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CD-Based Rotaxanes and Polyrotaxanes as Representative Supramolecules

Recent Reports

Rotaxanes

Structural analysis of rotaxanes consisting of alkylene backbone and α-CD or permethylated α-CD has been performed [6]; halogen bonding rotaxane to sense anions in water [7] and rotaxane for detection of toxic metals [8] also have been reported. Moreover, the [3]rotaxanes emitting blue light, which consist of alkynylpyrene and permethylated α-CD, were described [9]. One should mention here also a series of four reviews of our group concerning syntheses and properties of rotaxanes [10–13].

Pseudorotaxanes

Among pseudorotaxanes, the motion of the two rings in palindromic [3]pseudorotaxanes [14] and the photooxygenation of multiply threaded pseudorotaxanes have been investigated [15]. Also, the complexation of CD-based pseudorotaxanes with isoprenoid compounds, for example, with the reduced coenzyme Q_{10}, and with squalene for improvement of the pharmaceutical properties of obtained CD-pseudorotaxane-like supramolecules was studied [16], as well as the fabrication of poly(ε-caprolactone)/α-CD pseudorotaxane nanofibers [17].

Polyrotaxanes

The first fast radical end-coupling synthesis of polydimethylsiloxane (PDMS)-γ-CD-based polyrotaxanes (PRs) [18] and the preparation of PRs by copper-free click chemistry [19] have been reported. Cationic Pluronic-based PR$^+$s threaded with 2-hydroxypropyl-β-CD (HPCD) have been synthesized for pDNA delivery; they can be used as potent vectors for pDNA-based therapeutics [20].

Pseudopolyrotaxanes

Among studies concerning pseudopolyrotaxanes (PPRs), one should mention the report on PPRs consisting of poly(*p*-dioxanone) and CDs, which were

obtained via heat–cool cycles; it was found that they have better thermal stability than their backbone [21].

In this chapter, selected examples of CD-based rotaxanes (Section 1.1), PRs with triblock and pentablock backbones (Section 1.2.1), and PRs with other backbones (Section 1.2.2), as well as PPRs (Section 1.3), are presented.

1.1 CD-Based Rotaxanes

Rotaxanes are often multifunctional due to the combination of their properties, for example, photochromic [22], photoconductive [23], or electronic [24], which is promising for their use in sensors, molecular switches, or molecular machines. The viologen-based rotaxanes containing azobenzene groups and CD rings are such examples [25]. These systems are an important class of dyes because of the properties of azobenzene moieties [26] and of the photoactivity and strong electron acceptor character of viologens [27]. They also deserve attention due to the presence of the azo group, showing solvatochromism and nonlinear optical (NLO) properties [28]. One should note that in rotaxanes the CD units can undergo controlling shuttling movements, induced by various stimuli, often by irradiation [25].

In the experiments [2]rotaxanes have been synthesized; their dumbbells consist of azobenzene and viologen moieties stoppered with pentacyanoferrate groups; they are threaded by α- and β-CD units [29]. The pentacyanoferrate stoppers act as strong electron donors; they are connected with the strong electron acceptor viologen, thus giving rise to an exceptionally intense solvatochromism. The work is a continuation of the previous study on ferrocyanide(II) complexes of 4,4'-bipyridines, serving for comparison of their solvatochromic properties to those of synthesized rotaxanes [30]. The starting compound of the process is the Zincke salt **1.**

For the synthesis of compounds 2 and 3, first **2** has been obtained by an improved, earlier used procedure [25]. The synthesis of **2** proceeds in the solid state, (solvent-free Zincke reaction); for this purpose, **1,** that is, the Zincke salt and *p*-azodianiline were dissolved in ethanol and heated in an open round bottom flask until the entire quantity of ethanol was removed. The remaining pasty mixture was heated under a condenser overnight in the absence of any solvent. The 2,4-dinitroaniline formed as a by-product on the upper part of the flask was removed by sublimation, and the solid mass was dissolved in MeOH and treated with Et_2O to precipitate **2** as a red powder.

The aqueous solution of **2** formed by the reaction with the complex salt $Fe^{II}(CN)_5NH_3 \cdot 3H_2O$ (solid) turned blue. Addition of EtOH precipitated the deep blue dumbbell **3** (Figure 1.1).

For the synthesis of dyes 4a,b, the mixture of **2** with water was treated with α- or β-CD. In both cases the immediate dissolution of **2** occurred, indicating formation of pseudorotaxanes. Upon addition of $Na_3 [Fe^{II}(CN)_5NH_3] \cdot 3H_2O$, during the *in situ* stoppering, the color of both solutions turned deep blue. The reaction mixtures were stirred in the dark under an argon atmosphere at room
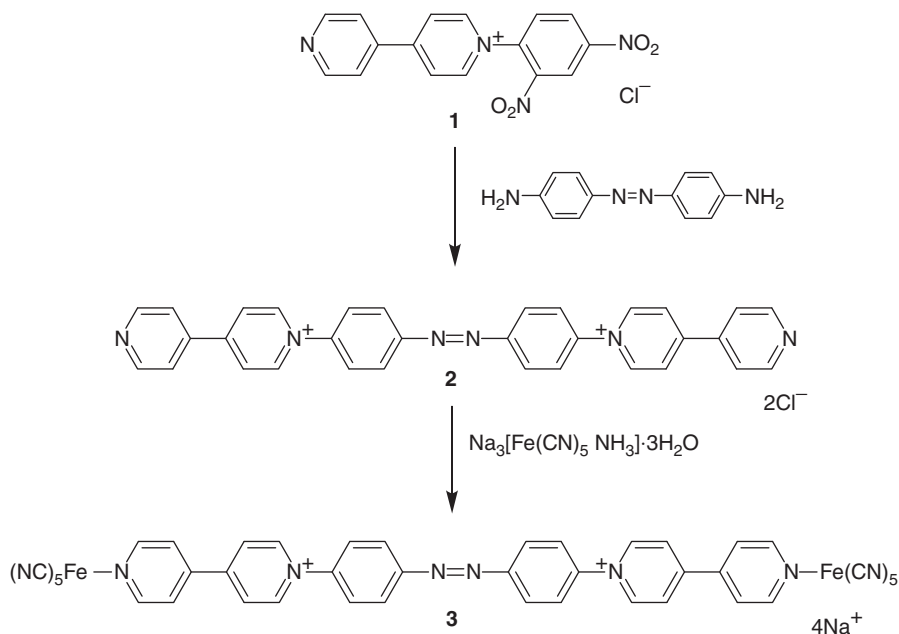
Figure 1.1 Synthesis of compounds **2** and **3**.

temperature, and the subsequent addition of EtOH precipitated **4a,b** as blue powders (Figure 1.2).

Compounds **3** (i.e., dumbbell of **4a,b**) and **5,** (i.e., dumbbell of **6a,b**) have the same precursor **2** [23, 25]. In **3** and **4a,b,** the strong electron-donating cyanoferrate groups are stoppers; in **5** and **6,** the strong electron-withdrawing 3,4-dinitrophenyl groups are stoppers. Intense charge transfer in **3** and **4a,b,** as compared to **5** and **6,** explains the intense solvatochromism of **3** and **4a,b** and the only negligible solvatochromism of **5** and **6** [30] (Figure 1.3).

The dumbbell **3** and rotaxanes **4a,b** are intensively solvatochromic. Solvatochromism involves the change in electronic spectra of a dye upon alteration of polarity of its solvent [31]; the changes in color often may be observed by the naked eye. Solvatochromic dyes receive growing attention today since they can be used as molecular sensors [32] and probes of solvent polarity. They became the basis of empirical parameters of solvent polarity, that is, of a Reichardt's dye and the corresponding polarity scale E_T (30) [33], serving for a scale of dipolarity/polarizability and Lewis acidity of solvents [33].

