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REVIEW ARTICLE

Recognition of exterior protein surfaces using artificial ligands based on calixarenes, crown ethers, and tetraphenylporphyrins

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Abstract Artificial ligands for recognition of exterior protein surfaces can be used for protein detection, protein modification/modulation, and protein separation. This article reviews recent developments of artificial ligands for complexation with exterior protein surfaces, with a focus on studies using calixarene-, crown ether-, and tetraphenylporphyrin-based ligands. Synthetic ligands that recognize amino acid residues can form *n*:1 supramolecules with proteins. 18-Crown-6 and calix[6]arene derivatives have been used for complexation with the lysine residues of proteins. By comparison, larger ligands that have a central core and multivalent functionalities at the periphery can form 1:1 supramolecules with proteins.

Keywords Protein recognition · Protein separation · Protein extraction · Calixarene · Crown ether · Tetraphenylporphyrin

Introduction

Because of rapid advances in proteomics, sophisticated synthetic molecules that can interact with specific target proteins are required. However, recognition of proteins is challenging because they contain multiple functional groups. Synthetic ligands, or receptors, that can detect specific proteins via complexation are useful for proteomics, medical diagonistics, and pathogen detection [1, 2]. Synthetic ligands that can specifically interact with target proteins in aqueous media can act as protein modulators as agonists of biological responses, and mediate protein function and dimerization [3–5]. Once bound to proteins, synthetic molecules can alter the physical and chemical properties of proteins. The modification of proteins by noncovalent interactions is favorable because it is a simple and relatively gentle reaction. Compared to recognition of proteins using biomolecules such as antibodies, recognition using synthetic ligands has higher chemical stability.

Protein recognition using ligands is also useful for protein separation. Generally, proteins are separated based on their different characteristics such as solubility, molecular weight, isoelectric point, and hydrophilic/lipophilic balance. If proteins have similar characteristics, separation becomes very difficult. In comparison to traditional separation systems, biological interactions have very high selectivity and specificity. This has facilitated the development of various separation systems based on bioaffinity. Affinity chromatography using highly specific biological interactions has been used for the purification of target proteins. Various biological interactions including lectin/sugar, enzyme/inhibitor, antigen/antibody, protein/protein, and DNA/protein interactions can be used for affinity chromatography [6, 7]. Synthetic ligands have also been developed as biomimetic ligands for affinity chromatography because of the high-capacity, chemical stability, durability, and cost-performance they provide. For instance, synthetic ligands based on synthetic dyes, such as triazine or triphenylmethane compounds, are used in dye-ligand affinity chromatography. These ligands interact with the active sites of many proteins and enzymes by mimicking the structure of substrates, cofactors, or binding agents. It is likely that the protein–synthetic ligand selectivity and specificity are lower than those in a living system. However, synthetic ligands are attractive alternatives to bioaffinity ligands.

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Protein recognition can occur on interior surfaces, such as active sites, or exterior surfaces [3, 8–10]. Proteins have concave and well-defined interior active sites that are shielded from solvents and solutes. By contrast, their exterior surfaces are flat, exposed, and are usually in direct contact with bulk water and solutes. Consequently, there are many challenges associated with the recognition of exterior protein surfaces. However, various strategies to overcome these challenges have been reported in recent years. This review summarizes recent progress in complexation between synthetic ligands and exterior protein surfaces. In particular, this review focuses on recognition of solvent-exposed regions or motifs on the protein surface using calixarene derivatives with multiple functional groups, crown ethers, and tetraphenylporphyrins (TPPs) (Fig. 1). Inclusion phenomena with macrocyclic compounds, calixarenes, and crown ethers are used for recognition of proteins. Additionally, the rigid structure and multivalency of synthetic ligands aid complexation with proteins. Complexation using synthetic ligands has been used in a number of applications. Because of rapid advances in the field, this review might not include all studies in the field. However, many challenges, including those faced in our recent studies, are summarized.

Complexation mode

Complexation between protein surfaces and ligands can be roughly divided into two types. Synthetic ligands that recognize amino acid residues can form *n*:1 supramolecules with proteins (Fig. 2a). Synthetic ligands that exhibit affinity for specific amino acid residues can be used for recognition and modification of proteins via noncovalent binding. Some residues can be targeted for specific complexation with synthetic ligands. For example, 18-crown-6 and calix[6]arene derivatives act as a host for the ε-amino group of the lysine residue, which is included into the host cavity [11–17]. Coordination between the imidazole group of histidine residues and transition metal ions such as Cu(II), Ni(II), Co(II), and Zn(II) can be used as a specific interaction in immobilized metal affinity chromatography [18–21]. This *n*:1 complexation can be applied for protein separation by discriminating between the number of amino acid residues in the proteins, and protein modification alters the protein functionality. Despite the research to date, many challenges remain in the recognition of amino acid residues using synthetic ligands. One of the most important limitations is the selective recognition of some residues, such as aliphatic residues, using synthetic ligands. Although complementary complexation between anionic ligands and guanidinium groups has been studied [22, 23], selective recognition of arginine over other amino acid residues has not been achieved. To date, selective complexation is possible only for lysine residues using macrocyclic compounds, histidine residues via coordination bonding with transition metal ions, and cysteine residues via covalent disulfide bonding.

By contrast, synthetic ligands that form 1:1 supramolecules with particular proteins via multiple interactions can recognize the entire protein structure (Fig. 2b) and modify its functionality. Synthetic ligands for the recognition of entire proteins are large (750–1,500 $Å^2$) and interact with many amino acid residues simultaneously via multiple interactions such as hydrogen bonding, electrostatic interaction, hydrophobic interaction [24, 25]. To recognize multiple amino acid residues that are widely distributed on the protein surface, the synthetic ligand should have a large central domain and multiple functional groups at the periphery. Multivalency refers to the ability of a synthetic ligand to bind the targeted species through multiple simultaneous noncovalent interactions [26–29]. Complementary multivalent interactions between ligand and protein functional groups result in powerful and specific complexation. Such ligands could potentially disrupt protein–protein interactions and lead to novel therapeutic

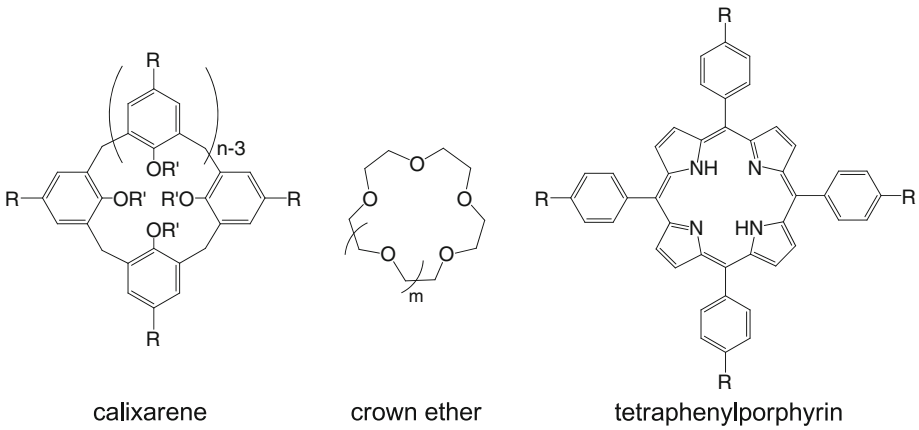

Fig. 1 Molecular structures of calixarene ($n = 4, 6, 8$ (typically)), crown ether ($m = 0 - 3$ (typically)), and tetraphenylporphyrin

