Molecular printboards: versatile platforms for the creation and positioning of supramolecular assemblies and materials

Manon J. W. Ludden,[a] David N. Reinhoudt[a] and Jurriaan Huskens*[ab]

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This *tutorial review* describes the development of molecular printboards, which are tailor-made surfaces functionalized with receptor (host) molecules. Such substrates can be used for the binding of complementary ligand (guest) molecules through multivalent interactions. Supramolecular multivalent interactions are ideal to attain a quantitative and fundamental understanding of multivalency at interfaces. Because of their quantitative interpretation, the focus is on (i) the interaction of cyclodextrin host surfaces with multivalent hydrophobic guest molecules, (ii) the vancomycin–oligopeptide system, and (iii) the multivalent binding of histidine-tagged proteins to NiNTA receptor surfaces. The review will be of interest to researchers in the fields of supramolecular chemistry, chemical biology, surface chemistry, and molecular recognition.

1. Introduction

The benefits of nanotechnology arise from the new materials properties that emerge when matter is structured at the nm scale. Whereas some properties already become apparent when inspected with a bulk technique, as for example the optical properties of nanoparticles in a solution, more sophisticated science, and therefore applications, are involved when (i) use is made of the properties of individual nanostructures, and/or when (ii) information processing occurs between nanostructures. In the former case, for example for the biomedical use of nanoparticles, the outer surface of the nanostructure needs to be equipped with specific functional groups which allow the

[a]*Laboratories of Supramolecular Chemistry & Technology and Molecular Nanofabrication, MESA+ Institute for Nanotechnology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands. E-mail: j.huskens@utwente.nl; Fax: (+31) 53-4894645*
[b]*MESA+ Strategic Research Orientation "Nanofabrication", MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands*

Jurriaan Huskens

Jurriaan Huskens (1968) obtained his PhD with Prof. Herman van Bekkum and Joop Peters from the Delft University of Technology, in 1994. After postdoctoral stays with Prof. Dean Sherry and Prof. Manfred Reetz, he became an Assistant, and later Associate, Professor in the group of Prof. David Reinhoudt in 1998 at the University of Twente. Since September 2005 he has been full professor Molecular Nanofabrication at the same university. Present research interests encompass: supramolecular chemistry at interfaces, multivalency, supramolecular materials, and nanofabrication methodologies.

Manon J. W. Ludden

Manon J. W. Ludden (1977) studied Chemistry at Leiden University, the Netherlands. In 2002 she obtained her undergraduate degree with Professor Jan Reedijk. She became a PhD student in the group of Professor David N. Reinhoudt in the same year. Her PhD work involves the selective and controllable attachment of proteins to surfaces.

David N. Reinhoudt

David N. Reinhoudt (1942) obtained his PhD in 1969 with Prof. H. C. Beijerman (Delft University of Technology). He was appointed as a part-time professor in 1975 at the University of Twente, and became full professor in 1978. His research is focused on supramolecular chemistry and technology with nanofabrication, molecular recognition, and non-covalent combinatorial synthesis as the major fields. He is the scientific director of the MESA+ Institute for Nanotechnology. Since 2002 he has been the chairman of the Board of NanoNed, the Dutch Network for Nanotechnology. He is the author of more than 800 scientific publications, patents, review articles, and books. He has been honored with the Izatt–Christensen award (1995), the Simon Stevin Mastership (1998) and Knight of the Order of the Dutch Lion (2002).

formation of desired interactions. In the latter case, for example in molecular electronics, surface attachment and positioning are of paramount importance in order to predetermine how and in which directions the information processing can take place.

Thus in all cases, the interface chemistry of the nanostructures and/or the substrates onto which they are placed is the key to the functioning of the individual nanostructures or of the devices that are composed of them. Fine tuning of the specificity and of the interaction strength of the interactions that occur at this interface between the nanostructure and its environment are of utmost importance. Usually, when designing a functional nanosystem, the perspective is from the nanostructure and its properties, and the interface is changed *ad hoc* to go as easy and quickly as possible to the functional device structure. Conceptually, however, it is attractive to decouple the interface design from the nanostructure fabrication in order to have the best engineering control over the interface properties. This implies the development of generally applicable interface chemistries for the fine tuning of assembly and interaction properties of the nanostructures.

Having the concept of controlled positioning of molecules, assemblies and particles on substrates in mind, parameters such as binding stoichiometry, binding strength, binding dynamics, packing density and order, and reversibility emerge as important tuning parameters. Covalent immobilization does not offer sufficient flexibility over most of these criteria. Physisorption or chemisorption do offer reversibility and error correction and therefore the potential of dense packing with high order, but the predictability of binding stoichiometry and thermodynamic binding parameters is small and thus the practical control is limited. Supramolecular interactions, for example of designed host–guest or receptor–ligand types, constitute a solution to the control of these parameters.

Self-assembled monolayers offer an easy way to the immobilization of receptors and ligands for such receptors. The fixation to a substrate automatically leads to a multivalent display of such supramolecular interaction sites, the density of which is a separate control parameter in the binding of (multivalent) complementary binding partners. Multivalency,[1,2] which describes the interaction between multiple interacting sites on one entity with multiple interacting sites on another, is therefore the underlying principle governing the stabilities and dynamics of such systems and offers the main way to control the binding properties of any entity binding to a substrate, *i.e.* through systematic variation and optimization of the number of interacting sites, the intrinsic binding strength of an individual interacting pair, and the geometry of the multivalent building blocks.

Apart from the nanotechnological implications described and reviewed here, multivalency has a profound impact on biology.[1] Contacts between cells and viruses or bacteria are initiated by multivalent protein–carbohydrate interactions. Whereas their monovalent parent interactions are fairly weak, the combined multivalent display at such biological interfaces makes the interactions strong, so that true recognition occurs which is the onset of endocytosis. Qualitatively this pathway is quite well understood, but quantitative details often lack for such systems. The supramolecular interface systems reviewed here can thus be seen as model systems for biological interfaces and their study can lead to a more quantitative understanding of multivalent binding at their biologically more relevant sister systems in the biochemical field.

In this review we cover only those systems for which quantitative data on multivalent binding have been described and interpreted. Apart from our own cyclodextrin-based host–guest chemistry at interfaces between substrates and aqueous solutions, the vancomycin–oligopeptide and the NiNTA–His-tag systems, reported by the Whitesides and Tampé groups, respectively, are covered here as well. Although the concept of multivalency at interfaces is introduced *via* the more well-spread multivalency in solution, we give only examples which have direct analogs with existing surface systems, in part also because such multivalent solution systems have been reviewed before.[1–3] After explaining how a quantitative interpretation of multivalent systems at interfaces can be obtained, which is the key to the control over the binding properties of designed supramolecular adsorbing entities, the more practical nanotechnological implications for positioning and materials assembly are reviewed.

2. Multivalency

Multivalency describes the multivalent interactions that occur between a multivalent host and a multivalent guest.[1–3] The most simple situation occurs when a divalent guest and a divalent host interact to form a divalent 1 : 1 complex. Multivalent systems are characterized by (an) intra-complex (further simply called intramolecular) assembly step(s) following an initial, intermolecular binding event. This makes such systems distinctly different, both thermodynamically and kinetically, from monovalent (between two monovalent entities) and multiply monovalent (between a multivalent and multiple monovalent entities, *e.g.* binding of oxygen by haemoglobin) systems which lack such intramolecular steps (Fig. 1).

