan each bearing 6 HS chains (see Fig 1a). Note the glycalyx potential is invariant with the HS chain polymerization degree as long as we do not consider the compression of the chains i.e. \( R < h_G \).

If we now consider the polymer brush made by the PEO chains and expressed on the nanoparticle surface, as the receptor binds to its ligand, the chains apply a steric repulsion to the receptor tip. For simplicity we assume the receptor tip with volume \( V_p \) and \( \sqrt[3]{V_p} < h_p \) we can write, using the same model for equation (2):

\[
\beta U_p = \frac{V_p (1 - \delta_p^2)^\frac{3}{4}}{(\sigma_P)^2} \tag{3}
\]

Due to the non-negligible curvature of the nanoparticle, here \( \sigma_p \), i.e. the area per PEO chain, changes along the brush height, \( h_p \), as a function of the nanoparticle size \( R \). Using the model proposed by Zhulina et al (48), we derive the area per chain \( \sigma_p \) as a function the interference parameter, \( \delta_p \) as:

\[
\sigma_p = \sigma_0 \left(1 + \frac{\delta_p h_p}{R}\right)^{-1} \tag{4}
\]

with \( \sigma_0 \) being the grafting density at the surface (i.e. \( \delta_p = 0 \)) and \( \gamma \) being a geometrical parameter to represent the packing of the chains on the surface, which is \( \gamma = \left(\frac{h_p}{R} + 1\right)^2 \) for \( \frac{h_p}{R} \leq (\sqrt{3} - 1) \) and \( \gamma = 3 \) for any other values, where \( h_p^0 \) is the value of the brush height on a planar surface. According to Zhulina et al (48), we can write the brush height as:

\[
h_p = R \left[1 + \frac{(\gamma + 2) N_{PEO} \varrho_{PEO} (\frac{3}{3} \sigma_0)^{\frac{1}{3}}}{3R} \right]^{\frac{3}{\gamma + 2}} - 1 \tag{5}
\]

where \( a_{PEO} \) is the PEO monomer size, \( \varrho \sim a_{PEO}^3 \) is the monomer excluded volume parameter and \( N_{PEO} \) is the PEO degree of polymerization. Note that \( h_p^0 = N_{PEO} \left(\frac{\varrho a_{PEO}^3}{3 \sigma_0}\right)^{\frac{1}{3}} \). If we substitute equation 5 in equation 4 we can write:

\[
\beta U_p = V_p \left[ \sigma_0 \left(1 + \delta \left(1 + \frac{(\gamma + 2) N_{PEO} \varrho_{PEO} (\frac{3}{3} \sigma_0)^{\frac{1}{3}}}{3R} \right)^{\frac{3}{\gamma + 2}} - 1\right) \right]^\frac{3}{2} (1 - \delta_p^2)^\frac{9}{4} \tag{6}
\]

Both equations 3 and 6 can be used to calculate the steric potentials (Figs.2f-g) as a function of the particle core radius \( R \) and two insertion parameters \( \delta_C \) and \( \delta_p \). In both cases we use experimental values using the
polymersomes model and the syndecan 4 brush expressed by brain endothelial cells. The resulting curves show considerable energetic barriers for both brushes. As shown in Fig.2f potentials up to \( \sim 200k_BT \) are required to overcome completely the PEO brush repulsion at \( \delta_p = 0 \). The graph in Fig.2g shows that the syndecan 4 brush act as an effective filter imposing prohibitive \( \sim 1000k_BT \) potentials for particles closer to 100nm. However, it is important to notice that for larger radii, the steric potential is no longer due to osmotic displacement of the chain but to their compression.

**Multivalent interactions.** Now that we have shown how both the polymer and the glycocalyx brush tune the binding strength of a given ligand-receptor pair, we need to describe the possibility to form multiple bonds at the same time affects the overall binding energy of the nanoparticle. In other words, we need a general model to describe multivalent effects. The latter arise from the fact that nanoparticles can use their ligands to bind the cell surface forming many distinct bond arrangements. Each of these constitute a possible micro-state of the system that should be taken into account when calculating the free-energy due to bond formation (7). Importantly, as first shown by Kitov and Bundle, there is a degeneracy, \( \Omega \), associated to each micro-state that can strongly contribute to its weight in determining the overall binding free-energy. This is simply due to the fact that this degeneracy translates in an associated entropy, typically named “avidity entropy” \( S_{\text{avidity}} = k_B \log \Omega \). In calculating the binding energy of a nanoparticle to a receptor-bearing surface, each micro-state should be properly taken into account, including its entropic contribution. Angioletti-Uberti et al showed (49) that when this is done a general analytical formula arises for the free-energy due to bond formation:

\[
\beta E_{\text{bond}} = \sum_i \left[ \ln(p_i) + \frac{1}{2}(1 - p_i) \right] \tag{7}
\]

where \( p_i \) is the probability that a ligand or receptor \( i \) is unbound, and the sum is all possible ligand and receptors. The values of \( p_i \) are given by the solution of the following set of self-consistent equations:

\[
p_i + \sum_{j \in \text{neigh}(i)} p_j \exp(-\beta \Delta G_{ij}) = 1 \tag{8}
\]

one for each ligand or receptor in the system, all coupled together. The sum in the left hand side of equation 8 runs over all possible neighbors \( j \) of a binder \( i \) ( \( i \) being either a ligand or receptor) and \( \chi_{ij} = e^{-\beta \Delta G_{ij}} \) is the bond strength for that specific ligand-receptor pair (47). In the case where all ligands bind to a single type of receptor only, and vice-versa, and considering that receptors are mobile on the cell surface, one can take an average over all receptors’ positions and substitute \( \chi_{ij} \) with its average value \( \langle \chi \rangle \), which would only depend on the type of ligand and receptor (50, 51). In this case, the equations leading to \( E_{\text{bond}} \) can be solved analytically (see the Supporting Information). At this point, it is important to discuss what are the various contributions to the bond energy, since this is crucial to understand how to engineer/design our targeting system. As shown in (50) the bond energy can be written as \( \Delta G_{ij} = \Delta G_{ij}^{0} + \Delta G_{ij}^{\text{conf}} \) with \( \beta \Delta G_{ij}^{0} = -\ln \frac{\rho^*}{K_D} \)