The experimental results show that the introduction of α- or β-CD units into **3,** affording **4a** or **4b,** does not decrease the solvatochromic character of **3;** all three compounds **3** and **4a,b** exhibit a very intense solvatochromism, even stronger than in the case of the Reichardt's betaine [33].

Dumbbell **3** and rotaxanes **4a,b** are highly soluble in polar hydroxylic solvents, predominantly in water, since they form, by their nitrogen atoms of CN groups, strong hydrogen bonds with molecules of these solvents [30]. Solvatochromism of **3** and **4a,b** was investigated in water/ethylene glycol binary mixtures.
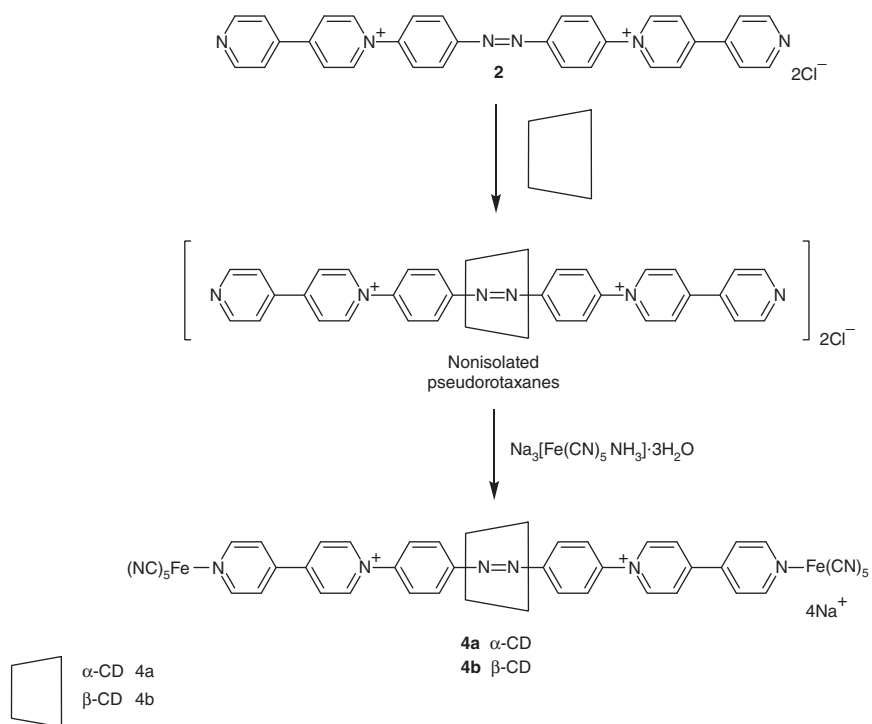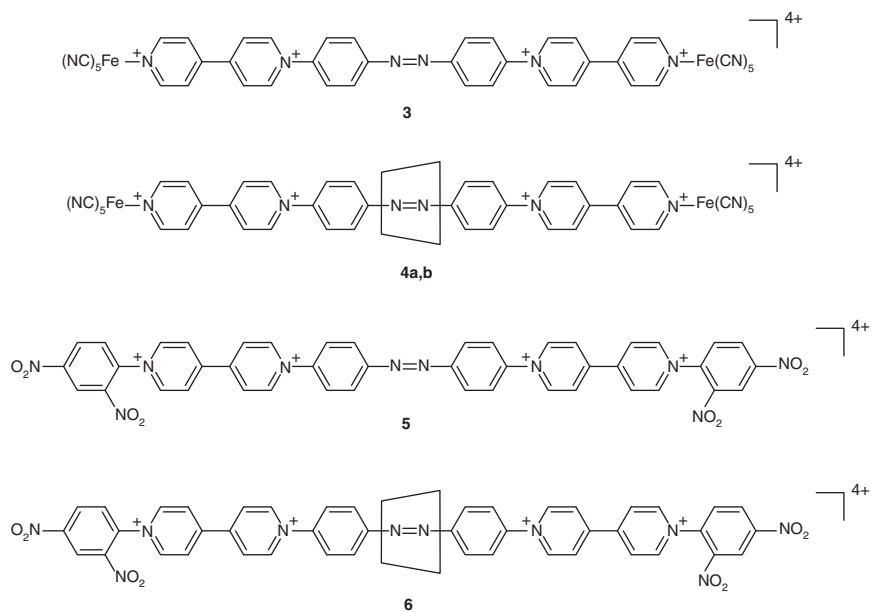
Figure 1.2 Synthesis of dyes **4a,b.**

Figure 1.3 Compounds **3, 4a,b, 5,** and **6.**

Water and ethylene glycol were chosen as two solvents not only because of their ability to dissolve **3** and **4a,b** but also because of the high stability of **3** and **4a,b** in these solvents and their mixtures. It was observed that **3** and **4b** show similar susceptibilities to medium polarity changes (in the region between the polar solvents water and ethylene glycol).

It is known that *azo dyes* can undergo photochemical trans/cis isomerization; due to this property, the azo dyes are promising for design of photoresponsive compounds and materials of a wide range of applications [26, 34]. However, in **3** and **4a,b,** the presence of the stopper groups —$Fe^{II}(CN)_5$ renders these compounds photochemically unstable. The irradiation of pentacyanoferrate(II) complexes results in the loss of the —$Fe^{II}(CN)_5$ groups [35]. But, on the other side, one should point out that these groups render these compounds strongly solvatochromic. It is noteworthy that **3** and **4a,b** are very stable in solution when they are not irradiated.

Today, the light-responsive drug delivery systems are intensively studied since they may enhance drug delivery efficiency and minimize side effects. It is known that light stimuli can easily be exerted with high precision at specific sites. There have been reports of several light-responsive drug carriers, upon irradiation undergoing cleavage of chemical bonds [36] or conformational changes, for example, *cis–trans* photoisomerization of azobenzene [37]. In these systems, UV or visible light is usually used as a trigger. However, due to the "water window" (700–1400 nm), the tissue penetration of UV and visible light is limited, resulting in inefficient deep-tissue drug delivery [38]. Therefore, to overcome this difficulty, the near-infrared (NIR)-responsive drug delivery systems are employed. NIR irradiation has high transmittance and attenuated cytotoxicity in living tissues and is of interest in noninvasive cancer therapy.

Thus was developed the NIR-responsive nanosystem for anticancer drug delivery [39]; it consists of the photo-switchable α-CD-based azobenzene rotaxane, immobilized onto an Au nanorod-mesoporous silica core–shell hybrid where the Au nanorods are silica covered.

The Au nanorods (core), which are widely used as a photothermal agent [40], serve as the energy converter to activate the isomerization of the azobenzene moiety. The mesoporous silica (shell) serves as a drug-storage reservoir [41] and as the substrate for postmodification by the rotaxane [42]. The rotaxane immobilized on the silica layer, formed of a thread containing the azobenzene group and α-CD encapsulating the *trans*-azobenzene, acts as a capping agent to control the drug loading and release.

The experiments concerning the design of the NIR-responsive nanosystem involve following procedures A–D.

A. For *the preparation of Au mesoporous silica-covered nanorods (Au@MSN),* four steps are necessary:

In the first step, the ultrasmall Au seeds were prepared by reduction of $HAuCl_4$ using $NaBH_4$ in aqueous environment. For this purpose, the $HAuCl_4$ aqueous solution was mixed with cetyltrimethyl ammonium bromide (CTAB) aqueous solution. Then this mixture was treated with

ice-cold NaBH$_4$ aqueous solution, and the ultrasmall Au seeds were formed immediately.

In the second step, the growth solution for Au nanorods was prepared; it is a mixture of CTAB, HAuCl$_4$, AgNO$_3$, H$_2$SO$_4$, and ascorbic acid solutions, added sequentially. The growth was initiated by treating this mixture with the above-obtained seed solution and was carried out at 30 °C for 6 h. The prepared Au nanorods were washed with water to remove the excessive CTAB, and then they were extracted by centrifugation and concentrated to 10 mg Au ml^{-1}.