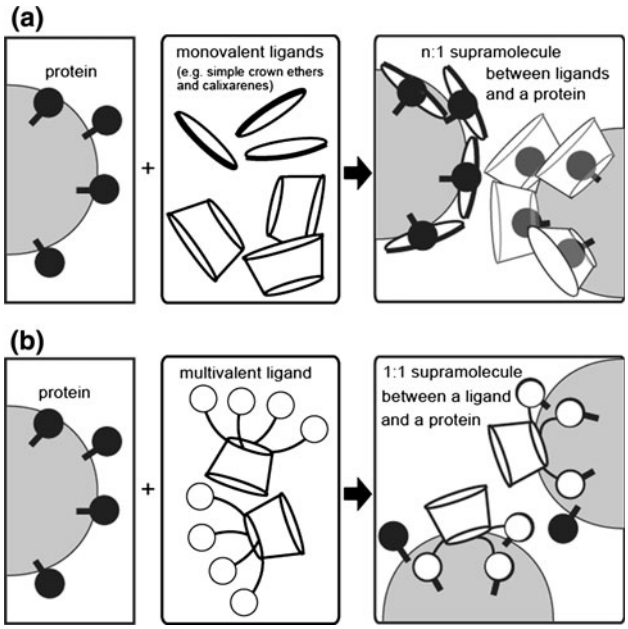

Fig. 2 Conceptual illustration of the recognition of protein using monovalent (**a**) and multivalent (**b**) ligands

effects. Multivalent ligands with fluorescent moieties can be used for protein detection based on fluorescence in response to complexation with a specific protein [8, 9].

Recognition of proteins using calixarene derivatives

Calixarenes are cyclic oligomers formed by multiple phenolic units linked by methylene bridges. These compounds have been used for preparing host molecules for inclusion of target species [30–33]. Calixarene derivatives bearing various functional groups have been developed for complexation with metal ions, organic molecules, and relatively small biomolecules [34, 35]. While some hydrophilic calixarene derivatives form complexes with biomolecules via hydrophobic interactions in aqueous media, hydrophobic derivatives form complexes with biomolecules in organic media through multiple interactions, including electrostatic interactions, hydrogen bonding, and cation–π interactions. Previous studies of protein recognition by calixarene derivatives have been limited, partly because the calixarene cavity size is too small for the large protein molecular structures. However, there have been a number of recent studies on complexation of calixarene-based artificial receptors and biomacromolecules [36]. Some calixarene derivatives are able to bind protein amino acid residues in a similar manner to smaller amino acids and peptides [12, 37]. Other calixarene derivatives possess multivalent functionalities for complexation with whole proteins [13]. Calixarene derivatives bearing structural units of biomolecules, such as peptides and saccharides, are particularly effective in this regard [28].

Complexation between proteins and water-soluble calixarenes

Water-soluble calixarene derivatives are useful complexation reagents for proteins. Coleman and co-workers have studied complexation between sulfonato-calix[*n*]arenes and proteins, especially bovine serum albumin (BSA) [38]. The biological activity of *para*-sulfonato-calix[*n*]arenes generally increases with the size of the calixarene. For example, *para*-sulfonato-calix[8]arene derivatives show high anticoagulant activity and strong interaction with the prion protein PrP [36, 39–41]. However, when binding of *para*-sulfonato-calix[*n*]arene derivatives (Fig. 3) to BSA was analyzed using electrospray ionization (ESI) mass spectrometry (MS) the opposite effect was observed [42]. The observed association constants ranged from 10^4 to 10^6 M^{-1} and varied inversely with the size of the *para*-sulfonato-calix[*n*]arenes as follows: calix[4]arene > calix[6]arene > calix[8]arene. This tendency is thought to occur because of steric hindrance of the interaction of larger calixarenes with the strong binding pocket.

Biomedical applications of *para*-sulfonato-calix[*n*]arenes have also been studied [37]. From screening of 57 water-soluble calixarenes, *para*-sulfonato-calix[*n*]arenes were found to exhibit anti-microbial activity [43]. Thrombin inhibition mediated by antithrombin and heparin cofactor II activation has been investigated [39]. Mono-(2-carboxymethoxy)-sulfonato-calix[8]arene and sulfonato-calix[6]arene exhibited higher antithrombotic activity than analogous compounds.

Cunsolo et al. developed a series of calix[8]arene derivatives with basic amino substituents for complexation with negatively charged tryptase clusters [44]. These calix[8]arene derivatives acted as indirect inhibitors through an antagonistic effect on the proteoglycan heparin, and direct competitive inhibitors of tryptase. Neri and co-workers developed calixarene derivatives that functioned as inhibitors for transglutaminase and histone deacetylase [45, 46]. A series of *N*-linked tetrakis(tetrapeptide)calix[4]arene diversomers were synthesized for complexation with transglutaminase, and derivatives bearing a Gly-Phe-Gly-Tyr

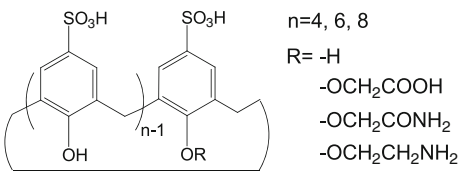

Fig. 3 Molecular structures of *p*-sulfonato-calix[*n*]arene derivatives [41]

tetrapeptide exhibited the most effective inhibition. Furthermore, inhibition of Yersinia protein tyrosine phosphatase by phosphonate derivatives of calixarenes has recently been investigated [47]. Some of them are potent competitive inhibitors of Yersinia protein tyrosine phosphatase with inhibition constants in the low micromolar range.

Complexation between lectins and glycocalixarenes

Sugar-binding lectin proteins bind to glycoproteins specifically through simultaneous multivalent interactions known as the "glycoside cluster effect". To date, many glycocalixarenes have been developed for specific binding to lectins [28]. Ungaro and co-workers prepared glycocalixarenes using thiourea groups as spacers (Fig. 4) [48–51]. Turbidimetry showed that tetraglucosyl and tetragalactosyl calixarene derivatives specifically bound to concanavalin A and peanut lectin (*Arachis hypogaea*), respectively. Consoli et al. synthesized *N*-acetyl-D-glucosamine clusters using amino acid–calixarenes [52]. The obtained glycosamino acid–calixarenes bound to lectin and increased inhibition of erythrocyte agglutination. Dondoni and co-workers also prepared glycoclusters based on calixarenes [53, 54]. Submillimolar concentrations of tetra- and octavalent sialoside clusters inhibited hemagglutination and viral infectivity mediated by BK and influenza A viruses. Roy and co-workers prepared calixarene derivatives with sialic acid and *N*-acetyl-galactosamine units on their lower rims [55, 56]. Dendritic, water-soluble, carbohydrate-containing *p-tert*-butylcalix[4]arene was synthesized to study the lectin-binding properties. The amphiphilic calix[4]arenes with 16 carbohydrate units displayed affinity for carbohydrate-binding proteins and polystyrene surfaces. Vidal and co-workers prepared seven topologically isomeric calix[4]arene glycoconjugates by the attachment of sugar moieties to a series of alkyne-derivatised calix[4]arene precursors [57, 58]. The trivalent conjugate exhibited higher affinity to a bacterial lectin than the monovalent conjugate.