being the binding energy or affinity from association of ligand \( i \) and receptor \( j \) in solution, as measured by the ligand/receptor equilibrium dissociation constant, \( K_D \), that can be measured experimentally and \( \rho^* = 1M \) is the standard concentration. \( \beta \Delta G_{ij}^{\text{conf}} \) is a configurational contribution due to the constraints imposed by binding (50). In our system, there are two contributions we need to include in \( \beta \Delta G_{ij}^{\text{conf}} \). The first arises due to the mobility of the receptors (51):

\[
\beta \Delta G_{ij}^{\text{conf,mobile}} = -\ln \left( \frac{A_{\text{bind}}}{A_{\text{free}}} \right) \tag{9}
\]

This contribution accounts for the fact that in order to interact and bind to a ligand, receptors need to be in its proximity. This limits their position within an area \( A_{\text{bind}} < A_{\text{free}} \), where \( A_{\text{free}} \) is the area they can span in the free, unbound state. To estimate \( \beta \Delta G_{ij}^{\text{conf,mobile}} \) hence, we take \( A_{\text{bind}} = 2\pi(\delta h_r)^2 \) i.e. approximately the area spanned by a rigid ligand, whereas for \( A_{\text{free}} \) we take the surface area exposed by a typical cell, of about 400\( \mu \text{m}^2 \). The second important contribution to \( \beta \Delta G_{ij}^{\text{conf}} \) comes from the fact that in order to bind a ligand,
receptors need to penetrate the PEO brush. We calculate this latter contribution assuming that the equilibrium adsorption distance between nanoparticles surface and receptors is the average ligand length, i.e. we set:

\[
\beta \Delta G^{\text{conf,PEO}}_{ij} = U_P(x = h_P(1 - \delta_P))
\] (10)

Given the free-energy for adsorption defined by equations 7-10, as in (11) we use a simple Langmuir-Hill model to describe the binding of the nanoparticles to a cell, considered as a multivalent surface. By using this model, we implicitly assume that: (i) we have a fixed number of adsorption sites and the number of receptors on each is given by a Poisson distribution with average \( <N_R>\); (ii) different nanoparticles do not compete for the same receptors and (iii) a surface can only be occupied by one nanoparticle at a time. Hence, we can write the fraction of bound nanoparticles, \( \theta \) as:

\[
\theta = \frac{a_{aq}}{\langle 1 + a_{aq} \rangle}
\] (11)

where \( \langle \rangle \) is an average over all possible distributions of receptors on the adsorption sites, weighted by their Poisson probability. In equation 11, \( q \) is the ratio between the bound vs. unbound partition function for a single nanoparticle on an adsorption site while \( a \) is the nanoparticle activity, which under the experimentally relevant dilute conditions can be approximated as:

\[
a \approx \langle P \rangle N_A v_B
\] (12)

where \( \langle P \rangle \) is the molar concentration of the nanoparticles in the bulk solution. The volume \( v_B \) can be derived for a spherical particle with radius \( R \) and ligand tether length, \( d = \delta_p h_P \), approaching a surface as:

\[
v_B = \frac{\pi}{3} [3(R + d)^3 - R^3]
\] (13)

The single-site bound state partition function is related to the adsorption free-energy by (52)

\[
q = \exp(-\beta U_G) \left[ \exp(-\beta E_{\text{bond}}) - 1 \right]
\] (14)

where \( E_{\text{bond}} \) is the free-energy due to bond formation, properly summed up over all possible bonding combinations given by equation 7. The additional \(-1 \) takes into account the fact that the nanoparticle is considered bound only in the case where at least one bond is present [16] and the factor \( \exp(-\beta U_G) \) accounts for the fact that in the bound state the particle gain an energy contribution of \( U_G \) due to the repulsion caused by the glycocalyx. We can thus combine equations 12, 13 and 14 to obtain:

\[
\theta = \left( \frac{\pi \langle P \rangle N_A [3(R + \delta_p h_P)^3 - R^3] \exp(-\beta U_G) \exp(-\beta E_{\text{bond}}) - 1}{\exp(-\beta U_G) \exp(-\beta E_{\text{bond}}) - 1 \} \right)_{p}
\] (15)

Equation 15 associates binding to several design parameters and hence allows to identify the most effective combinations. To facilitate the identification of super-selective regimes we use, from now on, the same selectivity function defined by Martinez-Veracoechea and Frenkel (11) as

\[
a = \frac{\delta \log \theta}{\delta \log N_R}
\] (16)
For monovalent binding the selectivity \( \alpha \leq 1 \) and under super-selective conditions for multivalent interactions. Equation 11 can be also expressed as an Hill function \( \theta = \frac{N^I}{K_B + N_L^I} \) with \( n \) being the Hill coefficient that defines the binding cooperativity (53) and indeed at very low surface coverage \( \alpha \sim n \). However, here we consider all possible bonds making \( \alpha \) not constant. We thus define the receptor (or ligand) number where \( \alpha \) takes its maximum value as the onset density \( N_{\text{onset}} \) (note that this number is actually the average value per site that controls the Poisson distribution) and the corresponding value of \( \alpha(N_{\text{onset}}) \equiv \alpha_{\text{max}} \) as the super selectivity parameter. As discussed previously (11) super-selective binding corresponds to quasi-step-like \( \theta(N_R) \) or \( \theta(N_L) \) functions where the fraction of bound particles rapidly grows from c.a. zero to c.a. 1 as the numbers of receptors (or ligands) goes above the onset density. Across this threshold value, a minimal change in \( N_R \) corresponds to changes of \( \theta(N_R) \sim N_R^{\alpha_{\text{max}}} \). Therefore, non-selective binding corresponds to \( \alpha_{\text{max}} < 1 \) whereas super-selective profiles will have \( \alpha_{\text{max}} > 1 \).