A quantitative comparison between the inter- and intramolecular binding events in a multivalent interaction can most clearly be accomplished by adopting the effective concentration or effective molarity terminology.[2] Effective concentration

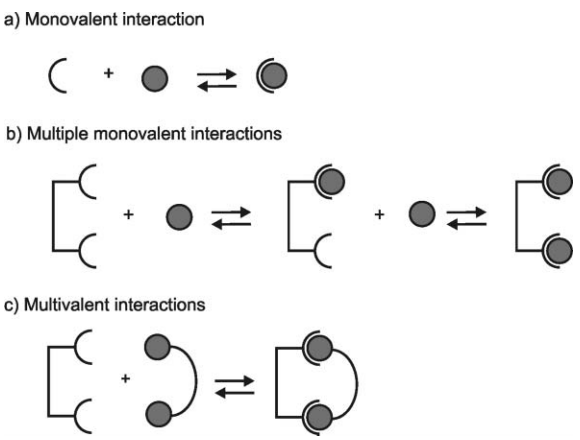

Fig. 1 Schematic modes of binding for monovalent, multiple monovalent, and multivalent interactions.

represents a probability of interaction between two complementary, interlinked entities and symbolizes a "physically real" concentration of one of the interacting functionalities as experienced by its complementary counterpart. The concept of effective concentration originates from the field of polymer chemistry where it was introduced to account for intramolecular cyclization reactions in polymer synthesis. Effective concentration is conceptually similar to the more generally used effective molarity (*EM*).[4] Whereas effective concentration is based on concentrations calculated or estimated from physical geometries of complexes, effective molarity denotes the ratio of intra- and intermolecular rate or association constants.[4]

We have argued before[2,5,6] that, although effective concentration and effective molarity are conceptually very closely related, it can be beneficial to keep the two terms separated in the analysis of quantitative thermodynamic data for multivalent systems. When effective molarity is used as an empirical quantity relating the overall stability constant of the multivalent system to the one of the monovalent parent system, the effective concentration is then the theoretical prediction, *e.g.* from molecular modeling incorporating linker lengths, flexibilities, *etc.*, of that quantity, and thus provides a theoretical estimate what *EM* should be when only statistical, entropic, multivalency factors are taken into account. Therefore the comparison between the two provides a handle to evaluate whether additional, cooperative effects occur: when $EM = C_{\mathrm{eff}}$, the data can be explained by assuming independent, non-cooperative interactions only, while when it is observed that $EM \neq C_{\mathrm{eff}}$, this may indicate the existence of positively ($EM > C_{\mathrm{eff}}$) or negatively ($EM < C_{\mathrm{eff}}$) cooperative effects. Note that cooperativity implies a change of interaction strength upon occupation of a neighboring binding site. Cooperativity effects or the lack thereof are otherwise notoriously difficult to ascertain quantitatively in multivalent systems compared to multiply monovalent systems for the latter of which tools such as Scatchard and Hill plots have been very useful. It has been shown that such tools fail to work for multivalent systems,[7] because of the occurrence of intramolecular binding events, as noted above.

Many quantitatively investigated solution systems have been described, but only in rare cases an attempt is made to dissect possible multivalency and cooperativity effects. An analysis as described above has been made to describe the thermodynamics of binding of the divalent complex between a bis-adamantyl calix[4]arene guest and a bis-cyclodextrin host (Fig. 2).[5] The comparison between the overall 1 : 1 binding constant of this divalent complex and the intrinsic monovalent binding constant yielded an effective molarity of approx. 3 mM, while a C_{eff} of (minimally) 2 mM was estimated from the linker lengths of the guest and host in the monovalently linked intermediate. This excellent agreement, together with the fact that the binding enthalpy of the divalent complex was twice the value for the monovalent complex, led to the conclusion that this divalent system could be well described with multivalency effects only, thus without cooperativity. A similar reasoning has been found to hold for other solution systems as well.[2]

The interaction between vancomycin (Van) and D-alanine-D-alanine and D-alanine-D-lactate and multivalent derivatives thereof has been thoroughly studied by Whitesides and

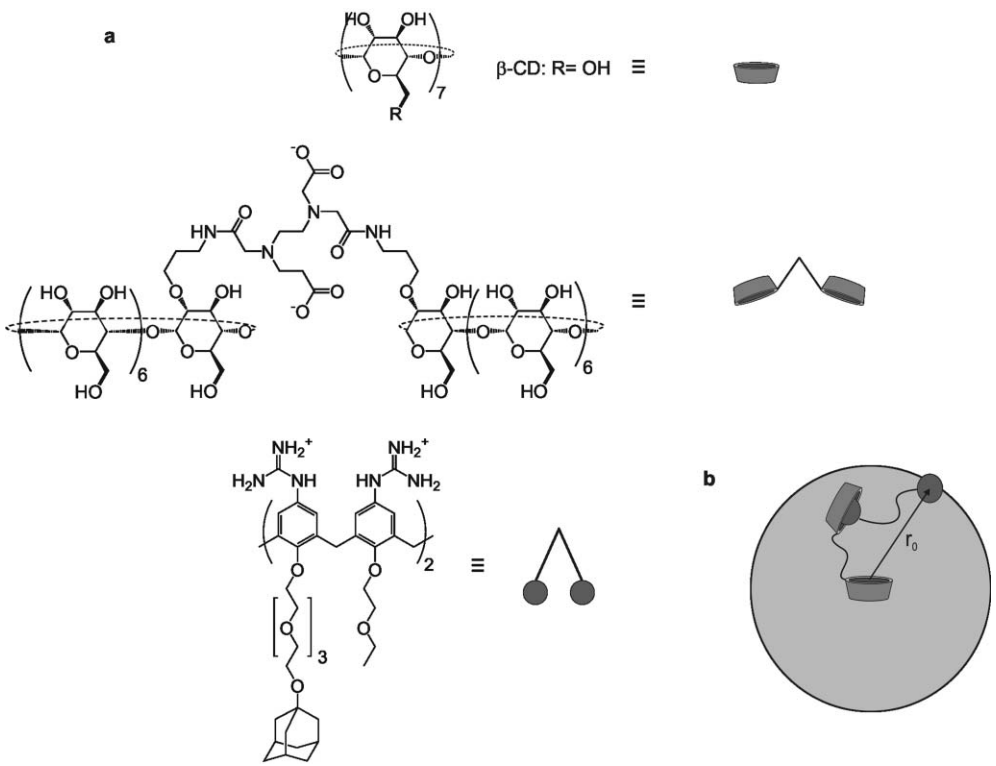

Fig. 2 (a) Host and guest molecules: β-cyclodextrin (CD), an EDTA-linked CD dimer, and a bis(adamantyl)-calix[4]arene;[5] (b) schematic representation of the concept of effective concentration for the interaction between the EDTA-linked CD dimer, and the bis(adamantyl)-calix[4]arene in solution.[5,6]

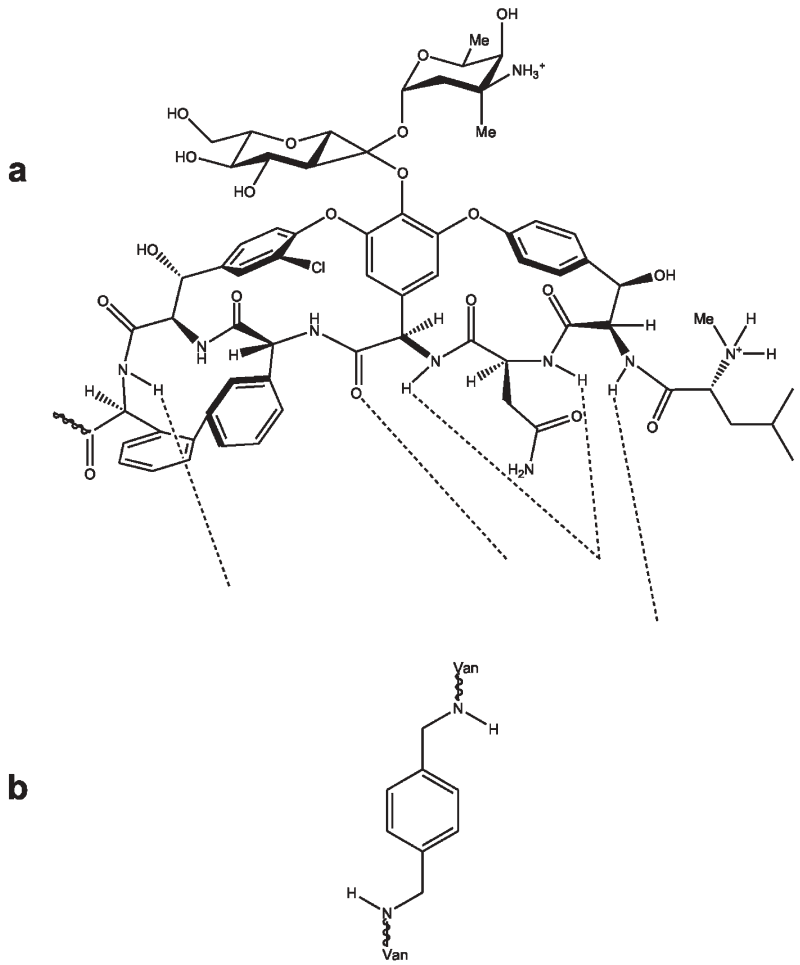

Fig. 3 Structures of vancomycin (a) and dimeric vancomycin (b). Dotted lines indicate possible hydrogen bonds for ligand interactions.

coworkers.[8–13] Vancomycin (Fig. 3) is an important member of the group of glycopeptide antibiotics that are active against Gram-positive bacteria. The D-Ala-D-Ala motif represents the carboxy-terminus of Gram-positive bacteria that are susceptible to vancomycin, whereas the D-Ala-D-Lac motif represents the carboxy-terminus of Gram-positive bacteria that are resistant to vancomycin. Solution studies revealed that the interaction between Van and D-Ala-D-Lac is much weaker than the interaction between Van and D-Ala-D-Ala, due to the absence of one hydrogen bond. It was also shown that a dimeric derivative of Van, Van-R_d-Van (R_d = $NHCH_2C_6H_4CH_2NH$; Fig. 3), binds more strongly to a divalent D-Ala-D-Lac derivative by means of multivalency.