In the third step, the mesoporous silica coating was performed via a template method. First, the concentrated Au nanorods solution (1 ml) was redispersed in aqueous CTAB solution (0.01 M, 100 ml) and the mixture was stirred for 15 min. The mixture was treated with ammonia water in order to adjust the solution pH to be slightly basic and then tetraethoxysilane (TEOS) was added. The temperature of the mixture was kept at 30 °C, and the reaction was carried out for 24 h. The Au nanorods coated with silica were extracted by centrifugation.

In the fourth step to remove the CTAB template, the prepared Au nanorods coated with silica were dispersed in ethanol (40 ml) containing hydrochloric acid (5 ml), and the mixture was stirred at 40 °C. After centrifugation and dehydration, the Au nanorods coated with mesoporous silica (Au@MSN) were obtained.

B. *For the synthesis of 7 containing azido group*, the reaction of azocompound **8** with bis(2-chloroethyl)ether in DMF in the presence of K$_2$CO$_3$ and KI in DMF was performed by stirring at 100 °C for 12 h. After filtration the solvent was removed under a reduced pressure to give compound **9.** The DMF solution of **9** was heated with NaN$_3$ and stirred at 70 °C under nitrogen for 12 h. After filtration and removal of the solvent, compound **7** was obtained (Figure 1.4).

C. *For grafting of propiolamide 10 onto Au@MSN surface affording Au@MSN-alkyne,* the anhydrous Au@MSN homogenously suspended in toluene was treated with **10** and refluxed at 120 °C for 24 h. The product was extracted by centrifugation and dehydrated under vacuum at room temperature to give Au@MSN-alkyne (Figure 1.5).

D. *For the synthesis of Au@MSN-rotaxane,* first the complex **7**/α-CD, containing azido group had to be obtained. To this end, **7** was stirred with α-CD in water at room temperature under nitrogen for 2 h affording the complex **7**/α-CD (Figure 1.6).

Then the mixed solutions of Au@MSN-alkyne and of the complex **7**/α-CD in water were treated with CuSO$_4$·5H$_2$O and sodium ascorbate, and under click reaction conditions were stirred at room temperature for 3 days. After centrifugation the Au@MSN-rotaxane was obtained (Figure 1.7).

In order to investigate the NIR-triggered drug release from Au@MSN-rotaxane serving as a nanocarrier, the fluorophore fluorescein isothiocyanate (FITC) was used as a model drug. Initially, the Au@MSN-rotaxane was loaded with FITC cargo molecules via diffusion at 40 °C, then the cargo-loaded nanocarrier was UV irradiated; in this process, the trans to cis isomerization occurs, along with
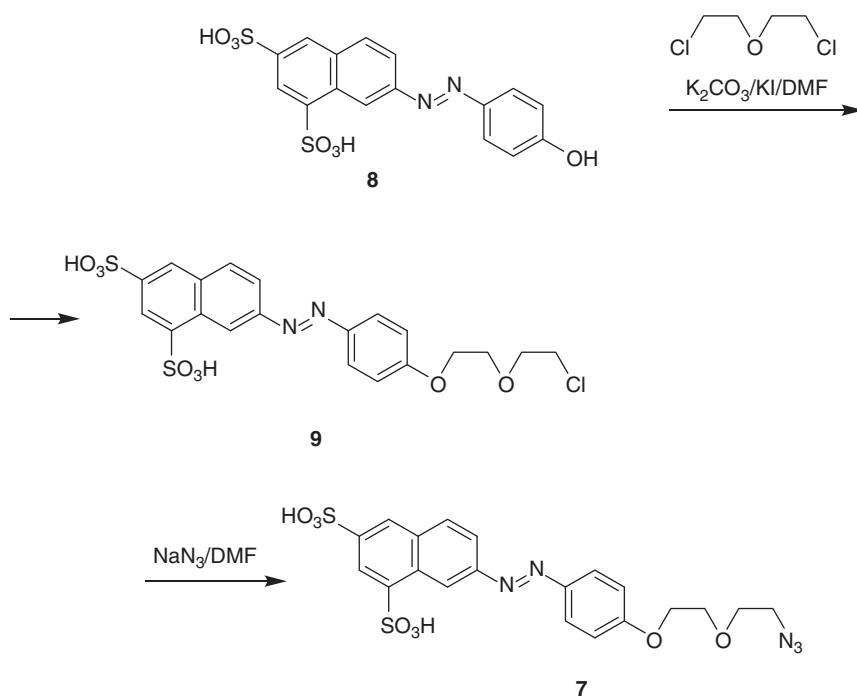
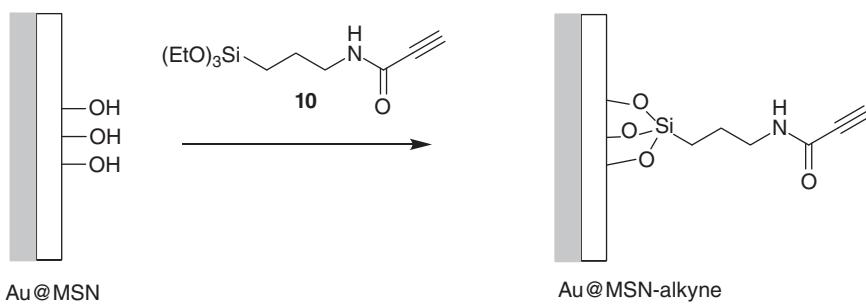**Figure 1.4** Synthesis of the compound **7**.

Au@MSN

Au@MSN-alkyne

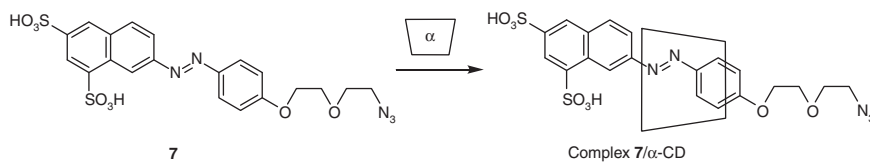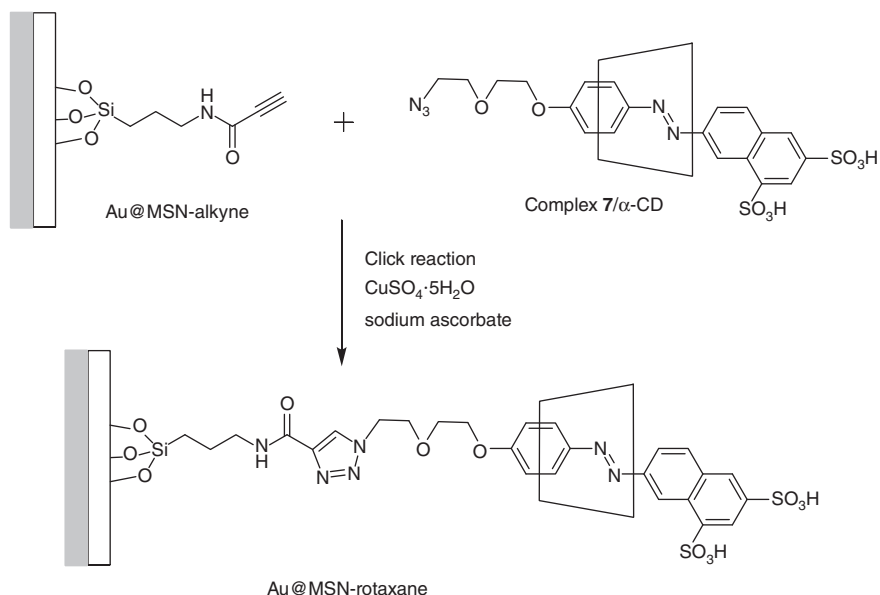**Figure 1.5** The grafting of propiolamide **10** onto Au@MSN surface.

Figure 1.6 Preparation of the complex **7**/α-CD.