The graphs shown in Fig.3 shows heat maps of \( \theta \) as function of the receptor average number per adsorption site \( < N_R > \) and different functional parameters. The maps were used to calculate the selectivity \( \alpha_{\text{max}} \) and the onset density \( N_{\text{onset}} \) as function of the same parameters. We used the angiopep-2 affinity to LRP1 as reported in literature (54), used the size of the LRP1 receptor and its insertion in the glyocalyx matrix as shown in Fig.1a.

Each variable was optimized to achieve high selectivity and tunable onset density. As shown in Fig.3a, \( \theta \) is extremely sensitive to the ligands number, \( N_L \), with selectivities always larger than 1 and even approaching 6. However, the higher the number of ligands, the higher the selectivity and the lower is the onset density. This varies with a normal-like trend with a peak at around 2 ligands and decaying to zero as \( N_L \to \infty \). The hyperbolic decay allows for precise tuning only between 2 and 10 ligands while for larger \( N_L \), the onset density goes to zero. For the ligand affinity \( \beta \Delta G_{ij} \) (Fig3b), we observe no interaction above \(-8k_B T \) where the binding energy is too low to overcome the steric potentials. For lower values, the selectivity parameter \( \alpha_{\text{max}} \) peaks between \(-10k_B T \) and \(-20k_B T \) to values close to 5 to then decay to non-selective values. In the similar range the high selectivity corresponds to a high onset receptors concentration with a fast decay in few \( k_B T \) units. A very similar trend is observed with the polymer insertion parameter, \( \delta_p \) (Fig5a) and the glyocalyx insertion, \( \delta_g \) (data no reported). It is also very important to note that the glyocalyx spacing, \( d_s \) needs to be large enough to allow the nanoparticle to access the receptor and indeed if this is too tight or the receptor is well hidden within the glyocalyx network, no interaction takes place. However (as shown in Fig5b), the extra steric potential enhances considerably the selectivity to 6 units while allowing for a good control on the onset receptor concentration. A similar argument can be made for the particle radius where small particles lack of selectivity and large particles cannot penetrate the glyocalyx network (see Fig3c). The two counteracting parameters thus lead to well defined size-controlled selectivity. Similarly, we can state that particles with larger radii have denser brushes and thus stronger repulsive contribution to the bond strength, which effectively decreases with increasing particle size. At the same time, however, the activity coefficient \( \alpha \) increase with particle size. However, it is important to emphasise that the radius dependence effectively is mixed with the dependence on the number of active ligands as well as details relating to the ligand tether length. If we increase the radius but keeping \( N_L \) fixed, we reduce the overall grafting density and/or we decrease the ligand length so that overall the same number can bind.

In our discussion, we also emphasize the exact values of \( \alpha_{\text{max}} \) and \( N_{\text{onset}} \), as well as the sweet-spots in terms of the optimal parameter range to tune these quantities, depend on the choice of the values for all other parameters involved (we have 6 and we fix 5 of them in each graph). However, the trends observed are not qualitatively affected by this choice.

**Multiplexing.** As showed in Fig3, we can vary different parameters to achieve the selectivity required for the targeted receptor ensuring that binding occurs above a certain therapeutic threshold. However, the very same physics determining the conditions for super-selectivity makes multivalent targeting of extremely high sensitivity and small changes of some of these parameters can lead to very different outcomes. In turn, this can lead to evolutionary responses that might simply render the system ineffective. For example, a small mutation in a receptor might make its binding energy towards targeting ligands weaker, shifting the required expression threshold at higher values than those experimentally achievable. We thus propose that to make multivalent targeting more robust and indeed precise towards such changes is to target more than one
receptor types using different ligands. In this way, one can make sure that more complex evolutionary adaptation responses must occur before targeting is made ineffective. In doing this, we can make use of the growing amount of bioinformatic data available about cancer-related receptors and their expression in different cancer lines, making a step closer to fulfilling the "big data" revolution expected in the treatment of cancer (55).

We thus propose the design of nanoparticles comprising $\zeta > 1$ types of multiple ligands where each $i$ type is expressed at numbers $(N_i)_i > 1$ on the surface with tether length $(\delta_P h_P)_i$. Each ligand is supposed to target a specific receptor type among those expressed on the surface. Considering that ligands are specific for one type of receptor only, hence competition between different ligands for the same receptor thus not occur, equations 7,8 show that the corresponding free energy of multiplexed and multivalent binding, $(E_{bond})_{MP}$ can be expressed as:

\[
(E_{bond})_{MP} = \sum_{\zeta} E_{bond}(\zeta)
\]  

(17)
where $\zeta$ is an index running over all possible ligand-receptor pairs in the system. The values of $E_{\text{bond}}^{\zeta}$ is given simply by solving equations 7 and 8 considering a sub-system where only that specific ligand/receptor pairs are present (and hence even in this case an analytical solution is available, see Supporting information). In this multiplexed case, one should also account for the different volumes of the various receptors, leading to $\Delta G_{\text{conf}}^{\zeta} = U_P^{\zeta}(\delta_P h_P)$ where we explicitly indicated that the two steric penalties $U_P$ and $U_G$ are different for different ligand-receptor pair $\zeta$. We can hence rewrite equation 15 as:

![Fig. 4 Multiplexing](image)

*Fig. 4 Multiplexing.* Heat maps showing the fraction of bound particle $\theta$ calculated for multiplexed multivalent nanoparticles targeting $\zeta=2$ (a) and $\zeta=3$ (b) for $\zeta>3$ the data are shown using a radar plots with a heat map (c) where multiple receptors can be combined in infinite combinations.
\[
\theta = \left( \frac{3}{\pi N_p [3(R+d\zeta)^2-R^2] \exp(-\beta u_G) \exp\left(-\beta \sum \epsilon_{\text{bond}}(\zeta)\right) + 1} \right)^{-1}
\]

(18)

Where we assumed that \(d\zeta = \delta_p h_p(1) = \delta_p h_p(2) = \ldots = \delta_p h_p(\zeta)\) or in other words that ligand tethers are equal for all the \(\zeta\) receptors/ligands couples. If we had ligands with tethers of different lengths, the situation would be more complicated, as the system would preferably stay at an intermediate distance from the surface. While multiplexing affects the single binding shifting it toward lower receptor densities, the clear advantage comes from the fact we can engineer holistic binding profiles where nanoparticles bind to surfaces only if they express all the targeted receptors at densities above a given threshold as shown in Fig.4 for \(\zeta = 2, 3\) and \(n\). This means that nanoparticles can be designed to target specific cell populations which over-express unique combinations and compositions of receptors. In other words, we can ‘bar-code’ targeting to information gathered from -omic screenings on the specific biological target hence potentially focus interaction to a single cell population. Note that this is different than the approach developed by Curk et al (56) where it is shown to design nanoparticles so as to target a specific distribution of receptors in terms of its relative composition, at a fixed number of receptors. In their case, any change around the targeted distribution would decrease the binding probability. In our case, we look for the design conditions where binding would occur when more than one receptor is expressed above a certain threshold, but anything above that number would still lead to binding, making the system robust towards any biological fluctuations among the different cell populations.

**Experimental validation**

As already anticipated, we decided to use EP polymersomes decorated with angiopep-2 peptides to target the LRP1 receptor (45) and responsible for the transport across the endothelial cells that make the blood brain barrier (34, 35) and with PMPC chains to target the scavenger receptor B1 (SRB1) (36). We prepared several polymersomes formulations which were all purified and characterized by transmission electron microscopy and dynamic light scattering (see Fig.S2). The Angiopep peptide was conjugated to POEGMA-PDPA copolymers and these were mixed at different concentration with pristine POEGMA-PDPA. The resulting arrangement of peptide expressed on the surface and immersed in the oligoethylene oxide chain \((N_p = 10)\) with an average interference parameter of \(\delta_p = 0.8\) as shown in Figs 1 and 2. The PMPC chains were co-polymerized with DPA to form PMPC24-PDPA70 and these were mixed with pristine POEGMA-PDPA chains at different concentrations. It is important to point out that while POEGMA-PDPA and PMPC-PDPA chains can undergo phase separation forming patchy polymersomes (57), the cellular experiments were performed right after preparation and hence without giving the sufficient time to separate (3-5 days). All the formulations had an average radius of 50nm (+/- 10) and the addition of the ligand did not alter the final structure as confirmed by both TEM and DLS. We also labelled about 5% of the POEGMA-PDPA chains with Cy5 dye to allow fluorescence quantification. We incubated different polymersomes formulations for an hour with brain endothelial cells (BEnd3), and macrophages (LADMAC). The total fluorescence per cells was measured using automated imaging analysis on the two cell types by confocal microscopy. We opted for short incubation times to ensure the nanoparticle/cell interaction is kinetically controlled by the binding and while endocytosis is present such a process accounts only for a negligible component of the overall process. In Fig.S3, an example of micrograph used for quantification is shown to illustrate the effective binding of ligand-modified polymersomes to brain endothelial cells. In Figs.5a-c, the average fluorescence per cell measured after 1hr incubation with brain endothelial cells and macrophages. To assess the ability of polymersomes to selectively target a given cell phenotype we define the selectivity index, \(s\), as:

\[
s = \log \left( \frac{F_{BE}}{\max(F_{BE})F_S} \right)
\]

(20)
where \( F_{BE} \) is the average fluorescence per cell in brain endothelial cells, here considered as target, and \( F_S \) is the average fluorescence per cells in macrophages herein considered as sentinel cells. Formulations with \( s > 1 \) interact preferentially with target cells than with the sentinels while \( s < 0 \) do the actual opposite, while those with selectivity index \( 1 \geq s \geq 0 \) are considered indifferent and incapable of distinguishing between target and sentinel. As shown in the data in **Fig. 5a**, the angiopep EP polymersomes interact preferentially with brain endothelial cells compared to the sentinel cells with selectivity peaking to 2.5 at around 30 of average ligand numbers. As expected at higher ligand numbers, the selectivity is lost and polymersomes interact equally with both cell populations. A very different outcome is observed for PMPC chains where, the macrophages show the highest uptake, and with the brain endothelial cells a lower value (albeit with magnitude similar to the angiopep polymersomes). Here the selectivity is negative with regard to the brain endothelial cells. It is worth mentioning that this is also due to the choice of target cells, if macrophages are considered as target, the PMPC chains will show high selectivity as we also demonstrated recently (58). In **Fig. 5c**, we report the cellular uptake of mixed angiopep and PMPC EP polymersomes where we varied the average number of angiopep ligands and fixed the PMPC number at 200. The data shows a similar trend to the angiopep alone, but with a shift of the selectivity from 30 of ligand average number down to 20 indicating that the PMPC chains increases the selectivity of the angiopep making the binding more synergistic the selectivity of the formulation. However, the same can also be said about the macrophages.
where an increase of angiopep ligands shift the binding at 200 PMPC chains a value that did not show detectable uptake with PMPC alone formulations (see Fig. 5b), the multiplexed polymersomes expand the selectivity range to lower numbers of ligands.