In immobilized metal affinity chromatography (IMAC), the *N*-nitrilotriacetate- (NTA-) His_6-tag chelator couple is a powerful tool for the purification of proteins. A protein with a (small) histidine tag, which is a short fused sequence of histidines, is purified by its ability of binding to a Ni^{2+}–NTA complex. NTA is a tetradentate ligand which forms a hexagonal complex with divalent metal ions like Ni^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}.[14] This way, four of the six binding sites at the metal ion are occupied, leaving two binding positions available for binding to a His_6-sequence, as depicted in Fig. 4. This interaction can be reversed by the addition of imidazole or EDTA. Especially this reversibility makes the system interesting because it allows the reversible attachment of proteins to surfaces. At present, the NiNTA–His-tag strategy is more and more applied in controlled immobilization, that is in orientation-specific binding and 2D organization of proteins at interfaces.[15–17] Multivalent attachment of histidine-tagged proteins is an important issue, since it allows for the stable and specific attachment of proteins to these surfaces.

High affinity receptors were designed by incorporating multiple NTA moieties into single molecular entities.[18] The binding process of oligo-histidine tags (His_6 or His_{10}) to such entities with 1–4 NTA headgroups, leading to 2–8 possible coordination bonds, was studied in detail.[18] His_6 and His_{10}

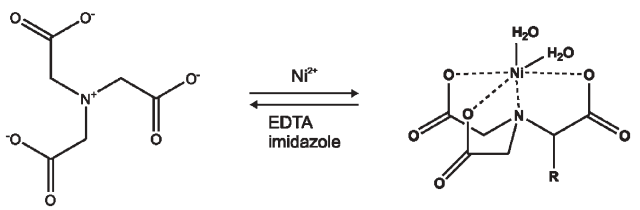

Fig. 4 *N*-Nitrilotriacetate is converted into the NiNTA complex by adding Ni^{2+}. Complexation can be reversed by adding EDTA or imidazole.[14]

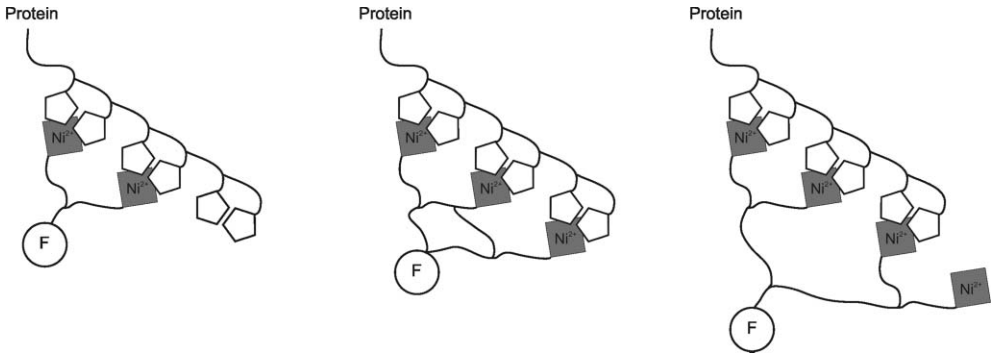

Fig. 5 Schematics of valencies and redundancy for interactions between a His_6-tagged protein and di-, tri-, and tetravalent NiNTA receptors: (a) redundancy of the His groups of the His_6 tag, (b) maximum valency, no redundancy, (c) redundancy of chelator groups.[18]

have 6 and 10 coordination possibilities, respectively. This means that theoretically the number of histidines on a tag are fewer or more than the demand of the chelators, which is called redundancy (Fig. 5). The multivalency principle dictates an increase of the complex stability with increasing valency. The system, however, remains switchable, as addition of EDTA or imidazole led to decomplexation.

Isothermal titration calorimetry (ITC) experiments revealed several issues. One of the consequences of redundancy is that complex stoichiometries depend on the absolute concentrations of the binding partners. Low concentrations of binding partners led to 1 : 1 complexes but at higher concentrations or a deficiency in one of the binding partners, other complex stoichiometries could not be excluded. ITC also showed that for higher valencies a substantial redundancy in histidines was required to reach an enthalpy of binding in line with the expected number of coordination bonds. This indicates that steric constraints interfered with full coordination. For the multivalent complexes, high entropy losses were reported. This is assigned to a huge loss of conformational freedom of the flexible spacers upon complexation.

3. Multivalency at interfaces

Several years ago, we reported self-assembled monolayers of a β-cyclodextrin (CD) heptathioether derivative on gold (Fig. 6),[19] at that stage a logical extension of the ongoing efforts to immobilize various receptors on surfaces, *e.g.* for sensor development. These CD SAMs have been extensively

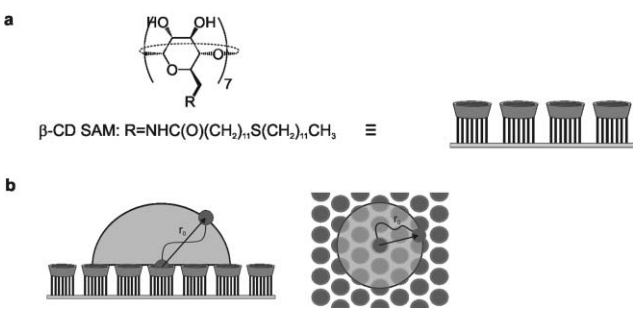

Fig. 6 (a) CD SAM on gold;[19] (b) schematic representation of the concept of effective concentration for the interaction between the bis(adamantyl)-calix[4]arene (Fig. 2) and the CD SAM.[5,6]

characterized with a plethora of analytical techniques. The main conclusions were that the molecules formed a monolayer with the secondary sides of the CD ring exposed to the solution, that the SAMs were comparatively well-ordered, and that they were densely packed in the alkyl regions of the adsorbate, leading to a CD cavity lattice periodicity of approx. 2 nm, which was confirmed by AFM.[19]

Initial host–guest studies, performed with small monovalent guests, showed that the molecular recognition properties of the CD cavities were unaltered by the surface immobilization as was shown (i) by the identical stability constants obtained for these guests in binding to the CD SAMs and to native CD in solution,[20] and (ii) by AFM pull-off experiments with a variety of guests immobilized on an AFM tip.[21] The association and dissociation were fast on the experimental timescales, as was to be expected for such monovalent systems, providing rapid reversibility to the system which was thought to be beneficial when sensor systems were envisaged.

Only for larger steroidal guests, an influence of the alkyl portion of the CD adsorbate was observed,[20] most likely due to the fact that, in the case of binding to native CD, such guests normally protrude from the cavity at both sides of the CD molecules. In this study,[20] when using a different SAM architecture in which a monothiol CD derivative was used, we observed for the first time indications for binding of (the same steroidal) guests by two immobilized CD cavities simultaneously.