Figure 1.7 Synthesis of Au@MSN-rotaxane.

the closure of silica mesopores and the robust encapsulation of FITC. The UV irradiation enables *trans* to *cis* photoisomerization of azobenzene; therefore, α-CD moves toward the nanopore orifice for the closure of nanopores [43].

The UV irradiation can induce the *trans*-to-*cis* photoisomerization of azobenzene, and the *cis*-azobenzene can undergo a thermal relaxation process to return to *trans* conformation. The α-CD can efficiently encapsulate *trans*-azobenzene, but not *cis*-azobenzene; in this way, the UV-/heat-controlled movement of α-CD unit in the rotaxane exists.

Upon UV irradiation of Au@MSN rotaxane, the *trans*-to-*cis* photoisomerization of azobenzene occurs, while under NIR irradiation the *cis*-azobenzene returns to its *trans* conformation, and the cargo release occurs, that is, the cargo release is performed under NIR irradiation [44] (Figure 1.8).

It was established that the UV/NIR reversibility between two azobenzene conformations maintained even after five cycles. These results confirm that NIR irradiation can efficiently trigger the *cis*-to-*trans* isomerization of azobenzene for controlled drug release.

In vivo drug release was carried out on zebrafish embryo models using the anticancer drug doxorubicin (DOX), itself having a strong red fluorescence, which facilitated confocal laser microscopy observation [45]. It was found that DOX release from the nanocarrier in zebrafish embryo models could be controlled remotely under NIR irradiation; a significant drug spreading to the adjacent tissues was established. The above study is a successful example of NIR-controlled drug release *in vivo*.

Oligoynes, that is, carbon-rich compounds containing conjugated triple bonds, are intensively studied due to their application possibilities; they show
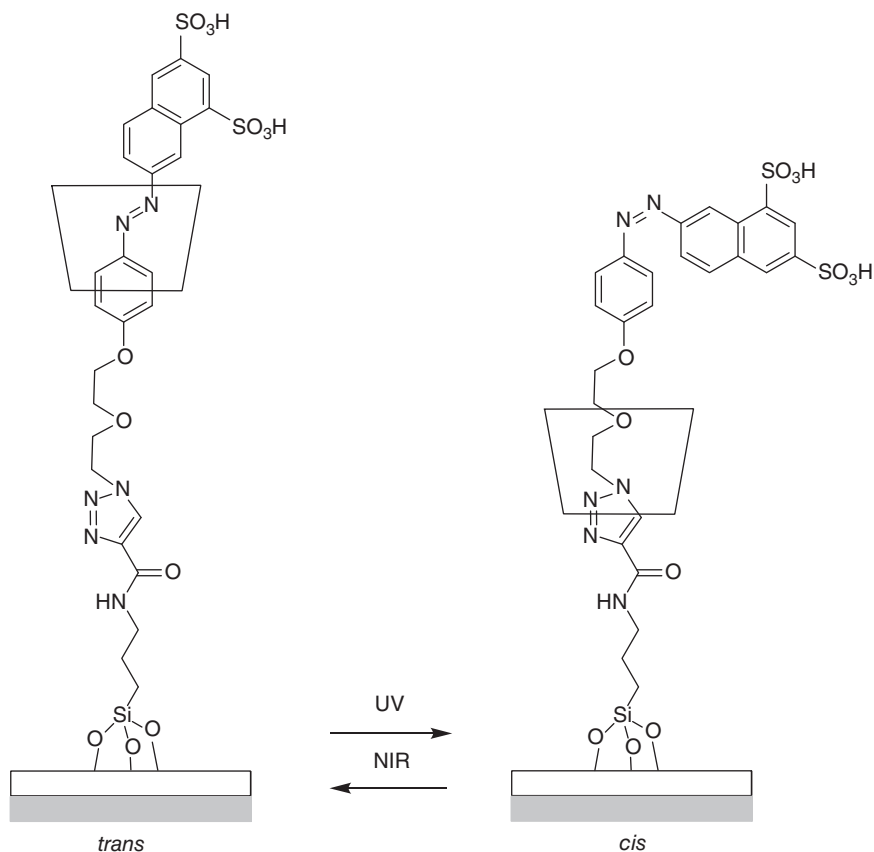
**Figure 1.8** The *trans*-to-*cis* photoisomerization of Au@MSN-rotaxane.

NLO properties [46] and have been recently used as molecular precursors for the preparation of carbon nanomaterials at room temperature [47–49]. It is also noteworthy that spectroscopic studies of oligoynes may be considered as an approach toward the properties of the carbon allotrope carbyne ($C\equiv C)_n$ [50, 51]. Although the stabilization of oligoynes by encapsulation via rotaxane formation is known [52], few examples have been reported [24, 53].

In the study of oligoynes, it was found that the amphiphilic nature of the TMS-protected triyne, **11** can be of use in the facile preparation of the CD-based hexayne [3]rotaxane **12** by simple reaction of **11** with α-CD in water [54].

The synthesis of rotaxane **12** begins with the deprotection of **11** by MeONa in an ether/methanol mixture (4 : 1), followed by Amberlite (H$^+$), which results in the deacetylation and simultaneous desilylation leading to the amphiphile **13**. The formed **13** was not isolated, but subjected *in situ* to the oxidative homocoupling with α-CD in water by the addition of CuBr$_2$ and TMEDA to give hexayne [3]rotaxane **12** (Figure 1.9). It was established that the **12** isomer with a tail-to-tail arrangement of α-CD units is formed exclusively.
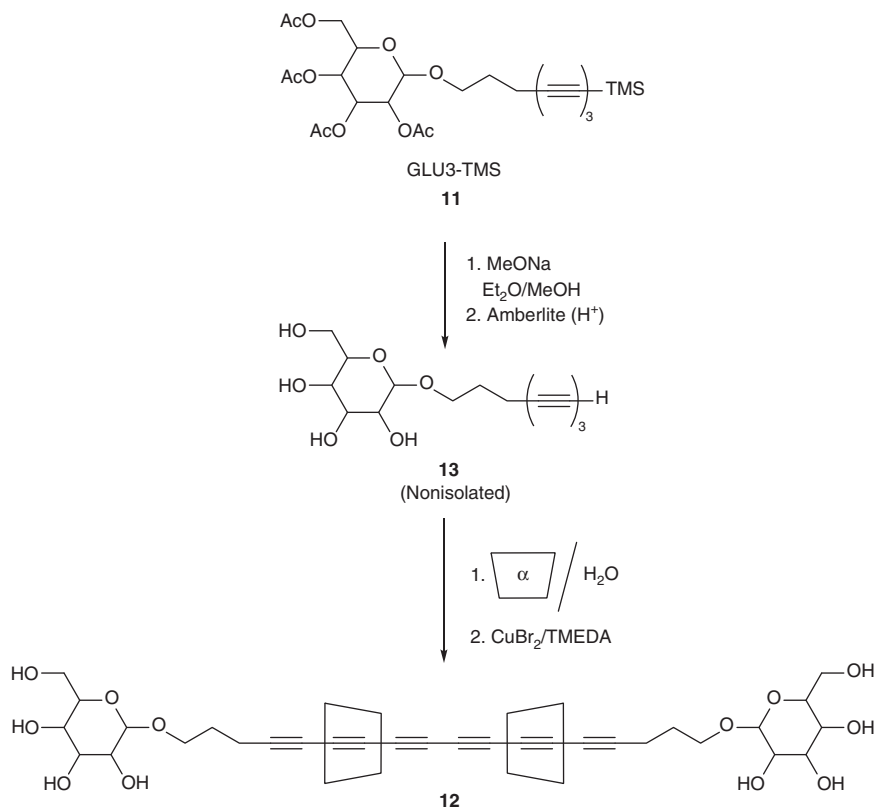
Figure 1.9 Synthesis of rotaxane **12**.
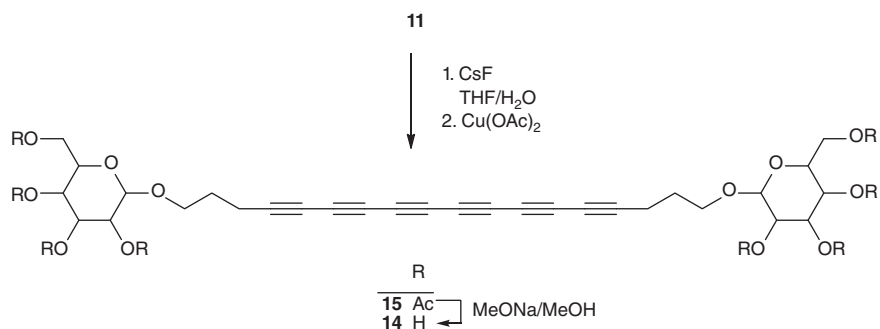
Figure 1.10 Synthesis of the compound **14**.