If we normalized the data in Figs. 5 for the maximum value, we can reinterpret the data using Hill binding assay measuring cell receptor saturations as a function of the number of ligands. This allows us to fit the experimental data using equation 16 or 19 fixing the number of receptors \( \langle N_R \rangle \) and varying the average number of ligands \( N_L \). As shown in Fig. 6a, the angiopep functionalization results in binding in both cell models.
with uptake of following a sigmoidal trend with the number of ligands confirming the expected high selectivity. The brain endothelial cells reached saturation at lower number of ligands than macrophages suggesting that they express higher number of LRP1 receptor. Experimentally, we have access to the polymersomes radius and the average number of ligands $N_L$. Using the reported dissociation constant of the angiopep with LRP1 (45) i.e. $K_D=313\text{nM}$, we also know that $N_{PEO}=10$, $a_{PEO}=0.35\text{nm}$, $\sigma=3.14\text{nm}$, $R = 50\text{nm}$ (experimentally measured), from Fig. 1, we can estimate $d = 5\text{nm}$, $\delta_P = 0.25$, $\delta_G = 0.7$, LRP1 tip volume $V_P$ =188.4 nm$^3$. Finally, we assume for all cell types a syndecan interchain distance $d_3=20\text{nm}$, in agreement with previously reported data (38). We can thus estimate the LRP1 density for Brain Endothelial cells $<N_R>_{BEC}$ = 18 molecules $\mu m^{-2}$ and Macrophages $<N_R>_M = 13$ molecules $\mu m^{-2}$. We repeated the same experiment using PMPC functionalized EP polymersomes and the result here is rather different with macrophages expressing the highest numbers of SRB1 and the other cells requiring higher number of ligands to reach saturation (Fig.6b). Similarly to angiopep, for PMPC we can assume $\delta_P = 0.1$, $\delta_G = 0.1$, $d_3=20\text{nm}$, SRB1 volume $V_P = 68.4\text{nm}^3$ to estimate the PMPC/SRB1 dissociation constant $K_D = 350\text{nM}$, and the SRB1 for Brain Endothelial cells $<N_R>_{BEC} = 17$ molecules $\mu m^{-2}$ and Macrophages $<N_R>_M = 25$ molecules $\mu m^{-2}$.

This results confirms our previous observations that macrophages (as well as other antigen presenting cells) express high level of SRB1 receptor and that PMPC functionalized polymersomes target them with high selectivity (58). Finally, we formulated three EP polymersomes with both ligands expressed and incubate them with brain endothelial cells. The results, plotted in Fig.6c, show a good agreement between equation 19 and the experimental data. Most importantly, we show that the two ligands act synergistically allowing targeting using ligands numbers that alone will not correspond to any interactions. This result clearly show that, albeit at short incubation times, our theoretical model fits well experimental data and indeed proposed an effective way to design nanoparticles.

Conclusions

We present here a general discussion on how exogenous material interact with a complex biological system presenting a very simple potential term that account for specific and unspecific interaction. We use this as 'rules of engagement' for the design of selective targeting, we thus derive a model adapting the SST theory to a defined multivalent nanoparticle (see Fig.1) equipped with realistic binding energies introducing a non-specific repulsive potential by inserting the ligand within a PEO polymer brush and from the insertion of the nanoparticle into the endogenous expressed glycoprotein network that characterize most cells. Such a strategy was partially validated by Wang and Dormidontova using Monte Carlo simulation where it was shown that the shielding ligands by long chains leads to the an extra loss of entropy at the onset density (59).

Here we build on this and show, using established models for polymer brush steric repulsion to proteins, that we can tune the interaction so as to create the low affinity necessary for super-selectivity as showed in Fig.3. We show that particle size, ligands number, polymer brush length can be computed together with ligand affinity and receptor volume to identify the most efficient formulations to achieve super-selectivity. Finally, we show that the combination of multiple ligands into a multiplexed system can indeed create purely super-selective targeting where multiple over-expressed receptors would be required for binding, increasing the robustness of the proposed targeting platform. We thus use cellular uptake of multivalent and multiplexed polymersomes to validate our theory showing a good agreement between our model and the experimental data. Overall, the model we present provides not only a very powerful tool to design personalized nanomedicines but also give important insights into how biological systems can achieve such high selectivity. Indeed, one can easily extrapolate from the theory herein presented 'rules-of-thumb' to how cells, viruses, bacteria, protein and nucleic acid interact between each other hence adding a powerful tool to the existing system biology approaches.

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Experimental methods

**Polymersomes preparation.** \(P[(\text{OEG})_{10}\text{MA}]_{20}\text{-PDPA}_{100}, \text{Cy5-P[(\text{OEG})_{10}\text{MA}]_{20}\text{-PDPA}_{100}}\) angiopep-P[(\text{OEG})_{10}\text{MA}]_{20}\text{-PDPA}_{100} and PMPC_{25}\text{-PDPA}_{70} copolymers were synthesized as reported in (34). To make 10mg/ml polymersomes, the copolymers were weighed and dissolved using pH 2 PBS. Once the film dissolved the pH was increased to 5.0. Peptide-functionalized copolymers can be added at this point, in order to avoid acidic degradation. The pH was gradually increased to pH 6.8-7.0, eventually stopping at pH 7.4-7.5. Prolonged stirring at pH 6.8-7.0 allowed polymersomes to form. Polymersomes were then ultrasound sonicated for 15-30mins, at 4°C. The purification of polymersomes was finally performed by passing through a gel permeation chromatography column pre packed with Sepharose 4B (Sigma Aldrich). For long-term storage, polymersomes can be kept at 4°C and when conjugated to dyes protected from light. The peptide-functionalized polymersomes were freshly made just before use. Polymersomes were characterized by transmission electron microscopy (FEI Tecnai G2) using phosphotungstic acid as staining agent and dynamic light scattering (Malvern Nanosizer).