A paradigm shift of the view of these CD SAMs occurred upon the initial work[22] using adamantyl-functionalized poly-(propylene imine) (PPI) dendrimers (Fig. 7). Slower dissociation kinetics, and thus a shift from reversible to irreversible binding was observed upon increase of the dendrimer generation and thus the number of interactions with the CD SAMs. For the larger dendrimer generations, even individual dendrimer molecules could be visualized using AFM showing that they were attached strongly enough to withstand the forces exerted by the AFM tip. At that moment it became apparent that such receptor-functionalized surfaces, rather than being rapidly interchanging sensor substrates, could be used as assembly platforms for larger entities with considerable complexation lifetimes. Hence, we coined the term "molecular printboards".[22]

It will become clear that the ultimate key to the control over binding thermodynamics and kinetics and even to

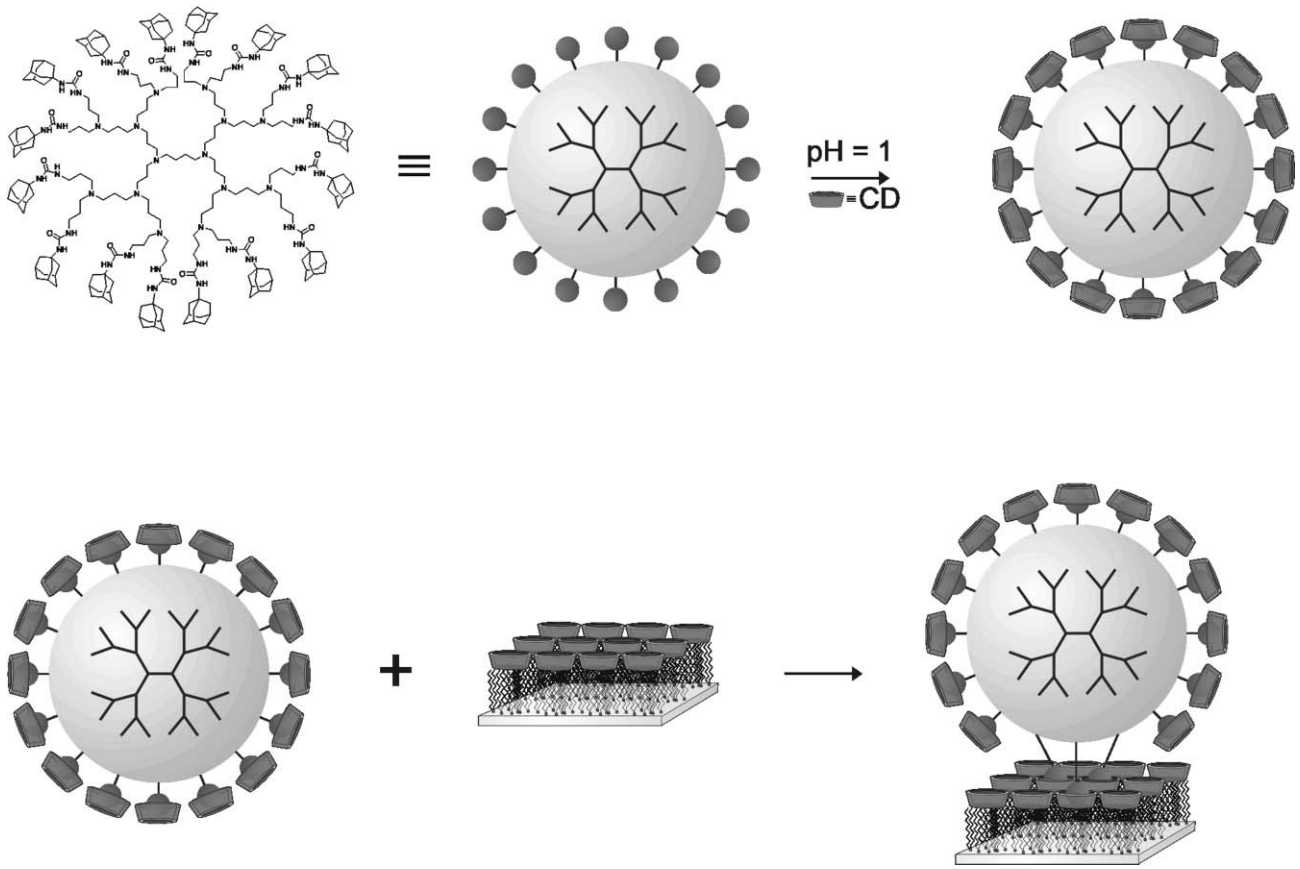

Fig. 7 Generation-3 adamantyl-functionalized poly(propylene imine) dendrimer and the formation of water-soluble assemblies by CD complexation (top), and the adsorption of these assemblies onto a CD SAM on gold (bottom).[22]

stimulus-dependent control arises from multivalency. By tuning the type of monovalent interaction, the number of such interactions, and the epitope density and their geometry on both multivalent guest and host platforms, one can vary the association and dissociation rates practically at will so that the whole range from labile to stable complexes can be accessed. Somewhat to our initial surprise, the number of interactions needed to obtain kinetically stable assemblies can be rather low, even for interaction motifs with moderately weak intrinsic interaction strengths.

The divalent calix[4]arene guest described above in the solution systems was also studied regarding its binding to the CD SAMs.[5] A clear distinction with the solution system was the fact that the overall binding constant at the CD SAM was 2–3 orders of magnitude larger than in solution. This effect was, however, again fully attributable to multivalency: the stability constant increase (from about 10^7 M^{-1} for binding to the CD dimer, shown in Fig. 2, to 10^9–10^{10} M^{-1} for binding to the CD SAM) was fully due to a higher effective concentration (of approx. 0.2 M) at the CD SAM because in the (smaller) probing volume, compared to solution, resided a larger number of accessible host molecules (Fig. 6).

The interaction between vancomycin (Van) and D-Ala-D-Ala and D-Ala-D-Lac has also been studied at surfaces. Whitesides and coworkers developed mixed SAMs on gold that consist of adsorbates with N^{α}-Ac-L-Lys-D-Ala-D-Ala (L*) and with carboxylic acid groups, the mole fractions of which were both about 0.5.[10,23] To these monolayers, the adsorption of Van was compared to the adsorption of a divalent Van derivative (Fig. 3) by means of surface plasmon resonance (SPR) spectroscopy.[24–26] These SPR experiments indicated that the binding of Van to L* at a SAM was comparable to binding in solution, a similar conclusion observed above for small guests binding to CD SAMs. It was also established that binding of the divalent Van derivative to the SAM was much stronger than the binding of the monomeric Van derivative, and that the interaction was biospecific.

Similar mixed SAMs on gold, consisting of adsorbates with N^{α}-Ac-L-Lys-D-Ala-D-Lac (L*[2]) and with carboxylic acid groups, were prepared in order to mimic the surfaces of cells that are resistant to Van. The affinity of Van for such SAMs was 300-fold less than for SAMs consisting of L*. The divalent Van derivative, however, interacted much more strongly with these surfaces; the dissociation rate was about 100 times slower than that of Van. This supports the hypothesis that multivalency contributes to the antibacterial activity against vancomycin-resistant bacteria.[27]

Tampé and coworkers combined the possibilities of the NiNTA–His-tag couple with self-assembled monolayers (SAMs). They designed chelator lipids for the reversible immobilization of engineered proteins at self-assembled lipid interfaces.[28] Chelator-lipid monolayers have some advantages for the immobilization of proteins at surfaces, such as (i) the possibility of coating nearly every surface by various

techniques, (ii) the lateral organization and pattern formation because of the phase behavior of these lipid films,[29] and (iii) their biocompatibility. When Langmuir monolayers were formed of the NTA lipids, complex formation between Ni^{2+} and NTA was demonstrated at the air/water interface. The Ni^{2+} binding capability of the NTA groups was not compromised by the presence of the lipid. Since His-tagged proteins interact with the Ni^{2+} center through the imidazole ring of the histidine groups, imidazole is a suitable model compound for the testing of ligand binding to the NiNTA complex. The binding between imidazole and the NiNTA complex appeared to be specific, and the binding could be reversed by adding EDTA, because of its superior binding to Ni^{2+}. Since two histidines are required for stable complex formation with one NiNTA group, there is hardly any nonspecific interaction expected between proteins without a His_6-tag and a NiNTA-modified surface. This was confirmed by epifluorescence studies and film balance measurements.[14] From these studies, it could also be concluded that the binding process and pattern formation of histidine-tagged molecules were directly triggered by complex formation of the chelator lipid, and that the binding was specific.

Multivalency was first observed in a system in which Green Fluorescent Protein (GFP) was immobilized through specific binding on chelator–lipid-rich domains of a phase segregated monolayer.[17] When GFP was immobilized at such a surface, EDTA concentrations well above 1 mM were needed in order to desorb GFP from the surface. This very stable immobilization was attributed to the high surface concentration of binding sites present in the condensed lipid domains, and thus a high effective concentration. Approximately nine binding sites were present underneath a single GFP so that rebinding to unoccupied chelator lipids could easily occur. Further evidence for multivalent interactions between a His_6 tag and the NTA groups came from experiments involving immobilization of His_6-tagged proteins on chelating lipid membranes with chelators at different surface concentrations.[30] Pair formation between a rhodamine-labeled, His-tagged peptide by an NBD-labeled chelator lipid was demonstrated using fluorescence energy transfer spectroscopy at the monolayer interface as well as in solution. Also the dissociation of the complex by adding EDTA was shown this way. A very low percentage of nonspecifically bound protein was observed. FRET kinetic studies on this system showed that the binding (dissociation) constant was 3.0 ± 0.4 μM. From fluorescence correlation spectroscopy (FCS), a dissociation constant of 4.3 ± 0.8 μM could be deduced. Kinetically stable immobilization of the proteins at the chelator interfaces for at least 60 min was observed.