For comparison purposes, the dumbbell **14** of rotaxane **12** was obtained. The synthesis begins with the desilylation of **11** by cesium fluoride and simultaneous homocoupling by Cu(OAc)$_2$, leading to hexayne **15,** which upon deacetylation with NaOMe/MeOH affords the dumbbell **14** (Figure 1.10).

It was found that the encapsulation of **14** by CD units stabilizes the resulting rotaxane **12** against UV irradiation, while sole **14** does not show stability

against UV irradiation. The effective prevention of **14** by formation of rotaxane **12** against photodegradation or polymerization is promising for preparation of shielded molecular wires of the **12** type.

1.2 CD-Based Polyrotaxanes

1.2.1 CD-Based Polyrotaxanes with Triblock and Pentablock Backbones

The biocompatibility of biomolecules for implantation is closely related to collagen adsorption and subsequent fibrillization on implants. Important steps for the body to adapt to the biomaterials for implantation are the initial adsorption rearrangement and infiltration of collagen fibrils onto the biomaterials [55]. The inadequate interaction of collagen with the implant may lead to its rejection.

The control of collagen adsorption and fibrillization was investigated using surface mobility, that is, molecular mobility on the surface. The surface mobility represents the dynamic motion of molecules under hydrated conditions. The dynamic motion of the surface molecules is an important parameter in the regulation of nonspecific biological responses [56–58]. Therefore, the protein molecules, or the cells, continuously move on the surface until they achieve a thermodynamic equilibrium for their final conformation.

In the experiments, the relations between surface mobility, fibrillogenesis of collagen molecules, and the inflammatory response have been investigated *in vitro* and *in vivo* [59]. The study concerned *in vitro* adsorption and fibrillogenesis of collagen on a surface with dynamic properties and how this surface influences the inflammatory response *in vivo.* The investigation of collagen–surface interactions is related to the control of wound healing where collagen adsorption, fibrillization, deposition, and maturation occur.

Polyrotaxanes (PRs) **16a,b** consisting of the ABA-type block copolymers as backbones, threaded along poly(ethylene glycol) (PEG) by mobile α-CD units α-CD and MeO-α-CD, respectively, and end capped by hydrophobic terminal groups were used to prepare mobile surfaces with representative dynamic properties (Figure 1.11).

PRs **16a,b** are convenient models to establish specific biorelevant interactions involving collagen adsorption and fibrillization, with surface mobility as one of the functional parameters. The surface dynamism represented by **16a,b** has shown that differences in protein adsorption and fibroblast morphology may occur [56, 57]. The difference in mobility of α-CD unit within the PEG chain is a crucial parameter in the regulation of a nonspecific biological response.

It was found that increasing the mobility of the polymer on the surface resulted in the formation of the soft collagen layer. The collagens in this layer rearrange, leading to the formation of thicker collagen fibrils by lateral aggregation, that is, by their maturation. The obtained results show that the surface mobility on an implant is important for wound healing.

With the use of PRs **16a,b** it was found that a loop structure was formed on the surface. This allowed to determine the role of molecular mobility on collagen adsorption and fibrillogenesis, and to see how it affects the healing
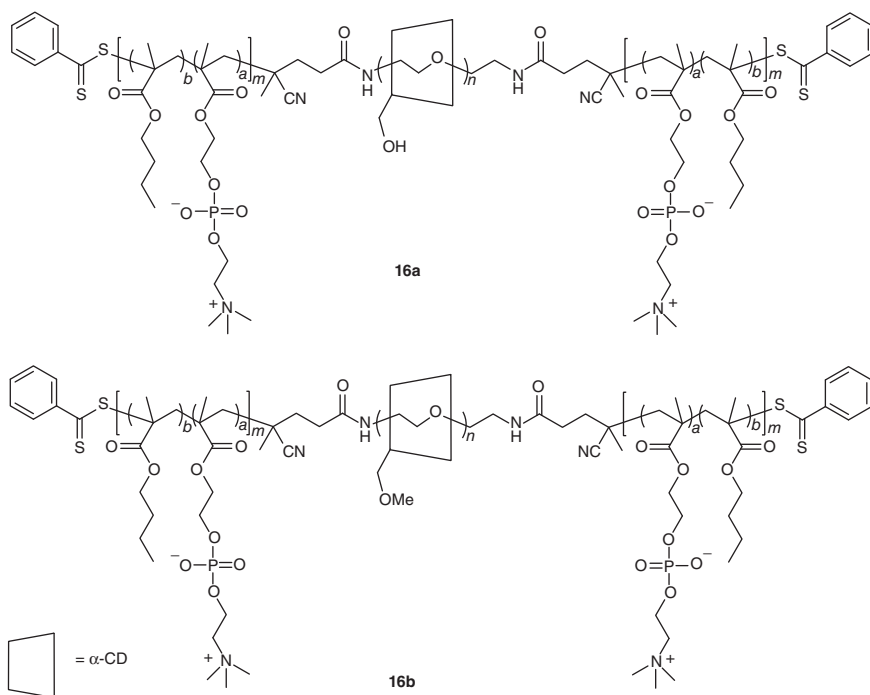
Figure 1.11 Polyrotaxanes **16a,b.**

process. The presence of methoxy groups in **16b** promoted the adsorption of collagen onto the surface. Although the mobility of the polymer surface did not influence the amount of proteins adsorbed, it influenced the formation of a soft-dissipative layer of collagen on the surface. The collagen in this layer had reaggregated to form thicker fibrils aligned in a specific direction. This affected *in vivo* responses, where the high molecular mobility facilitated by **16b** induced faster molecular rearrangement leading to the formation of a new collagen layer at the implant–tissue interface. The obtained results suggest that wound healing can be controlled by modulation of the surface property of implants, and that the surface mobility plays an important role in this process.

Today, the CD-based PRs have been widely investigated in various areas, including biomedical applications [60]. PRs consisting of ABA triblock copolymers threaded by α-CD units have been synthesized; they form flower polymeric micelles (PMs), which are promising for delivery of anticancer drugs [61]. Such copolymers may be obtained via atom transfer radical polymerization (ATRP); however, it is difficult to regulate the number of threading CDs in the synthesized PRs. It is known that the number of threading CD units in PRs is an important factor determining their properties; therefore, another synthetic procedure was necessary.

To this end, in the performed study the reversible addition-fragmentation chain transfer (RAFT) polymerization was used; in this procedure the PR-based macro-chain transfer agent, that is, macro-CTA is involved. This method enables

the regulation of both the molecular weight of the polymer chain and the number of threading CD units in PR segments. Moreover, the formation of self-assembled supramolecular flower micelles consisting of a core of hydrophobic polymers surrounded by hydrophilic loops of PRs was studied and the possibility of their use as a drug delivery carrier was shown.