**Cell cultures.** Brain endothelial cells bEND.3 cells (ATCC CRL-2299) were seeded on a rat-tail collagen Type I (SIGMA-ALDRICH, C3867) pre-coated T-75 flask, maintained in DMEM medium (Dulbecco’s Modified Eagle’s Medium-high glucose, D5671-SIGMA) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (FCS). Cultures were maintained at 37°C in an atmosphere of 5% CO2 and 95% air and sub-cultured routinely using 0.02% (w/v) EDTA trypsin (5ml, 5min 37 °C, 5% CO2 incubation) once 100% confluence was reached. LADMAC macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA) were cultured in Eagle’s minimal essential medium (EMEM) supplemented with 10% (v/v) FCS and 2 mM L-glutamine.

**Confocal laser scanning microscopy.** Cells were placed on 96plates with image-read bottom plastic, they were treated for 1 hr with the different polymersomes formulations and subsequently their media was replenished. The cells were imaged using confocal laser scanning microscopy equipped with an incubation chamber connected to ZEISS temperature control unit 37-2 and CO2 controller. (1-2 hours before the experiment was allowed for stabilization of the temperature and CO2 concentration). All the confocal laser scanning microscopy was performed on a (ZEISS LSM 510) microscope, equipped with the following lasers: Ar laser, 30mW; HeNe laser, 1mW and HeNe laser, 5mW. The laser excitation wavelengths used were: 405nm (DAPI), and 548nm (Cy5-Polymeromes). The quantification of the fluorescence intensity was performed on normally 30 micrographs per formulation over triplicate experiments. The images were analyzed by ImageJ by creating ad hoc region of interest around the nuclei (pre-stained with DAPI) and measuring the intensity in the Cy5 channel.

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Supporting information

Binding probability for a monovalent ligand on a multivalent target. We derive an equation for the binding probability of a monovalent ligand to a surface coated with multiple receptors. There are various ways to do this, each with a slightly different interpretation. For clarity and to understand the implicit assumptions, we will provide two different derivations, using first a grand-canonical and then a canonical description. One possible derivation can be done by treating ligand binding as a Langmuir adsorption problem (10) where a surface with \( N_T \) adsorbing sites, each of which carries \( N_R \) ligands. In this case, we interpret the fraction of sites with a ligand adsorbed, \( \theta = N / N_T \), as the adsorption probability. Note that since in the Langmuir adsorption problem each site is independent from each other, this is the same as calculating the probability that a single site has a bound ligand attached. Thus, following the classical Langmuir model, we write:

\[
\theta = \frac{aq}{1+aq}
\]  

(51)

where \( a \) is the ligand activity which for dilute solutions is given as \( a \approx N v_0 \). With \( \rho \) being there the number concentration of ligands \( \rho = [L] v_0 \), where \([L]\) is the molar concentration of ligands and \( N_A \) Avogadro’s number and \( v_0 \) the so-called binding volume, i.e. the volume in which a ligand must be confined to be considered bound to the site. Note that \( q \) is formally the ratio of the partition function for a bound ligand with respect to an unbound, one once the ligand has been confined within a volume \( v_0 \) around the receptor. In other words it is the change in free-energy if we turned on the interaction potential between the ligand and the receptor, keeping the ligand within the binding volume. If there are \( N_R \) receptor per site, and a ligand can only bind one of them at any one time, we have that:

\[
q = N_R \exp(-\beta \Delta G_0)
\]  

(52)

where \( \beta = 1/k_B T \) and \( \Delta G_0 \) is the ligand-receptor binding free-energy. In order to understand the exact meaning of these quantities, it is important to give a proper definition of this energy. The value of \( \Delta G_0 \) is linked to the internal partition function of ligands and receptors in the bound state, \( z_{LR} \), compared to the free ones, \( z_L \) and \( z_R \), respectively. More precisely, if \( M \) and \( m \) are the degrees of freedom of a ligand and receptor, respectively, \( z_{LR} \) is the \( m + M - 6 \) dimensional integral of \( \exp(-\beta \mathcal{H}(\{x\})) \), \( \mathcal{H} \) being the Hamiltonian of the system, over all the degrees of freedom \( \{x\} \) of the bound ligand-receptor complex once its centre of mass has been fixed and the ligand (or, by symmetry, the receptor) is furthermore confined within a volume \( v_0 \) around the receptor. Similarly, \( z_L \) and \( z_R \) are the \( m - 3 \) and \( m - 3 \) dimensional integral over the degrees of freedom of the ligand and receptor, respectively, once their centre of mass has been fixed in space. With these definitions, we have that:

\[
\exp(-\beta \Delta G_0) = \frac{z_{LR}}{z_L z_R}
\]  

(53)

How do the microscopic quantities in Eq.53 relate to experimentally measurable parameters? An equation connecting microscopic and macroscopic quantities can be derived by comparing the microscopic definition of the chemical potential for a bound ligand receptor pair and an unbound ligand or receptor to their thermodynamic definition. The derivation is a bit long and we will not reproduce it here but it can be taken from [Leunissen et al, J. Am. Chem. Soc. 2010, 132, 1903–1913]. This provides the following equation:

\[
\exp(-\beta \Delta G_0) v_0 = K_A = \frac{\exp(-\beta \Delta G_{bind})}{\rho^*}
\]  