The examples discussed above all described the binding of multivalent guests for which the binding stoichiometries followed logically from their structure and were thus predictable. In contrast, although a qualitative shift from kinetically labile to stable interactions had been observed,[22] the dendrimeric guest systems proved harder to study since their binding stoichiometry to the CD SAMs could only be estimated from basic molecular modeling. The key to a quantitative understanding of the multivalent dendrimer systems came from the use of the electroactive ferrocenyl-functionalized PPI dendrimers (Fig. 8) which provided an independent experimental measure of the numbers of interactions.[31,32] On the one hand, the overall binding constants, determined by SPR, of the still thermodynamically reversible dendrimer generations 1–3 could be evaluated in terms of multivalency. A model for describing the multivalency effects in a quantitative fashion for the binding of such highly multivalent molecules at the CD interfaces was developed incorporating the effective concentration concept as well as possible competition with monovalent hosts of guests in solution.[6] All dendrimer data pointed as well to the conclusion that all binding enhancement stemmed solely from the multivalency effect.

On the other hand, the surface coverages of electroactive ferrocenyl groups, after assembly of a full monolayer of these dendrimers on a CD SAM (Fig. 8), as determined by cyclic voltammetry, were compared to the known surface coverage of the CD host molecules. This provided ratios of bound *vs.* unbound ferrocenyl groups, and thus in a direct fashion the binding stoichiometries (Fig. 9).[31] This electrochemical stoichiometry determination method worked also for the higher generations 4 and 5 which showed dissociation rates that were too slow to allow stability constant determinations by SPR. Straightforward extension of the thermodynamic model nevertheless provides reliable K value predictions for such systems as well.

Comparison of the experimentally observed binding stoichiometries for these dendrimers to molecular models revealed a straightforward geometric rule for the binding stoichiometry. As long as a dendritic branch can stretch without violating common bond lengths and angles to reach a neighboring free CD binding site at the CD SAM it will bind, thus contributing to the overall stoichiometry. This was confirmed by modifying the dendrimer skeleton and the spacer length between the dendrimer amino endgroups of the parent dendrimers and the ferrocenyl groups attached to them.[32] These modifications led to different binding stoichiometries but always followed this geometric rule. Since replacing the ferrocenyl groups by adamantyls does not change the geometry of the dendrimers significantly, the stoichiometry data for the ferrocenyl dendrimers could be directly applied to the adamantyl dendrimers, thus allowing thermodynamic data for the latter to be interpreted quantitatively as well.

In conclusion, careful extension of the multivalency of the model guest systems, together with the well-defined properties of the CD SAMs, has allowed us to obtain quantitative thermodynamic data and interpretations for multivalent binding events occurring at these interfaces. The data and model lead to the conclusions[6] that (i) binding events at these CD SAMs are commonly explained by multivalency only, without the need for assuming cooperativity, and that (ii) crude molecular models (*e.g.* CPK) suffice to estimate whether an unused binding site of the guest can reach a neighboring free host site and thus provide easy estimates of the (maximal) binding stoichiometry even when these are not experimentally accessible. The mathematical model also provides a clearcut way to estimate dissociation rate constants,[6] although experimental dissociation measurements are bound to be convoluted with mass transport limitation effects.

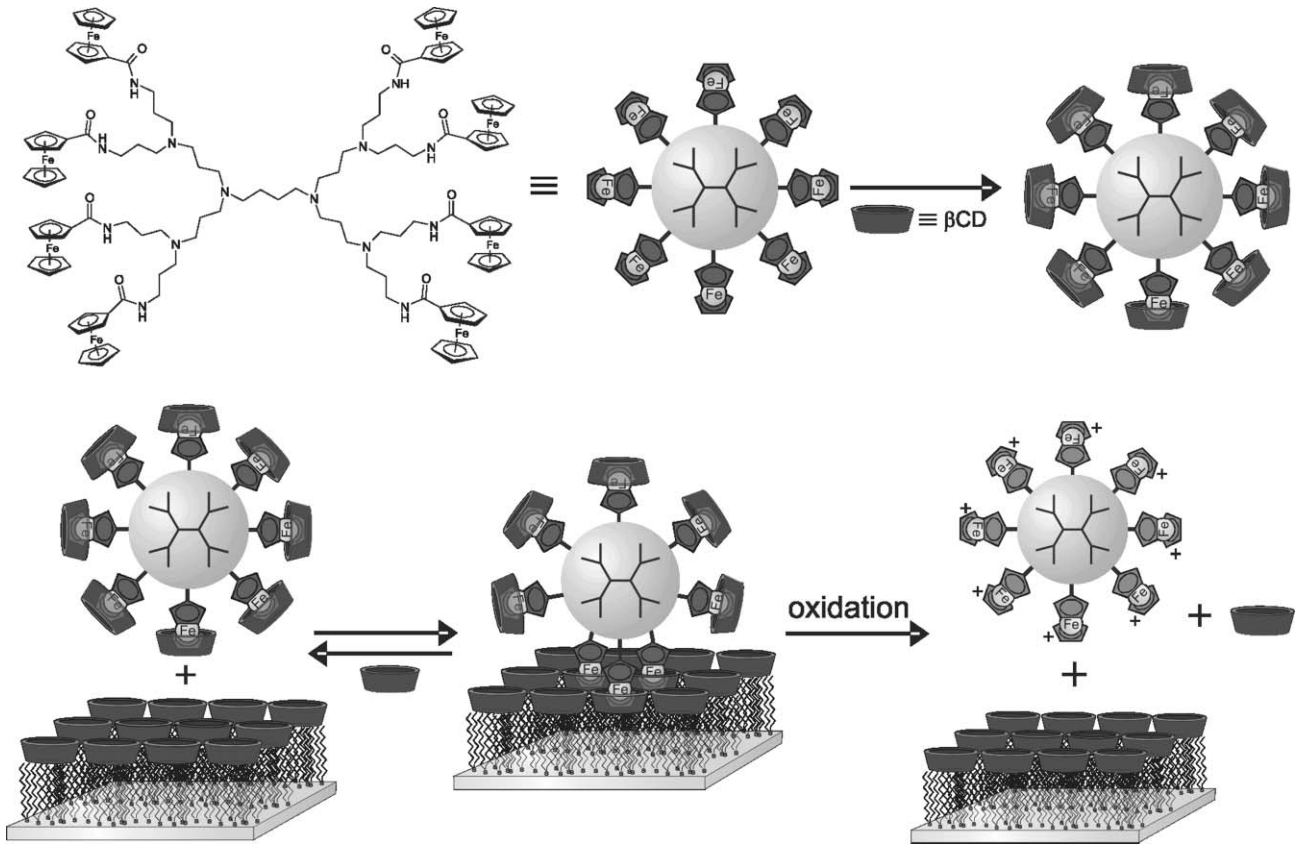

Fig. 8 Generation-2 ferrocenyl-functionalized PPI dendrimer and its formation of water soluble assemblies with CD (top); the adsorption of the dendrimer–CD assembly at the CD SAM (bottom) and the electrochemically induced desorption from the molecular printboard.[31,32]

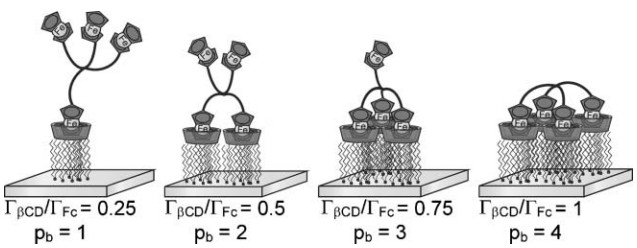

Fig. 9 Schematic representation of the four possible binding modes of the generation-1 ferrocenyl dendrimer to the CD SAM with the numbers of bound sites, p_b, and the predicted coverage ratios, Γ_{CD}/Γ_{Fc}, depicted below.[31]

4. Stable positioning and directed assembly at molecular printboards

Although perhaps unexpected initially, weak supramolecular interactions when used in a multivalent fashion can provide thermodynamically and kinetically stable assemblies, both in solution and at interfaces. This stability may be put to use by noting that it implies (i) that, when the assembly occurs at an interface, the complex stays at the position where it was originally formed, (ii) that directed assembly can therefore be applied to obtain patterns of such supramolecular complexes, and (iii) that additional building blocks with other or identical binding motifs can be employed in subsequent assembly steps to extend the supramolecular structure, thus leading to supramolecular materials. This paragraph deals with the stable assembly and stimulus-dependent reversal of various types of multivalent supramolecular entities, from molecules to polymers and biomolecules, onto the molecular printboards, and basic motifs for extending the assemblies to larger systems, while the truly materials systems, including nanoparticle assembly, are covered in the next paragraph.