Modulation of protein–protein interactions using calixarenes bearing peptide loops

Hamilton and co-workers prepared a synthetic protein binding agent containing four peptide loops connected to a calix[4]arene core (Fig. 5) [59–61]. The loops contained negatively charged GlyAspGlyAsp to complement the surface charge of cationic cytochrome *c*. This allowed strong binding of the antibody mimic to cytochrome *c*, which disrupted its interaction with reducing agents. The binding of the receptor also disrupted the protein–protein interactions between cytochrome *c* and cytochrome *c* peroxidase [62]. The receptor competed effectively with cytochrome *c* peroxidase for binding of cytochrome *c* by forming a 1:1 (cytochrome *c*:receptor) complex with a binding constant of around $10^8\ M^{-1}$. Other antibody mimics prepared with four peptide loops connected to a central calix[4]arene core were evaluated as inhibitors for the serine protease α-chymotrypsin [63, 64]. These antibody mimics showed slow binding kinetics in an analogous manner to natural protein protease inhibitors. Complexation between the receptor and α-chymotrypsin disrupted the interaction with proteinaceous inhibitors. These agents were particularly effective at blocking the soybean chymotrypsin–trypsin inhibitor complex. Furthermore, a synthetic molecule composed of a central calix[4]arene scaffold bearing four peptide loop domains (GFB-111) was found to bind to a platelet-derived growth factor binding molecule [65]. GFB-111 inhibited tumor growth and angiogenesis in nude mice with human tumors. These results indicate that receptors based on calixarenes will regulate enzymatic activity and modulate protein–protein interactions.

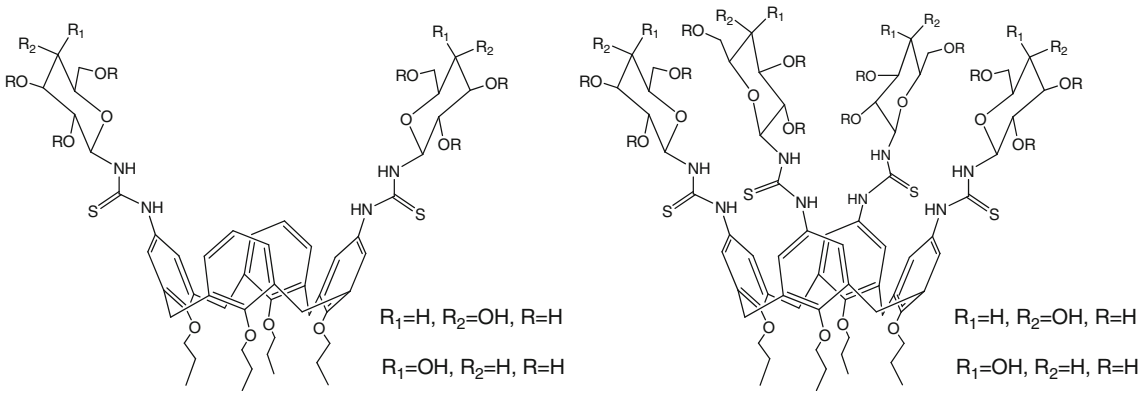

Fig. 4 Molecular structures of glycocalixarenes [46]

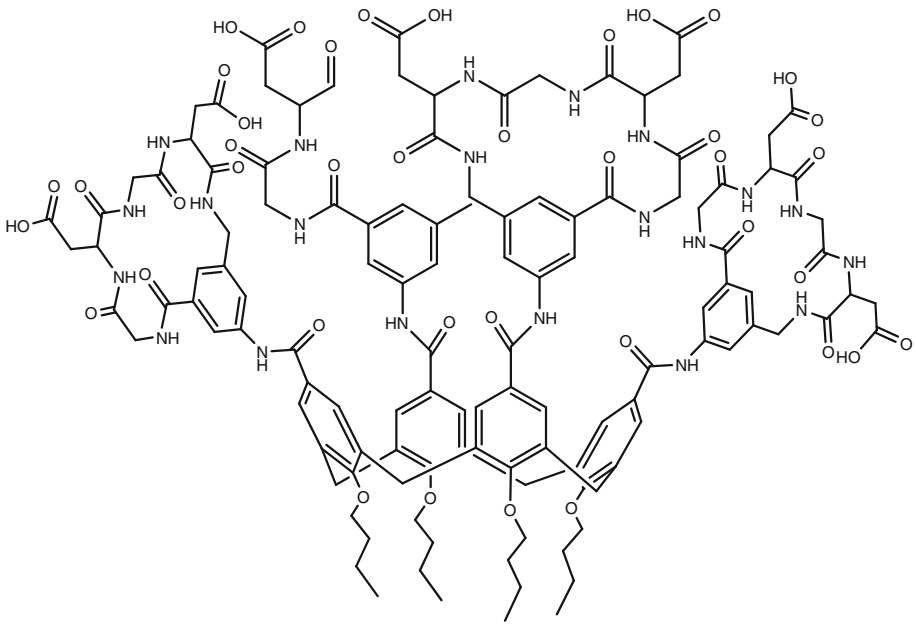

Fig. 5 Molecular structures of calix[4]arenes bearing peptide loops [57, 60, 61]

Liquid–liquid extraction of proteins using calixarenes

A *p-tert*-octylcalix[6]arene carboxylic acid derivative (Fig. 6) was found to extract proteins [66, 67]. Calix[6]arene is one of the most powerful host molecules for complexation with protonated amino groups (NH_3^+), because it has six ionizable carboxylic acid groups for electrostatic interactions, a large cavity to include the guest, and stereochemically favorable C_6 symmetry. Therefore, calix[6]arene forms stable complexes with various amino compounds such as amino acid esters [68–70], nucleobases [71], and catecholamines [72]. In the similar manner, calix[6]arene interacts with the ε-amino group of lysine residues in protein molecules as shown in Fig. 7. Complexation between calix[6]arene and lysine residues via electrostatic interaction makes the protein more hydrophobic. Cytochrome *c* is a cationic protein with 19 lysine residues that can simultaneously bind with multiple calix[6]arene molecules through their ^{t}Oct[6]CH_2COOH groups, and this forms a hydrophobic complex that is transferred into the organic phase.

The percentage of cytochrome *c* extracted into the organic phase is proportional to the concentration of the calix[6]arene, and quantitative extraction is achieved with around 20:1 calix[6]arene:protein. The complexation reaction is represented by the proton-exchange reaction shown in Eq. 1:

$$n\mathrm{H_6R} + \left(\mathrm{NH_3^+}\right)_n\text{-protein} = \left(\mathrm{H_5R \cdot NH_3^+}\right)_n\text{-protein} + n\mathrm{H^+} \quad (1)$$

where H_6R and $(NH_3^+)_n$-protein denote the calix[6]arene and cytochrome *c*, respectively. The number of calixarene molecules bound to each protein molecule has not been confirmed, but seven bound calixarene molecules were detected by MALDI-TOF MS (unpublished data).