(54)

where \( K_A = K_D^{-1} \) is the experimentally measurable equilibrium association constant for free ligands and receptors in solution (\( K_D \) being the dissociation constant), while \( \rho^* = 1 M \) is the standard concentration of ligands and receptors at which the equilibrium constant is measured. The presence of this concentration in
the equation connecting the binding energy and the association constant is very important but often creates confusion. We will come back to this aspect later, let us first comment on the meaning of equation S4 and provide a final expression for the binding probability \( \theta \). Equation S4 makes explicit that the thermodynamically-defined binding free-energy for the reaction binding between ligand and receptors \( L + R \rightarrow LR, \Delta G_{bind} \), is not exactly the bond energy \( \Delta G_0 \) as defined here, and it actually depends on the definition of the chosen value for \( v_0 \). This should not really come as a surprise. In an experiment, the equilibrium concentration of bound and unbound complexes can be measured to calculate the equilibrium association constant from the definition \( K_A = \frac{[LR]}{[L][R]} \), where \([LR]\) and \([L][R]\) are the equilibrium molar concentration of bound ligand-receptor pairs and unbound ligands(receptors), respectively. However, clearly what we define as bound or unbound will depend on what cutoff distance we decide to choose to decide that two reactants A and B are forming an AB complex, which defines our volume \( v_0 \). Now by substituting Eqs S3 and S4 into Eq. S1 we finally obtain:

\[
p_B = \theta = \frac{\rho v_0 N_R \exp(-\beta \Delta G_0)}{1 + \rho v_0 N_R \exp(-\beta \Delta G_0)} = \frac{\rho N_R K_A}{1 + \rho N_R K_A} \tag{S5}
\]

which is our final expression for Eq.1 in the main text. Let us now quickly see where the factor \( \rho^* \) comes from, which is the source of confusion and can make expression appear dimensionally not correct, if the exact definition or interpretation of concentrations or densities is not given. Binding between a ligand and receptor in solution can be described as a bimolecular reaction \( L + R \rightarrow LR \), for which the textbook definition of the equilibrium constant is:

\[
K_A^p = \frac{[LR]}{[L][R]} = \exp(-\beta \Delta G_{bind}) \tag{S6}
\]

This expression makes sense only if \( K_A^p \) (the superscript \( p \) will be clear in a second) is non-dimensional, which also means that all the concentrations \([L],[R]\) and \([LR]\) must be non-dimensional, which is true if they are intended as scaled by some reference value. However, the association constant is typically reported in inverse molar \( M^{-1} \), creating confusion. The difference is what has been sometime referred to as the ``physics'' vs the ``chemistry'' definition of the association constant. When writing Eq. S6, \([L],[R]\) and \([LR]\) in fact are not the molar concentration, but the molar concentration scaled by the reference value of the ``reactants'' \( L \) and \( R \) at which the equilibrium association constant is measured. By convention, this value is \( \rho^* = 1 M \). If we insist to interpret \([L],[R]\) and \([LR]\) instead as unscaled concentrations, the correct expression for Eq. S6 is:

\[
K_A^p = \frac{[LR]/\rho^*}{[L]/\rho^*[R]/\rho^*} = \frac{[LR]/\rho^*}{[L][R]} = \exp(-\beta \Delta G_{bind}) \tag{S7}
\]

or in other words:

\[
\frac{[LR]}{[L][R]} \equiv K_A^c = K_A^p/\rho^* = \frac{\exp(-\beta \Delta G_{bind})}{\rho^*} \tag{S8}
\]

where \( K_A^c \) is now what we called the ``chemistry'' definition of the association constant and has indeed the recognisable dimensions of an inverse concentration \( M^{-1} \).

We now shown another possible route to derive Eq.1, this time using a ``canonical'' approach rather than the grand-canonical one implicit in the derivation of the Langmuir isotherm, Eq S1. We do that because it helps
clarify some of the assumptions we are making in defining this binding probability. Consider $N$ monovalent ligands in a volume $V$ and a target bearing $N_R$ receptors. We assume that, if one of the $N_R$ receptors is occupied, then no other ligand can bind to the other $N_R - 1$ receptors present, e.g., due to steric repulsion. We consider dilute solutions, where we can assume ligands in the bulk are almost never in proximity hence their partition function will be that of an ideal gas. Ligands instead gain an energy $\Delta G_0$ upon binding with a receptor (i.e., when they are within a volume $V_0$ containing the receptor). Given these assumptions, the bound partition function $q_B$ can be calculated considering all configurations where one of the ligands is bound to any of the receptors, and in this case is confined in a volume $V_0$ around it, whereas the other $N - 1$ ligands are free to float in the rest of the volume $V - N_R V_0$. This is given by:

$$q_B = \frac{1}{(N-1)!} (V - N_R V_0)^{N-1} [N_R V_0 \exp(-\beta \Delta G_0)]$$ (59)

where the factor $\frac{1}{(N-1)!}$ comes from the fact that we consider indistinguishable ligands, although we can still tell apart whether a ligand is bound or unbound (for example, checking that it is not in the volume $V_0$ where it can interact with the receptor). Note that if we want to assume that all ligands are distinguishable nothing changes, as long as we do that consistently for both the bound and unbound state, and we would have exactly the same final expression, as shown later.