Whereas the earlier work on adamantyl dendrimers had already shown the imaging and thus strong binding of individual dendrimer molecules are the CD SAMs, these were arranged in an unordered fashion, randomly spread over the substrate.[22] Directed assembly was achieved initially with the divalent calix[4]arene guest molecule (Fig. 2) in which case microcontact printing (μCP) was applied to obtain micrometer-sized patterns of these molecules on the CD SAMs.[33] Comparison with adsorption onto OH-functionalized SAMs showed that the assembly on the CD SAMs was governed by specific, multivalent host–guest interactions. The guest molecules were found exclusively in the areas of preceding contact between the microcontact printing stamp and the substrate, even after extensive rinsing with water or salt solutions. Only rinsing with 10 mM CD, in order to induce competition for binding the adamantyl guest sites, led to appreciable, *i.e.* noticeable by AFM, desorption. Very similar results were obtained using the adamantyl-functionalized PPI dendrimers.[34]

In order to allow the application of fluorescent molecules, CD SAMs on silicon oxide and glass were developed.[35]

Although the monolayer formation employs in this case a three- or four-step covalent procedure, these SAMs showed a CD coverage and guest binding characteristics that were fully comparable to the CD SAMs on gold. Divalently binding fluorescent guests, with a fluorescent group fixed to a synthetic core to which branched adamantyl-functionalized linkers were attached, were applied to these SAMs, and similar binding behavior and specificity were observed as for the calix[4]arene guest on the CD SAMs on gold.[36] For one of these dye molecules, a fluorescent titration curve was obtained, from which a stability constant was derived that was fully in line with data obtained for the gold substrates.[35] The application of two dyes, one in a printing step and the second one in a subsequent solution assembly step, showed that alternating patterns of dyes could be obtained.[36] The second dye was found almost exclusively in the areas left vacant after the preceding printing step which showed that the first dye was bound in a stable fashion and that exchange of dyes in the subsequent solution assembly step did not occur to a noticeable extent. More high-resolution patterning, down to linewidths of approx. 200 nm, was achieved by dip-pen nanolithography, using the calix[4]arene, an adamantyl dendrimer, and the fluorescent dye guest molecules as the ink.

One of the divalent fluorescent guest molecules was also used in the binding to cyclodextrin vesicles of about 100 nm.[37] Binding constants found were similar to values obtained for flat CD SAMs. Vesicles consisting of both α- and β-cyclodextrin of varying ratios were employed to test the hypothesis of receptor clustering in these mobile layered architectures.[38] Indeed, binding of the divalent dyes showed that binding to vesicles with a fraction of β-CD yielded always significantly higher binding constants than expected when assuming random mixing of these receptors. Whether this clustering stems from demixing of the receptor molecules in the vesicles before guest binding or from active clustering upon guest binding is an unsolved issue.

Patterning the adamantyl dendrimers on the CD SAMs on silicon oxide provided one of the first cases of the use of two, orthogonal interaction motifs for the formation of more complex architectures.[39] The application of a solution of a negatively charged fluorescent dye to a substrate patterned by μCP with the adamantyl dendrimers led to localization of the dyes in the dendrimer-printed areas only. This two-step procedure therefore succumbed to an architecture where the dendrimers were bound by multivalent host–guest interactions whereas the dyes were immobilized inside the dendrimer cores by electrostatic interactions. An even more complicated architecture was developed by printing lines of dendrimers in one direction, subsequently printing one dye in an orthogonal direction, and finally assembling a second dye from solution (Fig. 10). This yielded a dicolored block pattern again showing good selectivity and directionality even for these electrostatically bound dyes.[39]

A more complex architecture with a well-defined stoichiometry was achieved in the stepwise buildup of supramolecular capsules on molecular printboards.[40] The capsule was composed of two calix[4]arenes which were held together by four charged groups on each building block. One of the calix[4]arenes was functionalized with four adamantyl groups on the opposite side of the molecule which allowed attachment to the CD SAMs (Fig. 11). The capsule formation in solution was confirmed by ITC. The capsule was assembled on the molecular printboards in a stepwise fashion: adsorption of the adamantyl-functionalized half of the capsule on the CD SAM followed by binding of the second half to complete the capsule (Fig. 11). Both steps were shown unambiguously to yield the capsule architecture at the surface. The architecture could also be disassembled in a stepwise fashion by first rinsing with a competitive calix[4]arene complementary to the top half of the immobilized capsule, followed by rinsing with a polar organic solvent to weaken the CD host–guest interactions (the tetravalently bound calix[4]arene was attached too strongly to be removed by rinsing with 10 mM CD).

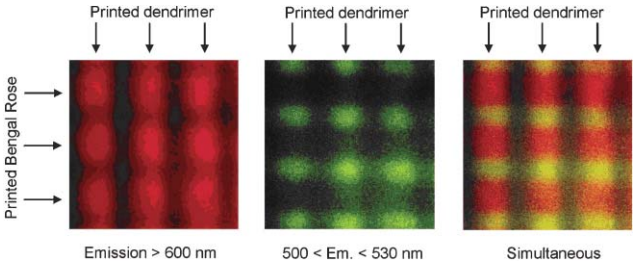

Fig. 10 Confocal microscopy images (50×50 μm^2) after μCP of the generation-5 adamantyl dendrimer on a CD SAM on glass, followed by cross printing of Bengal Rose, and subsequent filling with fluorescein.[39] The substrate was simultaneously excited at 488 nm and 543 nm and images were recorded by measuring the emission above 600 nm (left), between 500 and 530 nm (center), and simultaneous (right).

In the studies discussed above, the binding of adamantyl derivatives to CD SAMs can only be reversed by competition with a host in solution.[33] This becomes progressively more difficult with increasing numbers of interactions of the multivalent complexes, as was for example indicated by SPR titrations of the adamantyl-terminated dendrimers. Totally irreversible adsorption was also observed for polymers functionalized with *tert*-butylphenyl or adamantyl groups.[41] SPR and AFM showed that such polymers showed strong adsorption and a very efficient use of all or most endgroups. This behavior led to a drastic change of conformation of the polymers from an on average spherical shape in solution to a completely flattened thin layer of less than 1 nm when adsorbed on the CD SAMs. This adsorption proved completely irreversible by competition with monovalent guests or hosts from solution which was to be expected from the strong multivalency effect for such highly multivalent systems.