In the series of calix[*n*]arene carboxylic acid derivatives ($n = 4, 6, 8$), calix[6]arene extracted the most cytochrome *c*. Calix[4]arene and calix[8]arene also extracted cytochrome *c*, but to a lesser extent than calix[6]arene. The

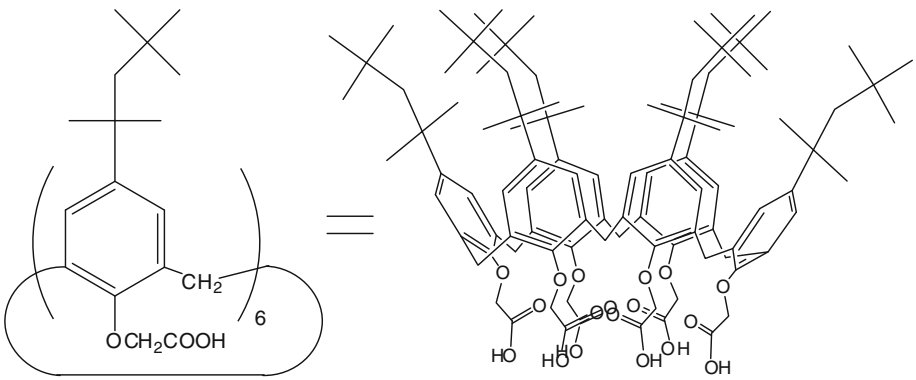

Fig. 6 Molecular structure of the calix[6]arene carboxylic acid derivative [64]

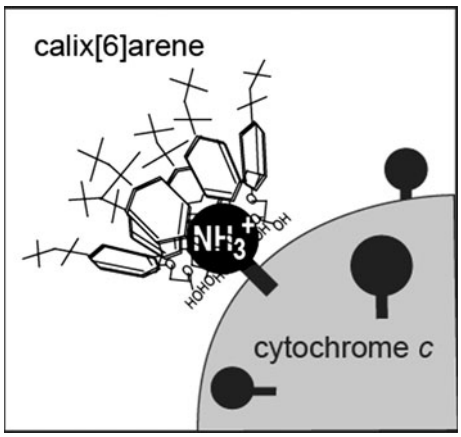

Fig. 7 Schematic illustration of complexation between cytochrome *c* and the calix[6]arene carboxylic acid derivative [64]

extraction order for the protein with the calix[*n*]arenes agrees with that for organic amines. The calix[6]arene derivative has the strongest affinity for the protonated amino group because of its ideal cavity size, C_3 symmetry, and preorganized carboxylic acid groups for inclusion. By comparison, recognition with the calix[8]arene is not as selective, because the cavity is too large for the protonated amino group. Calix[4]arene is not appropriate for the inclusion of the protonated amino group, because its cavity is too small. Because complexation between the calixarene carboxylic acid derivatives and the protein is based on the proton-exchange reaction, the protein is not extracted as well by phenolic calix[6]arene as it is by the calix[6]arene ethyl ester derivative under the same conditions.

For the extracted cytochrome *c* in the organic solution, the Soret band is shifted from that of native cytochrome *c*, and the ligand-to-metal charge transfer band disappears. The disappearance of the ligand-to-metal charge transfer band corresponds to cleavage of the coordination bond between Met 80 and heme iron. The circular dichroism (CD) spectrum of cytochrome *c* extracted using calix[6]-arene is also different from that of native cytochrome *c* in water. These spectral changes suggest that the tertiary structure of the protein complexed with the calixarene is different to that of native cytochrome *c*.

Recently, a biosensor based on supported lipid films with incorporated calix[6]arene for detection of cytochrome *c* was studied using electrochemical impedance spectroscopy [73]. Shuang and co-workers reported that carboxylphenyl-modified calix[4]arenes could also form complexes with cytochrome *c* [74, 75]. The binding affinity to cytochrome *c* in dimethylformamide using tetrakis-carboxylphenylcalix[4]arene was higher than those using the structural analogues bis-carboxylphenylcalix[4]arene and tetrakis-phenylcalix[4]arene, which indicates binding is dependent on the number of carboxyl groups. The fluorescence of the calix[4]arene derivative was quenched on binding with cytochrome *c*.

Separation of proteins by liquid–liquid extraction with calixarene

Generally, proteins are separated based on differences in their physical properties, such as molecular weight and isoelectric point (p*I*) [6, 7]. By contrast, calix[6]arene can separate cationic proteins that have similar molecular weights and p*I*s by discriminating the numbers of lysine residues on the surfaces of the protein molecules. Calix[6]arene shows extraction selectivity for lysine-rich proteins because its extraction of cytochrome *c* is based on complexation between the calix[6]arene and protonated amino groups of lysine residues. The cationic proteins, cytochrome *c*, ribonuclease A, and lysozyme have similar molecular weights but different numbers of lysine residues as follows: 19 for cytochrome *c*, 10 for ribonuclease A, and 6 for lysozyme. Because of calix[6]arene's selectivity for lysine-rich proteins, cytochrome *c* is quantitatively extracted but ribonuclease A and lysozyme are not extracted and tend to aggregate in the aqueous phase. This illustrates how calix[6]arene can selectively extract proteins based on the difference in the number of lysine residues on the protein surface.

Cytochrome *c* extracted using calix[6]arene can be quantitatively recovered into an aqueous solution under optimal conditions [76, 77]. Because the extraction is based on the proton-exchange reaction, the extracted protein is stripped using an aqueous acidic solution. The organic solvent and co-solvent should be selected carefully for the back-extraction. For example, the extracted protein is effectively stripped from isooctane containing 1-octanol (volume fraction 10%) as the co-solvent, but not from polar organic solvents such as chloroform [77]. The addition of co-solvents such as alcohol that distribute into both the aqueous and organic phases is effective for controlling the difference in the hydrophilicity/lipophilicity of the aqueous and organic phases. As a model case, the separation of cytochrome *c* and lysozyme by solvent extraction using calix[6]arene was examined under optimal conditions [77]. In the forward extraction stage, 93% of the cytochrome *c* in the aqueous solution was extracted with calix[6]arene, while lysozyme remained in the solution. In the subsequent stripping stage, 97% of the extracted cytochrome *c* was recovered in the aqueous solution, whereas lysozyme was not detected at all.

The tertiary structure of cytochrome *c* after the back-extraction is denatured because of the acidity and the presence of the alcohol in the aqueous stripping solution, but this structure is recovered after neutralization of the solution. The activity of cytochrome *c* as an electron

transfer protein is partly retained (67%) through the forward extraction and back-extraction after neutralization.

A *p-tert*-octylcalix[6]arene carboxylic acid derivative has been investigated for the extraction of the major serum antibody immunoglobulin G (IgG). Martinez-Aragon et al. reported extraction of the very large IgG protein (MW = 135,000) into isooctane containing 10% 1-octanol as co-solvent [78]. At higher pH values, up to 50% of the IgG was transferred to the organic phase. The IgG was back extracted from the organic phase to a fresh aqueous solution, but denaturation of IgG occurred through the extraction process.