The unbound partition function $q_U$ is when no ligand is bound, which means they are all free to float outside the volume where they interact with the receptor, thus giving:

$$q_U = \frac{1}{N!} (V - N_R V_0)^N$$ (S10)

Now the probability to observe the bound state is given by:

$$p_B = \frac{q_B}{q_B + q_U} = \frac{\frac{1}{(N-1)!} (V - N_R V_0)^{N-1} (N_R V_0 \exp(-\beta \Delta G))}{\frac{1}{N!} (V - N N_R V_0)^N + \frac{1}{(N-1)!} (V - N_R V_0)^{N-1} (N_R V_0 \exp(-\beta \Delta G))}$$

$$= \frac{\frac{1}{V - N_R V_0} N_R V_0 \exp(-\beta \Delta G)}{1 + \frac{1}{V - N_R V_0} N_R V_0 \exp(-\beta \Delta G)} = \frac{N_R V_0 \exp(-\beta \Delta G)}{1 + N_R V_0 \exp(-\beta \Delta G)} = \frac{\rho N_R K_A}{1 + \rho N_R K_A}$$ (S11)

where the approximation we used is that $V \gg N_R V_0$, or in other words the binding volume occupied by the receptors is a very small fraction compared to the bulk volume of the solution. Finally, we also replaced $N/V = \rho$ and $V_0 \exp(-\beta \Delta G) = K_A$. Clearly, Eq.S11 is exactly the same equation we had derived in the grand-canonical ensemble starting from Eq 1, which was interpreted as the probability for a binding site in the Langmuir picture to be occupied by a ligand (note the analogy between a single target in the canonical ensemble here and an adsorption site in the Langmuir description, each of which bears $N_R$ receptors). We note here that, as previously stated, if all ligands were considered completely distinguishable, nothing would have changed. In the definition of the bound partition function, Eq. S9, the factor $\frac{1}{(N-1)!}$ would have been replaced by a factor of $N$, the number of distinguishable configurations with one bound ligand. At the same time, the unbound partition function would have had no $\frac{1}{N!}$ factor at all, since this comes from indistinguishability of particles. Overall, this gives again the same final expression for $p_B$. One curiosity about this derivation is that it makes clear that we are not counting the contribution to the bound state coming from configurations where more than a single ligand is bound at the same time to the target (which is also an assumption in the derivation of the classical Langmuir model, Eq.S1). In practice, this means the expression we provide is valid for low bond energies / low ligand concentrations, where this probability is small. In any case, due to this approximation, Eq. S9 actually represents an upper bound to the probability of a single target with multiple receptors to have at least one ligand bound to it.
**Single bond derivation** We now specialise the case of equations 7 and 8 in order to provide an explicit value for the system under consideration. If a single ligand-receptor pair type \( \zeta \) is present, we obtain:

\[
\frac{E^{\zeta}_{\text{bond}}}{k_B T} = N_{L,\zeta}[\ln(p_{L,\zeta} + \frac{1}{2}(1 - p_{L,\zeta})] + N_{R,\zeta}[\ln(p_{R,\zeta}) + \frac{1}{2}(1 - p_{R,\zeta})]
\]

S.12

where \( N_{L,\zeta} \) is the number of ligands on a nanoparticle that can bind to the corresponding \( N_{R,\zeta} \) receptors on the surface of the target and the subscript \( \zeta \) specifies a possible pair. Note that \( N_L \) is not necessarily the total number of ligands on the surface of the nanoparticle, as also pointed out by Martinez-Veracochea and Frenkel, (11) but only those that can, due to the nanoparticle orientation, bind to the surface. In Eq. S.12, \( p_{L(R),\zeta} \) is the probability that a ligand(receptor) is unbound, which for a single type of ligand-receptor pair present in the system is given by the following system of coupled equations:

\[
\begin{align*}
    p_L + N_R p_R p_L \chi - 1 &= 0 \\
    p_R + N_L p_L p_R \chi - 1 &= 0
\end{align*}
\]

S.13

where we have dropped the subscript \( \zeta \) for simplicity. In writing Equation S13, we assumed that each ligand can bind to each of the receptors on the target *i.e.* the so-called radial topology of binding(7). Other binding topologies can be similarly considered without changing the qualitative features of the results obtained (11). The only physical solutions of the system in Equation S13 is:

\[
p_L = \frac{(N_L-N_R)\chi^{-1} + \sqrt{4N_L\chi + 1+(N_R-N_L)\chi}}{2N_L\chi}
\]

S14

\[
p_R = \frac{(N_R-N_L)\chi^{-1} + \sqrt{4N_R\chi + 1+(N_L-N_R)\chi}}{2N_R\chi}
\]

S15

whose substitution in Equation S.12 gives the binding free-energy. For the multiplexed case, equation S.12 is still valid for each ligand-receptor pair if one assumes that each ligand type can only bind a specific type of receptor. In other words, either no or weak cross-interactions with non-cognate receptors must be present. In this case, one can simply solve the same set of coupled equations for each ligand-receptor pair separately and sum up the resulting free-energy contribution over all possible pairs to provide the total binding energy.
Fig. S1 Scaling principles in super-selectivity continued. Heat maps showing the fraction of bound particle \( \theta \) as a function of the numbers of receptors \( <N_R> \) and number of the polymer interference parameter \( \delta_p \) (a) the glyocalyx spacing, \( d_G \) (b) and particle concentration \([P]\) (c). Each map was analysed to calculate the selectivity \( \alpha_{\text{max}} \) and the corresponding \( <N_R>_{\text{onset}} \) and the graph of these as function of the varying parameter are reported alongside.
**Fig.S2 Polymersome characterisation.** Particle size distributions measured by dynamic light scattering for POEGMA-PDPA/Angiopep (a) POEGMA-PDPA/PMPC (b) and POEGMA-PDPA/PMPC + Angiopep (c) polymersomes. Representative transmission electron micrographs of POEGMA-PDPA/Angiopep (25 ligands) and POEGMA-PDPA/PMPC (1000 ligands) formulations.
**Fig. S3 Polymersome cellular uptake.** Micrograph of polymersome bearing 22 angiopep2 ligands uptake in brain endothelial cells (BEnd3) after 1hr incubation. Note the polymersomes were labelled by Cy3 (Red) and the cell DNA by DAPI (Blue)