The experiments begin with the synthesis of the PR-based macro-CTA, **17,** followed by the synthesis of the PR consisting of triblock copolymer, threaded by α-CD units, that is, **18.**

- *For the synthesis of PR-based macro-CTA, **17,** first the aqueous solution of α,ω-bisphenylalanyl PEG was treated with the saturated aqueous solution of α-CD. After the freeze drying, the pseudopolyrotaxane **19** was obtained as a powder. The reaction of **19** with 4-cyanopentanoic acid dithiobenzoate (CPDTB) and 4-(4,6-dimethoxy [1,3,5]triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) afforded the precipitate, which was dissolved in DMSO. The received solution was freeze dried to give the PR-based macro-CTA, **17.***
- *For the synthesis of polyrotaxane, which contains triblock copolymer, that is, **18,** first **17,** benzyl methacrylate, and 4,4'-azobis(4-cyanopentanoic acid) (V-501) were dissolved in DMSO; then this solution was bubbled with nitrogen for deoxygenation. The reaction mixture was stirred at 70 °C for 24 h, and then the obtained polymer was purified by dialysis against DMSO, followed by water. The recovered solution was freeze dried to yield **18** (Figure 1.12).*

The number of threading α-CD units in **17** can be controlled by varying the α-CD/**19** ratio and the reaction time, as in the previous study [62]. Therefore, the synthetic method used in this work may be applied to prepare a variety of PRs which contain triblock copolymers.

It is known that the amphiphilic block copolymers (composed of hydrophobic and hydrophilic polymers) self-assemble into PMs with a shell of hydrophilic polymers. The PRs which contain triblock copolymers also form PMs [63].

The preparation of supramolecular PMs consisting of a hydrophobic polymer core and hydrophilic PR shell was attempted using **18.** However, the PR is not soluble in aqueous solution due to the intra- and intermolecular hydrogen bonding among threading α-CD units; therefore, the **18** precipitates in aqueous solution. To increase the solubility of the PR segments of **18** in aqueous media, the hydrophilic hydroxyethyl (HE) groups were introduced into the α-CD units of **18** to give **18** modified by HE groups denoted as **20** [64].
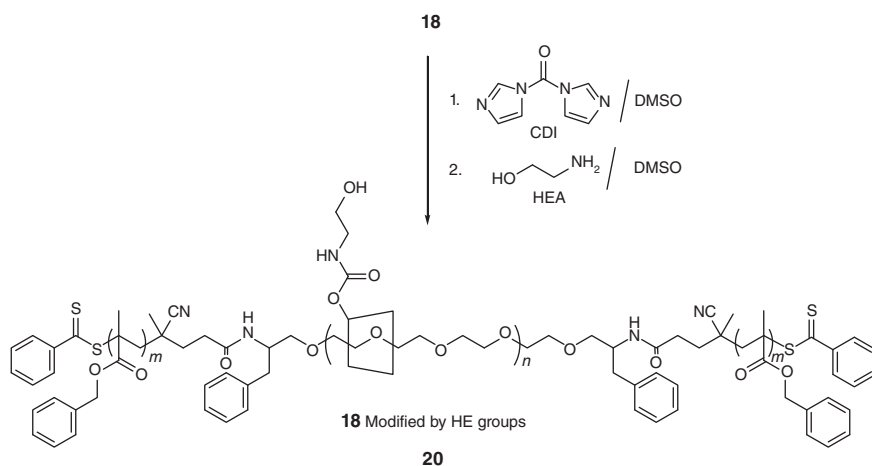
- *For the synthesis of **18** modified by HE groups, **20,** the DMSO solution of **18** and 1,1′-carbonyldiimidazole (CDI) was stirred at room temperature for 24 h. Then 2-hydroxylethylamine (HEA) was added and the reaction mixture was stirred at room temperature for a further 24 h. The formed polymer was purified by dialysis against water for 3 days and the recovered solution after freeze drying yielded **20** (Figure 1.13). The number of introduced HE groups, determined by* ^{1}H NMR, *was sufficient to solubilize PR segments in aqueous solution [64].* The ABA-type triblock copolymers with central hydrophilic segments form by self-assembly the PMs consisting of a core of hydrophobic segments surrounded by a shell of loops of hydrophilic segments.
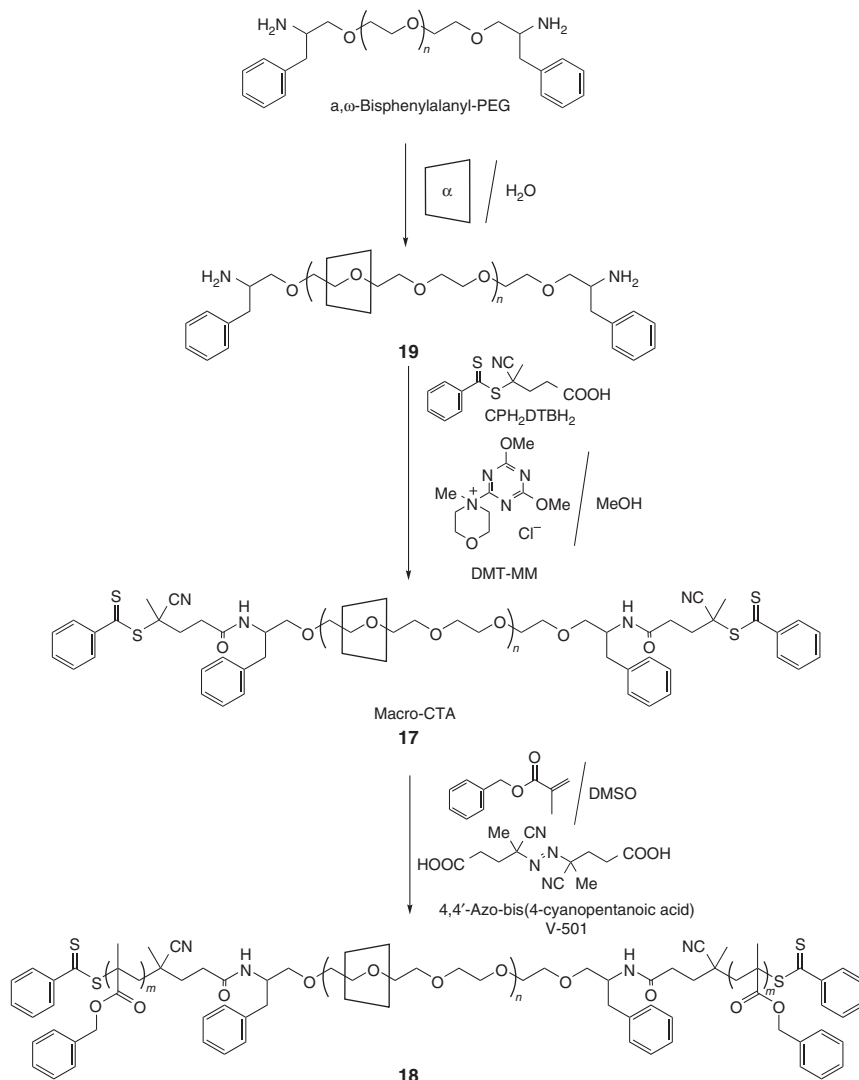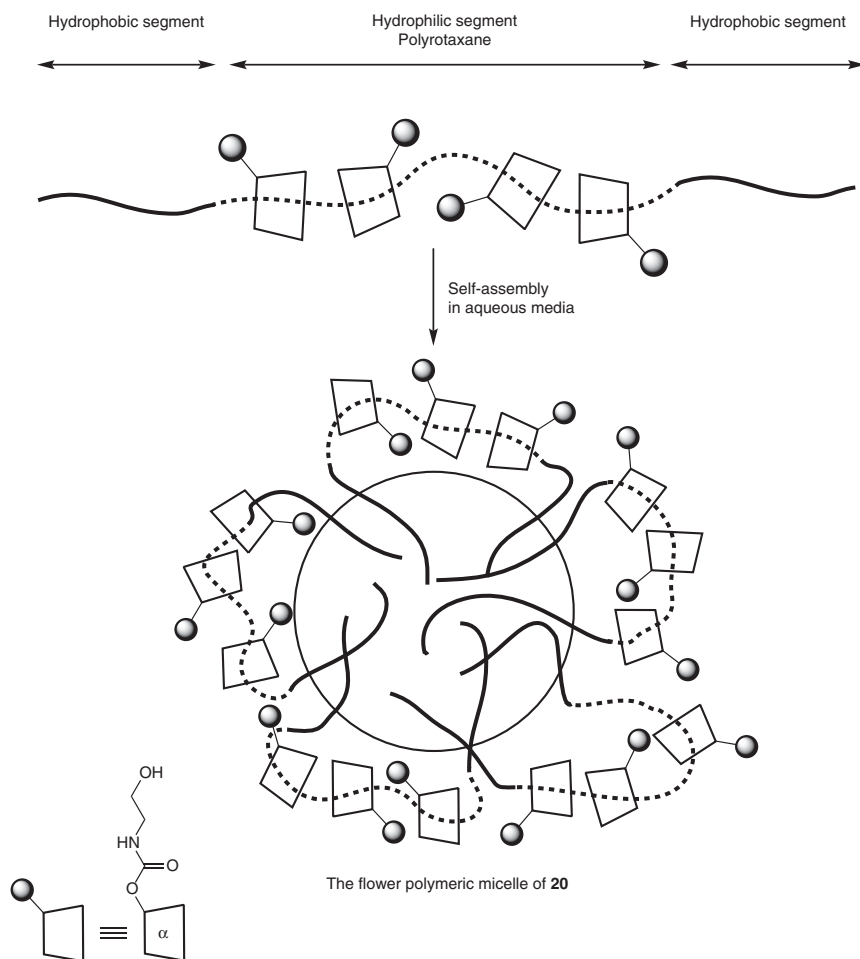
Figure 1.12 Synthesis of polyrotaxane **18**.