Protein refolding via liquid–liquid extraction using calixarene

The expression of recombinant proteins in bacteria often results in the formation of insoluble aggregates called inclusion bodies. The insoluble aggregate can be solubilized using denaturants to regenerate the active form of the protein. The solubilized protein is refolded by decreasing the concentration of the denaturant. To prevent reaggregation of the folding intermediate, the proteins must be isolated from the solution containing the denaturants and unfolded protein. The liquid–liquid extraction process for proteins using the calix[6]arene carboxylic acid derivative can also be applied to this protein refolding [79]. Denatured protein molecules complexed with multiple calixarene molecules are isolated from each other. Finally, the extracted protein is recovered into a denaturant-free aqueous solution and spontaneously refolded into a biologically active conformation. As a case study, cytochrome *c* was extracted from an aqueous solution containing 8 mol L^{-1} urea at pH 4.5–6.0. The extracted cytochrome *c* was recovered into an acidic solution (pH < 2.5) containing 1-butanol (volume fraction 30%) as co-solvent. Ultraviolet–visible spectroscopy, CD, and fluorescence spectroscopy confirmed the structure of the refolded protein was identical to that of native cytochrome *c*. These results suggest that the calixarene protein extraction system provides a chaperone-like function for denatured protein.

Catalytic oxidation by cytochrome c extracted from organic media

Cytochrome *c* complexed with the calix[6]arene carboxylic acid derivative exhibits peroxidase activity in organic media [66, 80]. The oxidation of 2,6-dimethoxyphenol proceeds in the presence of cytochrome *c* in chloroform. Similarly, the N-demethylase activity of cytochrome *c* is enhanced by complexation with calix[6]arene. Enzymatic polymerization of *o*-phenylenediamine by addition of cytochrome *c* and hydrogen peroxide has been examined. The *o*-phenylenediamine (MW > 5,000) was polymerized in chloroform or hexane, and the average molecular weight of the polymer was around 7,000. The peroxidase activity in organic media was influenced by various factors. The peroxidase activity of cytochrome *c* increased with an increase of the extraction pH, probably because of denaturation of cytochrome *c* at lower pH values. The oxidation activity also depended on the type of organic solvent. The calixarene functions as a modifier for proteins by dissolving the protein into organic solution and by enhancing the catalytic activity.

Solvent extraction of the major hemoprotein hemoglobin (MW = 64,500) using *p-tert*-butylcalix[*n*]arene carboxylic acid derivatives (*n* = 4, 6, 8) was investigated by Karmali and co-workers [81]. Hemoglobin extraction at pH 5.1 was in the order calix[6]arene > calix[4]arene > calix[8]arene. The hemoglobin–calix[6]arene complex exhibited pseudo-peroxidase activity in chloroform, and catalyzed the oxidation of syringaldazine in the presence of hydrogen peroxidase. The catalytic half-life of the complex ranged from 1.96 to 2.64 days.

Recognition of proteins using crown ethers

Dissolution of proteins in organic media using crown ethers

18-Crown-6 is a well-known complexing reagent for biologically important amino compounds [82–86]. 18-Crown-6 can be used for inclusion of protonated amino groups because of its cavity size and preorganized oxygen for cation–π interactions. Therefore, 18-crown-6 and its derivatives also act as complexing reagents for the recognition of protein lysine residues, and form supramolecular complexes. The resulting *n*:1 (crown ether:protein) supramolecular complexes are more hydrophobic than the native protein and is dissolve in organic solutions.

Odell et al. reported that proteins can be solubilized in organic solvents by complexation with crown ethers and cryptands [87, 88]. 18-Crown-6 and cryptand[2.2.2] are more effective for dissolution than smaller or larger macrocycles. Reinhoudt et al. studied the effect of crown ethers on enzyme-catalyzed reactions in non-polar organic solvents [89]. 18-Crown-6 and dicyclohexano-18-crown-6 (DCH18C6) enhanced the rate of α-chymotrypsin-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester with propan-1-ol in *n*-octane. The lyophilization of serine proteases with crown ethers enhances enzymatic activity in organic solution. Some crown ethers have the potential to enhance both the enantioselectivity and reaction rate in lipase-catalyzed hydrolysis of acetate [90–95]. Many possibilities for crown ether activation of enzymes have been considered. It is likely that both acceleration of substrate transport by the crown ethers, and changes in the

conformational characteristics of the enzyme because of complexation between the crown ethers and lysine residues occur. Additionally, crown ethers are thought to participate in conformational stabilization of enzymes because of specific interactions with lysine residues [14, 15, 96, 97].

Tsukube et al. demonstrated chemical activation of cytochrome *c* in methanol via complexation with crown ethers [98–100]. In a series of lariat ethers, an alcohol-armed 18-crown-6 derivative exhibited the highest solubilization efficiency for cytochrome *c*. The supramolecular complex between cytochrome *c* and the lariat ether catalyzed the oxidation of pinacyanol chloride with hydrogen peroxide. Spectroscopic and electrochemical characterization showed that the supramolecular complex had the structure of cytochrome *c* in alkaline aqueous solution. The supramolecular complex between cytochrome *c* and 18-crown-6 promotes asymmetric oxidation of organic sulfoxide in methanol at lower temperatures. ESI–MS, ultaviolet visible spectroscopy, CD, and Raman spectroscopy confirmed that four or five 18-crown-6 molecules bound to each cytochrome *c*.

Complexation between lysine residues and 18-crown-6 molecules has been confirmed by ESI–MS [16, 17, 101]. The most intense peak of a mixture of 18-crown-6 and tetralysine is for the quadruply-charged peptide with four crown ethers attached. Namely, there is a one-to-one correlation between the number of 18-crown-6 and lysine residues. Julian et al. proposed an electrospray ionization mass spectrometry method to probe the protein structure using the interaction between lysine residues and 18-crown-6.

Liquid–liquid extraction of proteins using crown ethers

Crown ether extracts proteins in an ionic liquid extraction system as well as an aqueous two-phase system (ATPS). Shimojo and Goto reported quantitative extraction of cytochrome *c* into a hydroxyl-group-containing ionic liquid ([C_2OHmim][Tf_2N]) solution using DCH18C6 (Fig. 8) [102, 103]. The protein dissolved in an aqueous phase was quantitatively transferred into the ionic liquid phase in the presence of 1,000 equivalents of DCH18C6. The extraction mechanism of the protein using the crown ether can be correlated to that using the calix[6]arene, with complexation between multiple ε-amino groups of the lysine residues and the crown ether molecules.

The structural changes in cytochrome *c* dissolved in an ionic liquid by complexation with DCH18C6 were probed using ultraviolet–visible spectroscopy, CD, and resonance Raman spectroscopy. The results show it is likely that the sixth ligand Met 80 in the heme group of the complex between cytochrome *c* and DCH18C6 in the ionic liquid is replaced by other amino acid residues and that a non-natural, six-coordinate, low-spin ferric heme structure is induced. The supramolecular complex in [C_2OHmim][Tf_2N] offers peroxidase activity, and accelerates the initial reaction rate 5.5-fold relative to that of native cytochrome *c*.

The crown ether DCH18C6 is effective for controlling the distribution of cytochrome *c* in Li_2SO_4/PEG ATPS [104, 105]. The lysine-rich protein cytochrome *c* is distributed in a hydrophobic PEG-rich phase by multiple interactions between DCH18C6 molecules and the lysine residues. The protein is quantitatively extracted into the PEG-rich phase in the presence of DCH18C6 and perchlorate ion. Addition of perchlorate ion was also required for the extraction of cytochrome *c*. Of the various crown ethers and their analogues that were investigated, only DCH18C6 was able to extract cytochrome *c*. As the complexation is based on cation–dipole interaction between the protein and the crown ether, the extraction proceeds under weakly acidic and neutral conditions, in which the protein is positively charged. Analysis using reversed phase HPLC showed cytochrome *c* complexed with the crown ether was sufficiently hydrophobic to be transferred into the hydrophobic PEG-rich phase.