Bifunctional polymers consisting of vancomycin and fluorescein were prepared by Whitesides *et al.*, and adsorbed to SAMs consisting of D-Ala-D-Ala and tri(ethylene glycol) groups (Fig. 12).[42] SPR studies revealed that the adsorbed polymer desorbed only very slowly from the surface (k_{off} = 10^{-6} s^{-1}). When soluble ligand was added, however, the dissociation increased by a factor of about 50. This very strong interaction to the surface was attributed to multivalent interactions between the multiple vancomycin groups at the polymer and multiple D-Ala-D-Ala groups present on the SAM. The fluorescein groups present at the polymer directed

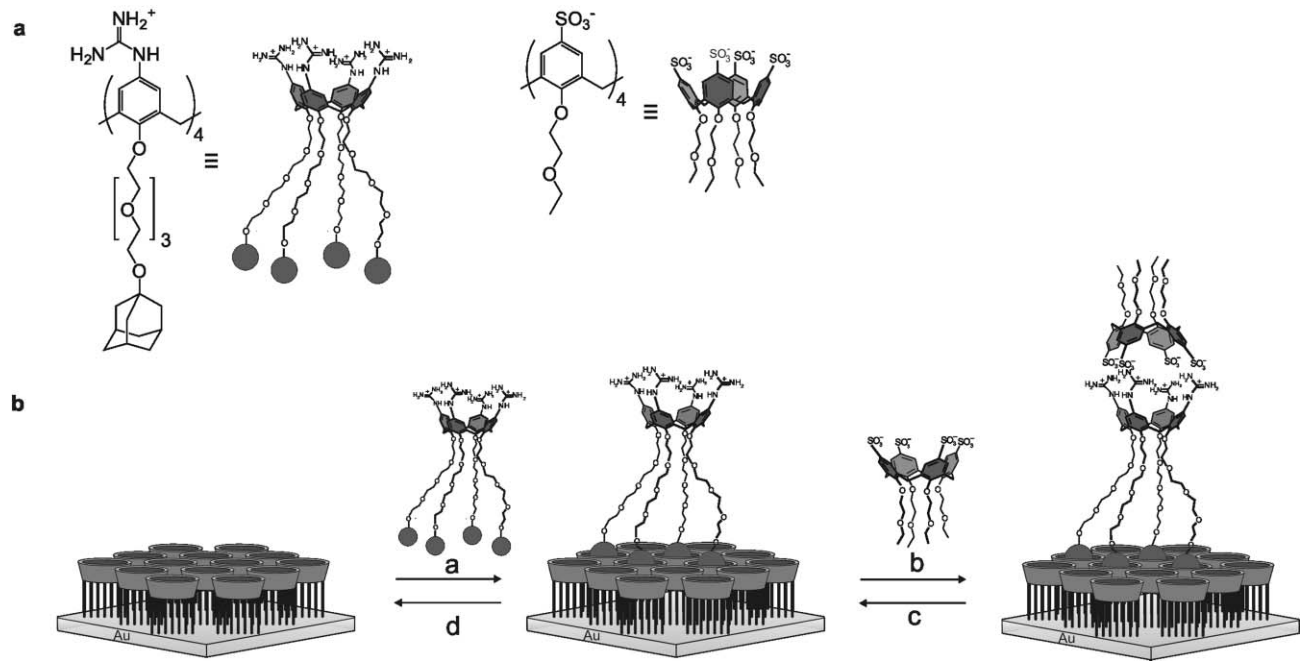

Fig. 11 (a) The capsule building blocks: a tetra(adamantyl)-calix[4]arene and a tetrasulfonate-calix[4]arene; (b) schematic representation of the stepwise build-up and subsequent break-down of the capsule at the molecular printboard.[40]

the assembly of anti-fluorescein antibodies towards the polymer (Fig. 12). It appeared that the affinity of the antibody for binding to the SAM was enhanced by a factor of 570 due to divalency. Thus the bifunctional polymer functions as a bridge between the SAM and the immunoglobin *via* two independent interactions, the polyvalent interaction between the SAM and the polymer, and the divalent interaction between the immunoglobin and the polymer. These polymers could thus be used for the direction of antibodies towards cell surfaces.

To introduce the possibility of desorption by an external stimulus, the electroactive ferrocenyl PPI dendrimers were subsequently explored.[31–33] It was noted before that oxidation of the ferrocenyl groups to ferrocenium cations leads to a strong reduction of the affinity of these groups for the CD cavity. Thus the externally triggered desorption by electrochemical oxidation of CD SAM-adsorbed ferrocenyl dendrimers was envisaged. Apart from the tool to study binding stoichiometry of such dendrimers as discussed above,[31] cyclic voltammetry also provided proof of the envisaged assembly and disassembly scheme (Fig. 8). Oxidation of the ferrocenyl groups of a full monolayer of Fc dendrimers on a CD SAM, which occurred for all ferrocenyl groups at the same potential, led to complete desorption of the dendrimers, as also indicated by the combination of CV with SPR.[32] Subsequent reduction showed that only part of the oxidized dendrimers were reduced back and readsorbed, thus leading to lower charge densities for subsequent CV scans. When the same dendrimers were added to the electrolyte solution which was in contact with the Fc dendrimer monolayer, oxidation led to complete desorption, but upon reduction the dendrimer monolayer was fully reconstituted by replenishment of Fc dendrimers from the electrolyte solution (Fig. 13). This procedure was found to be fully reversible at various scan rates.

Both the CD printboards and the NiNTA SAMs have been employed for the attachment of biomolecules. On the CD SAMs, orthogonal CD host–guest and streptavidin–biotin interactions have been used to attach streptavidin protein molecules onto the CD SAMs using heterofunctionalized

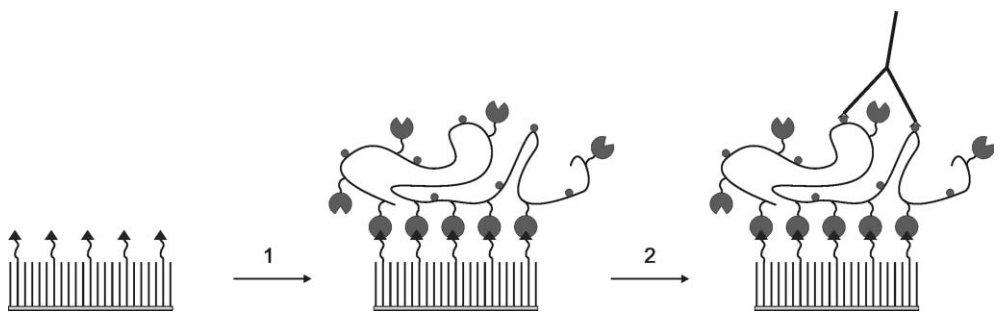

Fig. 12 The adsorption of a bifunctional polymer presenting vancomycin and fluorescein groups to SAMs consisting of D-Ala-D-Ala groups and tri(ethylene glycol) groups (1); the adsorption of an anti-fluorescein antibody to such SAMs to which the bifunctional vancomycin–fluorescein polymer was adsorbed (2).[42]

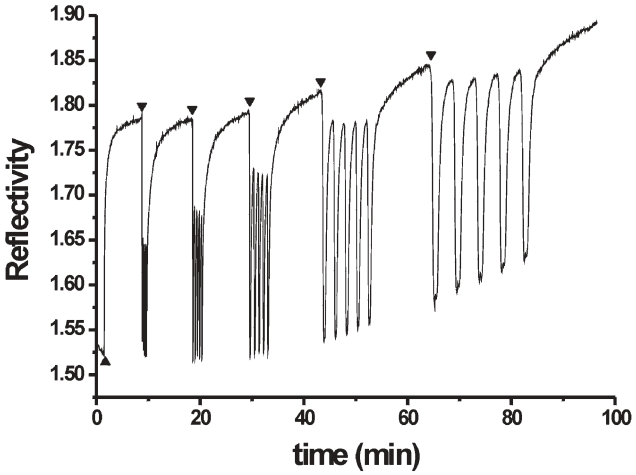

Fig. 13 SPR response of a CD SAM with the generation-3 ferrocenyl dendrimer in solution while perfoming cyclic voltammetry at different scan rates: ▲ = injection of 6.3 μM aqueous solution of the dendrimer with 10 mM βCD at pH = 2; ▼ = 5 scans at 100 V s^{-1}, 50 mV s^{-1}, 25 mV s^{-1}, 10 mV s^{-1}, and 5 mV s^{-1} from left to right.[32]

linkers.[43] Multivalency was found to play a role in the binding strength of the resulting complex, and the complex could be assembled in a variety of ways, again by variation of the order in which the interaction motifs were employed.