- *For the preparation of PMs*, the DMSO solution of **20** was dialyzed against water. The transmission electron microscopy (TEM) results have shown that **20** formed in aqueous solution uniform spherical PMs that were 15.3 ± 1.9 nm in diameter. In this way were obtained the PMs, named the flower PMs since they have the shape of a flower with hydrophilic segments resembling petals (Figure 1.14).

In the investigation of the ability of flower PMs to act as drug carriers, the loading of the hydrophobic anticancer drug, paclitaxel, was performed. It was found that the flower PMs can incorporate paclitaxel in their core, and therefore

18

1. CDI / DMSO

2. HEA / DMSO

18 Modified by HE groups

20

Figure 1.13 Synthesis of polyrotaxane **20.**

Hydrophobic segment

Hydrophilic segment
Polyrotaxane

Hydrophobic segment

Self-assembly
in aqueous media

The flower polymeric micelle of **20**

Figure 1.14 Formation of the flower polymeric micelle of **20.**

are promising for use in the delivery of anticancer drugs to targeted tumor tissues. This property is valuable for their application in the medical field.

PNIPAAm, that is, poly(*N*-isopropylacrylamide) has an interesting thermoresponse property due to its lower critical solution temperature (LCST) at around 32 °C in aqueous solution. Below the LCST, PNIPAAm is hydrophilic and has extended chains; but when temperature increases up to 32 °C, it becomes hydrophobic and phase-separated. Chemical modification of PNIPAAm may alter the LCST value. For example, the grafting of a hydrophilic polymer onto PNIPAAm usually enhances its LCST. The LCST is strongly increased due to the coverage of γ-CD units hindering the thermally responsive aggregation of the PNPAAm blocks.

PNIPAAm is used as end-capping polymeric blocks in the preparation of CD-based PRs; it not only inhibits the dethreading of α-and β-CDs but also imparts the thermoresponsive behavior to PRs [65–67].

In the experiments, the PR **21** containing PR PNPAAM-b-Pluronic F68-s-PNIPAAM pentablock copolymer "backbone" threaded by γ-CD units and terminated by β-CD units has been synthesized [68]. Before the synthesis of PR **21,** first the two following processes, that is, synthesis of the azido-terminated copolymer **22** and synthesis of propargylamine-β-CD **23** were performed.

- *For the synthesis of azido-terminated copolymer 22,* the DMF solution of Br-terminated copolymer **24** was treated with NaN_3 in DMF and stirred at room temperature for 30 h. After dialysis against water with the use of a cellulose membrane, the azido-terminated copolymer **22** was obtained [69, 70].
- *For the synthesis of propargylamine-*β*-CD 23,* the reaction of mono-tosyl-β-CD with propargylamine in DMF was performed at 70 °C for 24 h, and then the reaction mixture was treated repeatedly with cold acetone. The precipitates were subsequently dissolved in water/methanol mixture and poured into acetone for the removal of unreacted propargylamine. After drying at 50 °C in a vacuum oven, the propargylamine-β-CD **23** was obtained.
- *Synthesis of PR 21* proceeded via aqueous click chemistry. The azido-terminated copolymer **22** and aqueous solution of γ-CD were stirred at room temperature for 24 h to give PPR **25** (nonisolated). Then the obtained suspension of **22** underwent *in situ* the click reaction with propargylamine-β-CD, **23** in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate. The reaction temperature was maintained at 25 °C for 24 h, the crude product was dialyzed against water, dissolved in DMF, and precipitated with anhydrous ether to give PR **21** terminated by β-CD units (Figure 1.15).

It was observed that the higher the feed molar ratio of NIPAAm is, the PNIPAAm blocks become longer and therefore the molar ratio of γ-CD is lower because it is difficult for γ-CD units to include and slip over the longer PNIPAAM blocks to form PPRs. One should note that the molar ratio of γ-CD units is more than stoichiometric; this means that the γ-CDs not only form inclusion complexes with the flank PNIPAAM blocks but also slip over to the central poly(propylene glycol) (PPG) block of Pluronic F68.
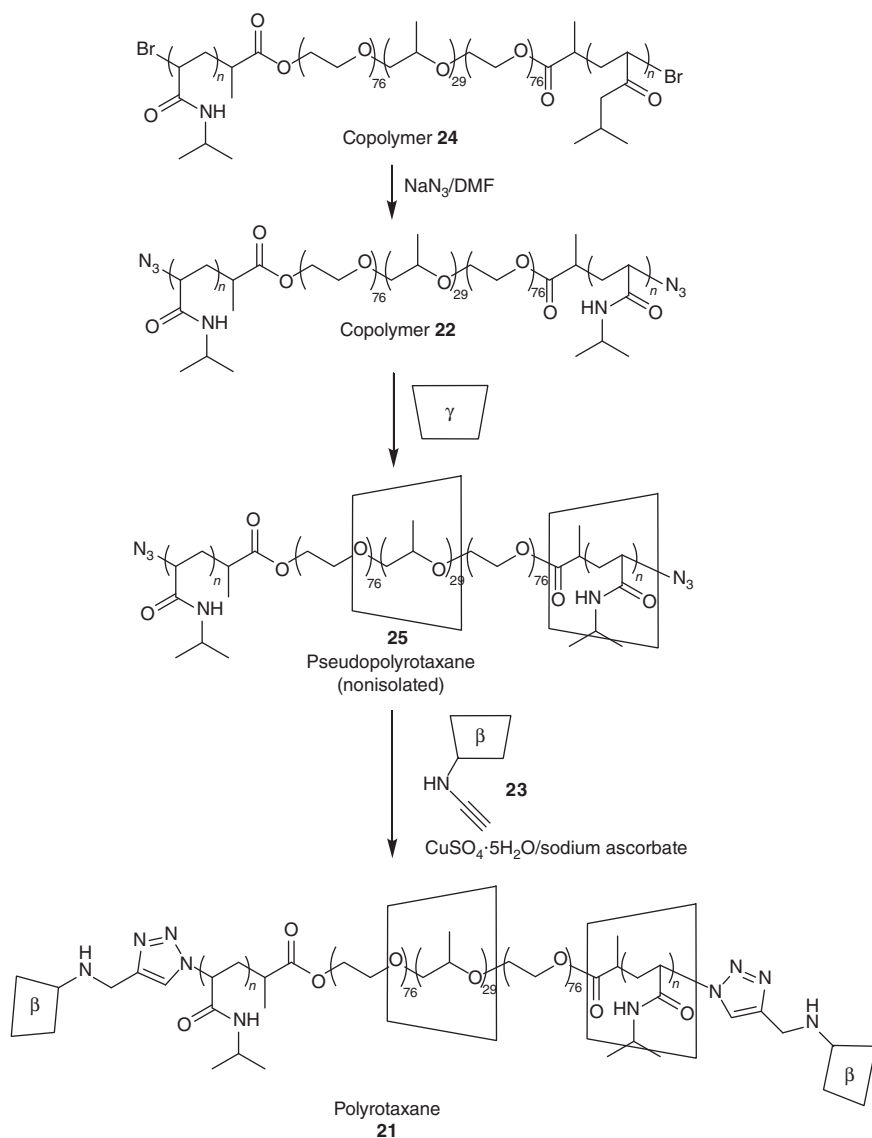
Figure 1.15 Synthesis of polyrotaxane **21.**