Cytochrome *c* complexed with DCH18C6 in the PEG-rich phase can be quantitatively recovered into a salt-rich phase using K_2SO_4 by ion exchange of the potassium ion and cationic protein. Furthermore, the extraction system using DCH18C6 is efficient for the separation of lysine-rich protein cytochrome *c* over other cationic proteins. Under optimum conditions, cytochrome *c* is selectively extracted using DCH18C6 over other cationic proteins (Fig. 9). The ATPS using the crown ether as a ligand is a novel separation system that discriminates on the basis of the number of lysine residues of the protein.

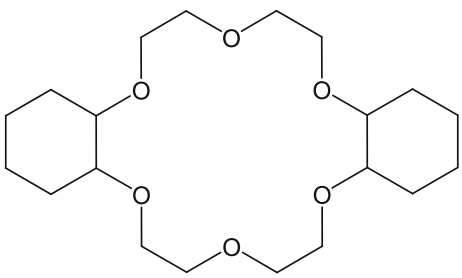

Fig. 8 Molecular structure of DCH18C6 [16, 17, 101]

Recognition of proteins using TPP derivatives

Porphyrin compounds are also attractive for preparation of receptors for proteins because of their rigid structures, multifunctionality, aromatic structures for hydrophobic and π–π stacking interactions, and photophysical properties. Jain and Hamilton and co-workers developed various TPP derivatives for recognition of various proteins by introducing multiple functional groups and amino acid residues

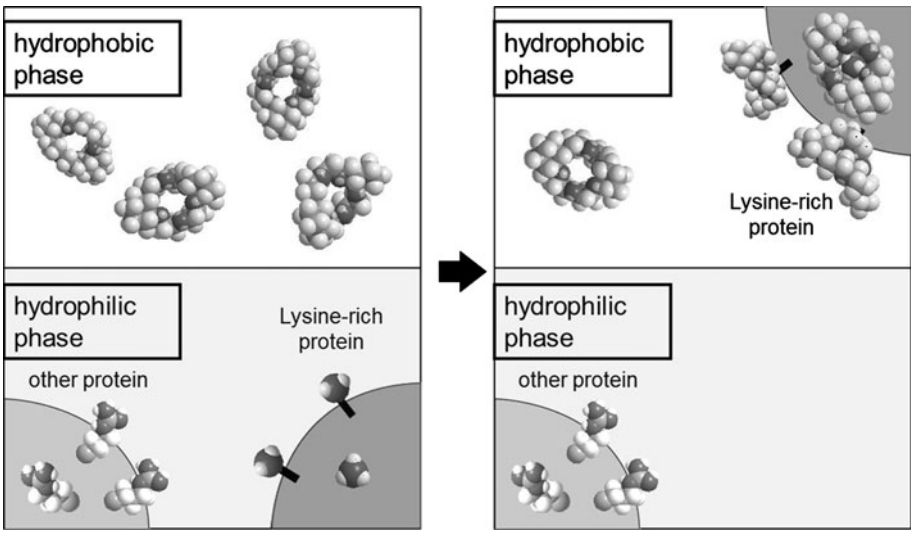

Fig. 9 Schematic illustration of protein extraction using crown ether in ionic liquid extraction and aqueous two-phase systems

at the periphery. Complexation with cytochrome *c* using a series of TPPs bearing various amino acid derivatives was studied [106]. Addition of cytochrome *c* to solutions containing the TPPs resulted in quenching of porphyrin fluorescence emission. Formation of the 1:1 complex was confirmed by Job's method. The TPP derivative bearing Tyr-Asp residues (Fig. 10), which has eight negatively charged groups and eight phenyl groups, was the strongest receptor for cytochrome *c*. The dissociation constant (K_d) was 20 ± 5 nM, which is much smaller than that of its natural protein partner cytochrome *c* peroxidase.

Complexation with TPP derivatives induces a conformational change in an active protein with subsequent loss of function. Complexation with the TPP derivative bearing four anionic Tyr-Asp residues lowered the melting point of cytochrome *c* to room temperature [107]. The receptor selectively bound native horse-heart cytochrome *c* rather than cytochrome c_{551} and acetylated cytochrome *c*, which lack cationic lysine residues. A family of tetrabiphenylporphyrin-based receptors has also been prepared for binding to proteins [108]. Tetrabiphenylporphyrin receptors bearing eight carboxyl groups showed moderate affinity to cytochrome *c* (K_d = 1.3–1.7 μM) in the presence of 0.05% Tween 20. By comparison, the receptor bearing 16 carboxyl groups (Fig. 11) had much stronger affinity (K_d = 0.67 nM) for complexation with cytochrome *c*, and enhanced protein unfolding. The receptor also shows selectivity to cytochrome *c* (12.5 kDa, p*I* = 10.4) over cytochrome c_{551} (9.3 kDa, p*I* = 4.7) and ferredoxin (6.2 kDa, p*I* = 2.75). Because the melting point of cytochrome *c* in the presence of the tetrabiphenylporphyrin-based receptor is influenced by ionic strength, electrostatic interactions contribute to the complexation. Metalloporphyrin derivatives have also been prepared for complexation with cytochrome *c* [109], and dimeric Cu(II)-porphyrins were effective for denaturation.

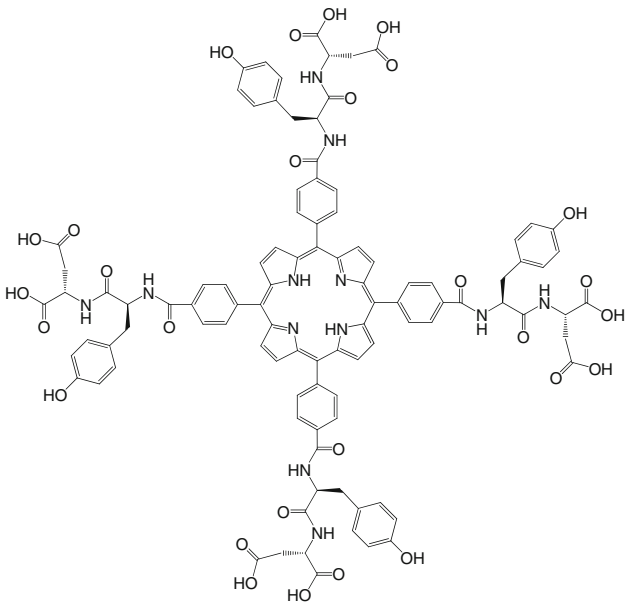

Fig. 10 Molecular structure of the tetraphenylporphyrin derivative bearing Tyr-Asp residues [103]

Complexation with the copper TPP derivatives accelerates denaturation and proteolytic degradation of heme proteins with trypsin [109–111]. In the presence of four equivalents of the dimeric Cu(II)-porphyrin, tryptic digestion of cytochrome *c* was advanced compared with the porphyrin free condition. Addition of the dimeric Cu(II)-porphyrin also accelerated proteolytic degradation of cytochrome *c* with trypsin. The induced conformational changes including disruption of native heme ligation and unraveling of α-helical secondary structure upon binding lowered the free energy of the protein conformation for proteolytic attack by trypsin by about 2.4 kcal/mol. Because the complete digestion of cytochrome *c* proceeded only in the presence of 0.1 equivalents of the dimeric