The stable immobilization and orientation of proteins on flat, biocompatible supports are a prerequisite for structural and mechanic studies of proteins. Therefore multivalent interactions play a major role in surface attachment of these proteins.[17,44] It was already shown that the chelator–lipid NiNTA surfaces are highly biocompatible, and that multipoint attachment of proteins to these layers is possible.[45,46] The proteasome 20S (Fig. 14) was his-tagged specifically at the α-positions,[16] and immobilized at a chelator–lipid interface. SPR measurements showed the specific immobilization of these proteins to the lipid chelator surface. The proteasome remained stable at the surface, and the protein complex could only be removed from the surface by 0.1 M EDTA. Lateral mobility of the proteasome on the surface was also proven in these experiments. Also the biological activity of the attached proteasome was demonstrated by SPR. This stable, specific immobilization of the proteasome to the metal chelator surface led to elucidation of the molecular mechanism of the catalytic activity of these protein complexes.[15,16]

So far, only surfaces consisting of *monovalent* chelator lipids have been discussed. However, SAMs were also prepared with

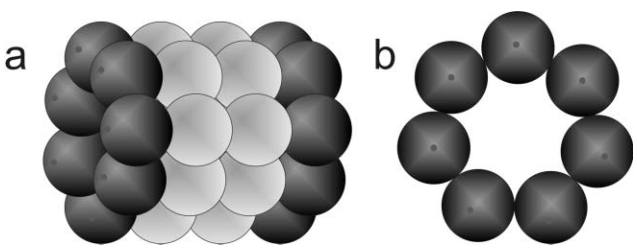

Fig. 14 Proteasome 20S, His-tagged at the α-terminus: (a) side view, (b) front view.[16]

multivalent chelator lipids. There was a considerable decrease in dissociation rate for the dissociation of a his-tagged protein (His_6 infar2) from the multivalent surface compared to the monovalent surface.[47] The low dissociation rate constant allowed orthogonal protein immobilization. The high stability of the multivalent NTA SAM towards His_6 tags also allowed the patterning of these SAMs.[47]

5. Towards supramolecular materials and devices

The (latent) power of multivalent host–guest interactions at interfaces lies in part in the easy conceptual transfer to various building blocks and substrate types. Apart from flat surfaces, also 3D objects such as nanoparticles can be functionalized with host or guest motifs with the aim of assembling materials.

From the stoichiometry data discussed above, it was clear that the adamantyl dendrimers have many unused guest groups when adsorbed on a CD SAM. Consequently, such dendrimer monolayers can be viewed as guest-functionalized layers which in turn allow the adsorption of host-functionalized species. On the other hand, the spherical nature of CD gold nanoparticles ensures that their subsequent adsorption again leads to a host-functionalized surface. Thus, the repeated application of dendrimers and nanoparticles has led to a supramolecular layer-by-layer assembly scheme (Fig. 15),[48] where each step was self-limiting and in which uncontrolled aggregation was prevented since both building blocks were applied from separate solutions. SPR, UV/Vis, ellipsometry and AFM all indicated a linear growth of the multilayer structures upon increase of the number of assembly steps, with a thickness increase of about 2 nm per bilayer.

As shown above, the use of multivalent interactions can lead to both thermodynamically and kinetically stable assemblies at interfaces. This stability has been shown to allow localized surface assembly, *i.e.* patterning, as well as materials buildup in a layer-by-layer fashion. The combination of the two holds a powerful paradigm for the construction of three-dimensional nanostructures of supramolecular materials. Various surface patterning strategies were applied to make patterned CD monolayers with adsorption-resisting monolayers in the areas in between, with the aim of directed layer-by-layer assembly of the adamantyl dendrimers and CD gold nanoparticles.[49] This proved impossible mainly because of limited selectivity of adsorption of the adamantyl dendrimers due to nonspecific interactions with the inert SAM areas. Two other nanofabrication schemes, however, were successful. First, supramolecular LBL assembly was performed on PDMS relief stamps.[49] Subsequent nanotransfer printing led to the transfer of the complete, intact assemblies from the stamp–substrate contact areas only onto the CD SAM substrate. This allowed the formation of nanostructures with lateral dimensions in the μm range and a height in the nm range. Truly 3D nanostructures, with sub-100 nm sizes in all three dimensions, were obtained through the use of nanoimprint lithography (NIL).[50] NIL was used to create polymer templates with nm lateral dimensions onto which CD SAM formation and supramolecular LBL assembly was performed (Fig. 16). As a last step, lift-off of the polymer template allowed the concomitant removal of any

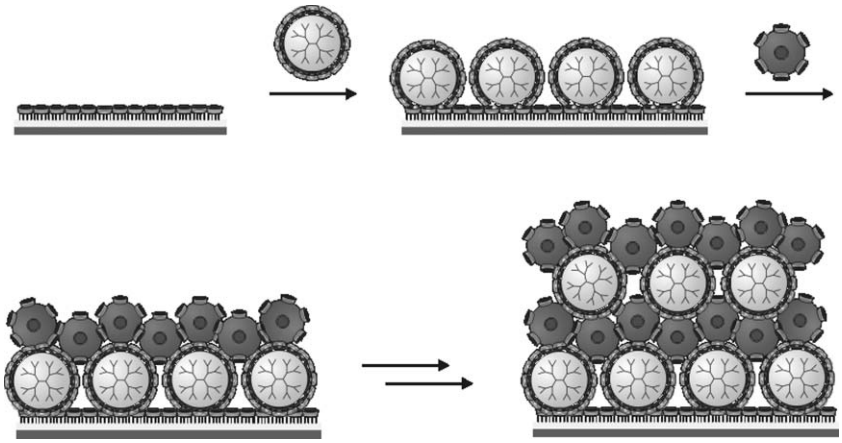

Fig. 15 Layer-by-layer assembly scheme for the alternating adsorption of generation-5 adamantyl-terminated PPI dendrimers and CD-functionalized gold nanoparticles onto CD SAMs.[48]

nonspecifically adsorbed material, while the structures assembled on the CD SAM areas remained intact.

Dendrimer-stabilized gold nanoparticles have been used for the localized growth of metal structures using electroless deposition.[34] To this aim, CD complexed, dendrimer-stabilized gold nanoparticles were microcontact printed onto CD SAMs. Consecutive electroless deposition of copper, initiated by the localized gold nanoparticles, led to the controlled growth of metallic copper structures in the contacted areas with a height of over 60 nm. Current activities are in the area of nanotransfer printing of gold top electrode structures onto ferrocenyl dendrimers for the construction of molecular electronic devices.

5. Conclusions and outlook

The different systems discussed in this review all show, each in their own way, that multivalency is an important tool for the understanding of different biological processes, of the interactions between proteins and receptor surfaces, and for the application of non-biological nanostructures at surfaces. Multivalency allows for the stable interaction between molecules to enhance the effectiveness of antibiotics, the elucidation of the mechanism of protein complexes, and the stable positioning of molecules on surfaces to create nanostructures.

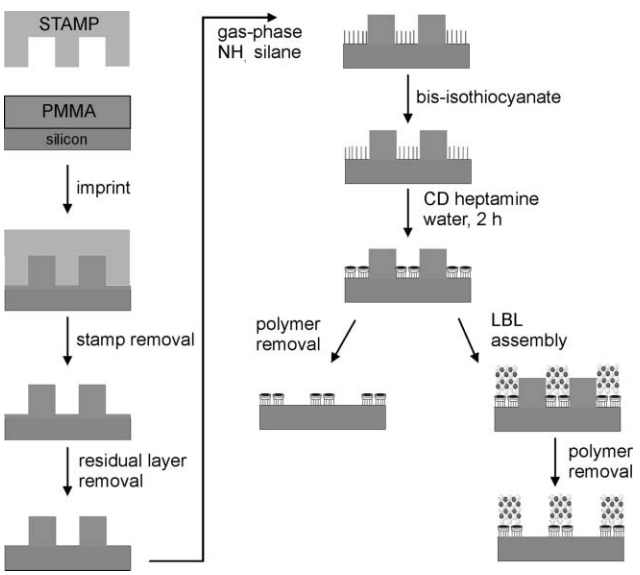

Fig. 16 Integrated nanofabrication scheme incorporating nanoimprint lithography, (patterned) CD SAM formation, and layer-by-layer assembly of adamantyl dendrimers and CD gold nanoparticles.[50]

Molecular printboards offer a host of applications, especially when employing multivalent interactions for assembling all kinds of building blocks. Whereas the thermodynamics is now well understood for the few printboard systems discussed here, the quantitative interpretation of multivalent interactions at biological interfaces, such as real cell membranes, is conspicuously lacking. Also reliable data on multivalent kinetics at interfaces are absent today, but inherently new surface diffusion mechanisms can be envisaged the implications of which for science and applications can at this moment only be speculated upon.

A clear trend is also envisaged in the direction of orthogonal multivalent interactions to create more complex nanostructures at interfaces. Limited numbers of examples are at hand, such as the electrostatic interaction of negatively charged dyes in the positively charged cores of adamantyl dendrimers adsorbed to CD SAMs,[39] the capsule formation of calix[4]arene building blocks on CD SAMs,[40] and the construction of protein assemblies through host–guest and streptavidin–biotin interactions.[43] More examples are needed for a better quantitative understanding of heterotropic multivalency, both in solution and at interfaces, but without doubt their development will lead to much more complex systems and materials.

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