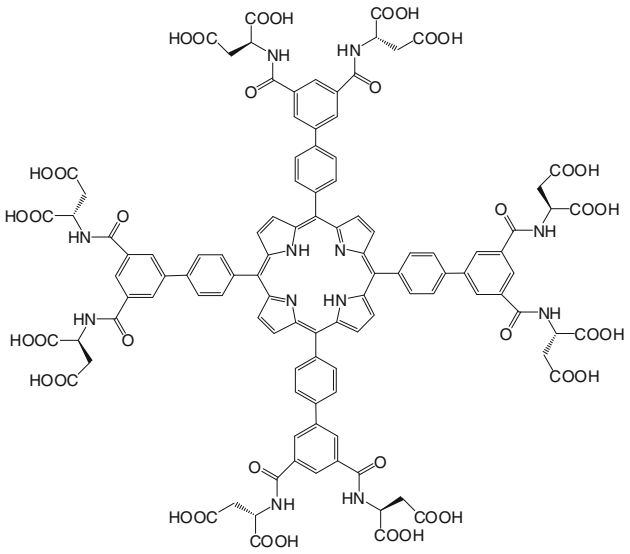

Fig. 11 Molecular structure of the tetraphenylporphyrin derivative bearing 16 carboxylic acid groups [105]

Cu(II)-porphyrin, the dimeric Cu(II)-porphyrin catalytically accelerated the digestion. Thus the synthetic porphyrins act as cocatalysts by conformationally activating protein cleavage by protease. Furthermore, a catalytic quantity of the dimeric Cu(II)-porphyrin catalyzes trypsin-mediated digestion of the larger proteins myoglobin and hemoglobin.

The charge, size, hydrophobicity, and symmetry of the peripheral substituents on TPP derivatives can be used to target the binding characteristics of the derivative towards various proteins. TPP derivatives are highly fluorescent and will show changes in the emission intensity on binding to the target protein. This has been used to develop a series of TPP derivatives for protein fingerprinting [112]. TPP derivatives with different amino acids or amino acid derivative (Fig. 12) peripheral substituents were prepared using a mixed condensation strategy. Thirty-five fluorophores were produced by repeated porphyrin functionalization and combination of the two peptidic components, followed by separation and deprotection. The fluorophores had every possible charge combination from +8 to −8 and four to eight hydrophobic groups. Eight members of the library with different functionalities were selected and arrayed in a 96 well plate. The fluorescence emission changes of the TPP derivatives on binding to four different proteins were assessed. Fluorescence or a lack of fluorescence was observed depending on the complexation between the TPP derivatives and proteins, and each column of the plate corresponded to a unique fingerprint that was characteristic to a specific protein. The fluorescence quenching accompanying complexation with the TPPs is

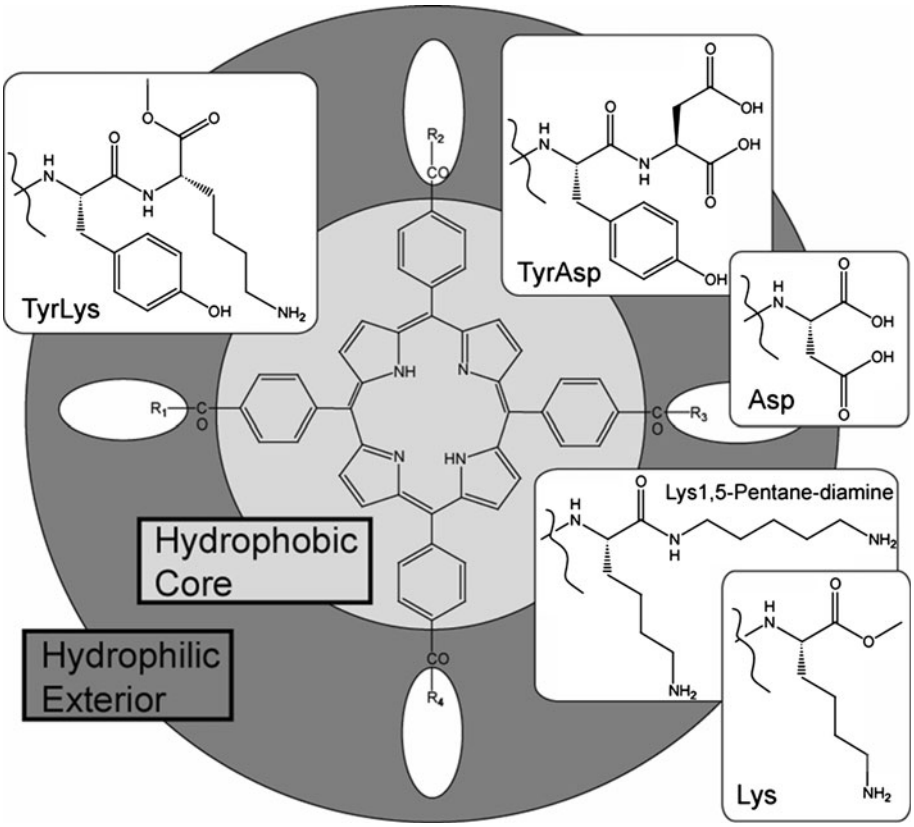

Fig. 12 Molecular structures of tetraphenylporphyrin derivatives bearing different amino acids or amino acid derivatives [109]

mainly based on the complementary charges and hydrophobic interaction. For example, the cationic protein cytochrome *c* is bound by negatively charged fluorophores, whereas the anionic protein ferredoxin is bound by positively charged fluorophores. The fluorescence emission changes of the TPPs on binding to proteins are complicated and versatile, because of the diverse complexation sites and mechanisms. The identification of both metal- and non-metal-containing proteins and protein mixtures has been demonstrated using the porphyrin array combined with pattern recognition techniques [113]. Unambiguous identification of a variety of proteins was achieved using an eight-porphyrin array, after processing the data matrix by principal component analysis clustering. The resolution of principal component analysis mapping for the proteins was improved using a sixteen-porphyrin array. The protein-detecting array allows facile detection without labeling of the sample proteins.

Other artificial ligands for protein recognition

As discussed above, various ligands based on calixarenes, crown ethers, and TPPs have been developed for the recognition of proteins. Artificial ligands have also been developed using other platforms, and these studies are briefly summarized in this section.

Ruthenium trisbipyridine complexes ($[Ru(bpy)_3]$) (Fig. 13) have been developed as substrates for cytochrome *c* [114]. The $[Ru(bpy)_3]$ complex with carboxylate groups on the periphery was selectively for cytochrome *c* complexation. Wilson and co-workers also studied selective protein-surface sensing using a ruthenium trisbipyridine complex [115, 116]. One of the functionalized Ru(II) complexes showed high affinity ($K_d = 2$ nM) to cytochrome *c*. Additionally, the affinity of functionalized Ru(II) complexes to cytochrome *c* was found to be dependent on their geometrical configuration. A series of anthracene-derived receptors has also been developed for protein recognition [117]. Receptors bearing multiple carboxylic acid groups exhibited affinity to cationic proteins such as lysozyme and cytochrome *c*, rather than cytochrome c551, α-lactalbumin, myoglobin, and RNase A.

Transition metal ions such as Cu(II), Ni(II), Co(II), and Zn(II) are also available for complexation with histidine residues of proteins. Mallik and co-workers developed multidentate ligands bearing multiple transition metal ions for recognition of histidine residues distributed on protein surfaces [118]. Trivalent ligands with Cu(II)-iminodiacetate arms exhibited higher affinity for carbonic anhydrase, which has six histidine residues located at specific distances from each other. One of the ligands (Fig. 14) bound to carbonic anhydrase with a K_a of 2.99×10^5 M^{-1} (Fig. 15).

Dendrimers are globular, large, monodisperse macromolecules with a central core and branched multivalent surface. They have been used as platforms to prepare sophisticated ligands [119]. Hirsch and co-workers prepared polyanionic fullerene dendrimers for complexation with cytochrome *c* [120]. The association constants between Zn-cytochrome *c* and the dendrofullerenes were approximately 10^5 M^{-1}. Tsukube and co-workers prepared a series of "proteo-dendrimer"-type receptors for complementary recognition of cytochrome *c* [121]. These receptors included asymmetrically distributed polyanionic hepta(glutamic acids) for interaction with cytochrome *c*, a zinc porphyrinate core, a hydrophilic polyether surface, and nonpeptide dendric components. The dendrimers exhibited affinity for cytochrome *c* and blocked complexation of cytochrome b_5 with cytochrome *c*.

Recently, nanoparticles have been considered as platforms for protein recognition [122–124]. Nanoparticles (size 1.5–10 nm) are comparable in size to proteins, which

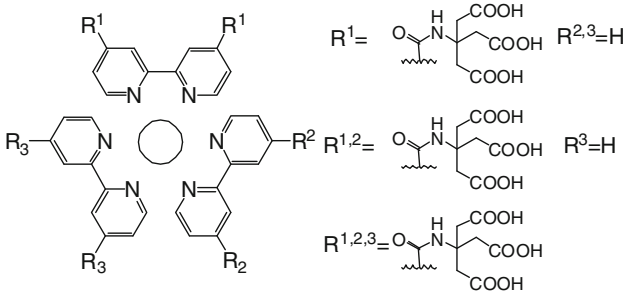

Fig. 13 Molecular structures of ruthenium trisbipyridine complexes [111]

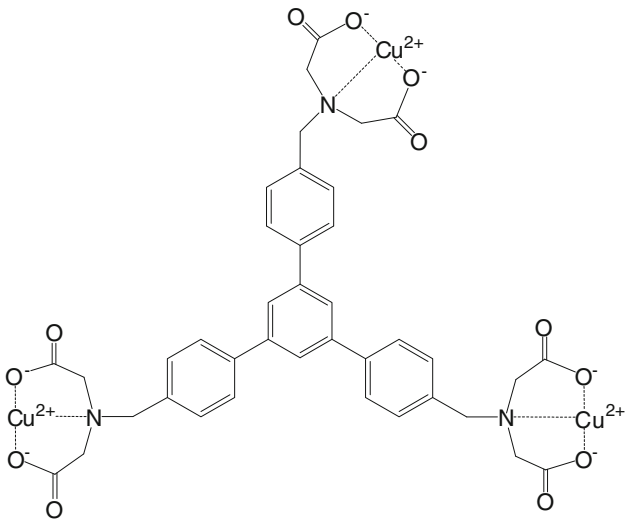

Fig. 14 Molecular structure of a trivalent ligand bearing Cu(II)-iminodiacetate arms [115]

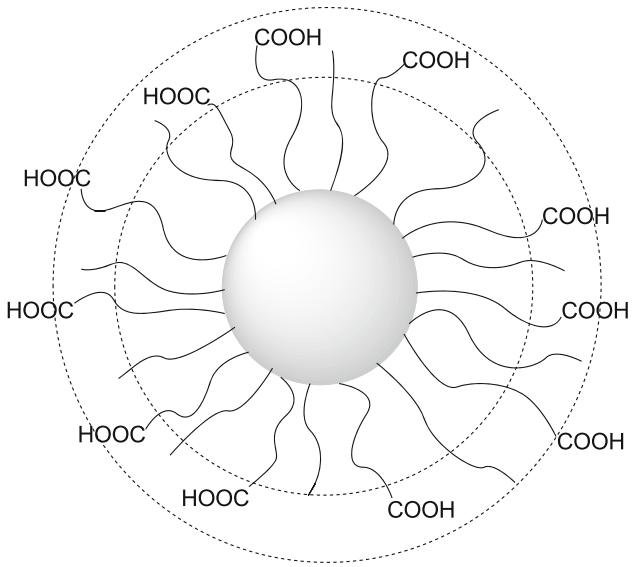

Fig. 15 Conceptual illustration of carboxylate-functionalized mixed monolayer protected clusters (MMPCs) [122]

is favorable for the recognition of protein surfaces. Rottelo and co-workers reported recognition of proteins using gold nanoparticles functionalized with anionic groups. Carboxylate-functionalized mixed monolayer protected clusters (Fig. 15) interacted with the model protein α-chymotrypsin, resulting in complete enzyme inhibition [125, 126]. Cytochrome *c* and cytochrome *c* peroxidase also interacted with functionalized gold nanoparticles based on their complementary charges [127]. A sensor array containing noncovalent gold nanoparticle–fluorescence polymer conjugates was developed to detect, identify, and quantify proteins [128]. The fluorescence of the polymer was quenched by complexation with cationic nanoparticles. Addition of proteins disrupted the nanoparticle-polymer interaction and resulted in a variety of fluorescence responses, which could be used for rapid identification of the proteins.

Perspective

The field of artificial ligand development for the recognition of proteins has rapidly advanced in recent years. Some ligands have been produced that show high affinity to target proteins, and specific recognition of target proteins has been achieved. These ligands have a variety of uses, including detection of the target protein, disruption of protein–protein interactions, denaturation of proteins, and modulation of protein functionality. Additionally, the artificial ligands used for recognition of proteins are valuable for protein separation. These ligands can trap a target protein from a mixture of similar proteins. Calixarenes and TPPs, which have a rigid scaffold and multiple peripheral ligation sites, are useful for preparation of multivalent ligands for proteins. Dendrimers and nanoparticles are also potent candidates as platforms for ligands to recognize proteins.

However, the use of artificial ligands for discrimination of specific amino acid residues and entire proteins from other residues/proteins is limited. To date, synthetic ligands have been developed that are selective for lysine, histidine, and cysteine. Ligands need to be developed for selective complexation of other residues, and these could be used in a number of applications.

Despite recent advances, development of ligands for recognition of whole proteins is still not easy. Ligand multivalency provides high affinity to the target protein, but selectivity for a specific protein in a complex mixture is difficult to achieve. It is also not easy to form a ligand–protein complex and retain the protein's native tertiary structure. Complexation with affinity ligands often alters the conformation of the protein to a non-native state. Retention of the protein native conformation during recognition via complexation is the ultimate goal in this area. Suitable ligands will need to have multiple, complementary mild interactions with the target protein. Rational design of the ligand structure considering complementary interactions and combinatorial preparation technology will be important for ligand preparation [9]. From the viewpoint of cost performance, the strategies used to prepare the ligands should be simple and versatile.

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