After the click reaction with propargylamine-β-CD **23**, the γ-CD units are entrapped in the whole main chain showing a loose-fit structure. Most γ-CDs begin to slip over to the middle block of Pluronic F68 to choose more suitable blocks for creation of a characteristic channel-type crystal structure. It was established that γ-CD units form the inclusion complexes with both PPG and PNIPAAM blocks. The obtained results are expected to enable preparation of stimuli–response intelligent new materials.

### 1.2.2 CD-Based Polyrotaxanes with other Backbones

PRs may be obtained by end capping the reaction of the corresponding PPRs with a bulky stopper [71]. In this aspect, the amine-terminated polymers have been used as a backbone of PPRs, threaded by CD or by permethylated α-CD units to achieve an efficient end capping by reactions of amino groups [72].

There exists also a method employing for end capping the nitrile N-oxide; this reaction proceeds in the absence of a catalyst [73]. The use of nitrile N-oxide as a stopper enables the catalyst-free, high-yield synthesis of [2]rotaxanes from pseudo [2]rotaxanes terminated by unsaturated C=C, C≡C, and C≡N groups.

In the experiments, the α-CD-based PRs have been obtained by the end capping of α-CD-based allyl-terminated PPRs with nitrile N-oxide **26** employing 1,3-dipolar cycloaddition reaction, performed in the solid state [74]. For this purpose, two methods may be used: the first method, denoted as *the sonication followed by solid-state process* and the second method denoted as *the all-solid-state process.*

The first method, that is, the sonication followed by solid-state process uses for threading α-CD units the sonication of diallyl PEG **27** or diallyl poly(tetrahydrofuran), that is, PTHF **28** with α-CD in water, affording allyl-terminated PPRs **29a,b,** respectively. In the following end-capping solid-state process, the PPRs **29a,b** were ground with nitrile N-oxide **26** in a mortar at 70 °C for 1 h to give by 1,3-dipolar cycloaddition reaction the PRs **30a,b** (Figure 1.16). Similar reactions were performed using permethylated α-CD, that is, PM-α-CD; however, the yields were lower than in the case of α-CD.

The second method, that is, the all-solid-state process uses solid-state grinding for threading and for end capping. The process begins with the solid-state grinding of the mixture of diallyl PEG **27** or diallyl PTHF **28** with α-CD in a mortar
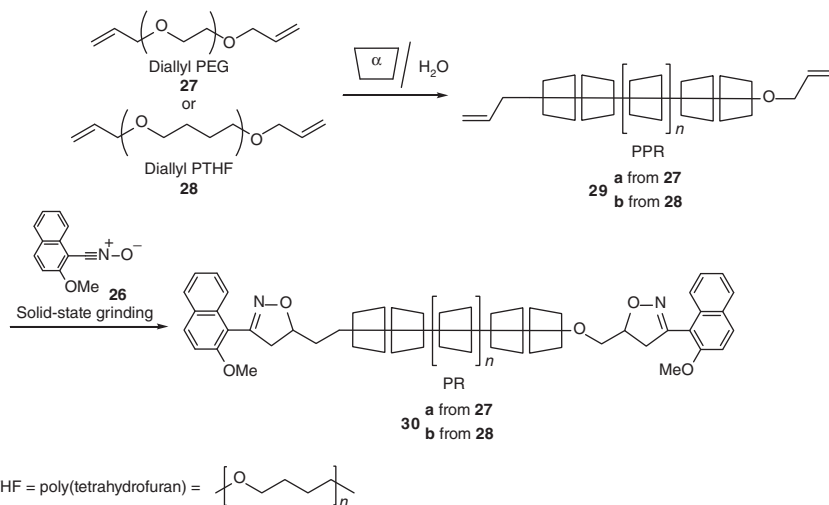
Figure 1.16 Synthesis of polyrotaxanes **30a,b** by the sonication followed by solid-state process.

at room temperature for 1 h in the absence of a solvent. The obtained solid product was treated directly with **26** in the same mortar and the mixture was ground at 70 °C for 1 h in the same mortar to give PRs **30a,b.** Similar reactions of PM-α-CD with **27** or **28** afforded PRs in higher yields than those obtained by the first method.

One should mention that in the case of β-CD and PPG instead of PEG, the same procedure did not lead to the corresponding PRs. This fact results probably from the favorable formation of the inclusion complex of β-CD with **26**; this formation of the inclusion complex of β-CD with **26** decreases the reactivity of **26** as a stopper.

The above-presented simple and smoothly proceeding reactions are promising for preparation of CD-based PRs which are free of by-products.

Biominerals are hard tissues produced by living organisms; $CaCO_3$ is the most abundant biomineral. It is known that $CaCO_3$ is used by living organisms to build materials with significant mechanical and optical properties; they are closely correlated with the hierarchical structures of the $CaCO_3$ biominerals. The morphology and orientation of $CaCO_3$ crystals in biominerals are directed by organic molecules.

Inspired by this, many researchers have synthesized $CaCO_3$ minerals using biomimeting approaches [75]. In the study, both soluble and insoluble polymers were used as templates to control crystallization of $CaCO_3$ [76]. By this method, thin films and 3D materials were obtained [77]. However, the templates employed in the above investigations were covalent polymers, whereas the use of supramolecular templates would be more valuable.

It was observed that the presence of carboxylic groups on polymer materials is important for initiation of $CaCO_3$ crystallization. In PRs, threaded by CD units bearing carboxyl groups, the CD units are mobile on the polymer chain [78]; therefore, morphologies of $CaCO_3$ may be influenced.

In the experiments, the carboxylated PRs were employed as supramolecular templates for formation of $CaCO_3$ thin films [79]. PRs consisting of PEG and of carboxylated α-CDs have been obtained as the inducers of $CaCO_3$ crystals. One should note that the use of PRs in the synthesis of inorganic materials, presented below, is rather rare.

The synthesis of PRs **31** and **32** begins with the reaction of poly(ethylene glycol) bisamine (PEG-BA) with α-CD. The aqueous solution of both these components was ultrasonicated for 10 min and then allowed to stand overnight. The precipitate was freeze dried and vacuum dried at 60 °C to give PPR **33.**

The reaction of **33** with 2,4-dinitrofluorobenzene in DMF afforded PRs **34** and **35,** which upon stirring with succinic anhydride in pyridine yielded carboxylated PRs **31** and **32.** For comparison purposes, the same carboxylation reaction of α-CD afforded carboxylated α-CD **36** (Figure 1.17). PRs were modified with carboxyl groups to interact with Ca^{2+} ions. PRs are insoluble in water, but the carboxylation resulted in their water solubility.

Crystallization of $CaCO_3$ was performed with the use of poly(vinyl alcohol) matrices by slow diffusion of ammonium carbonate into $CaCl_2$ solutions
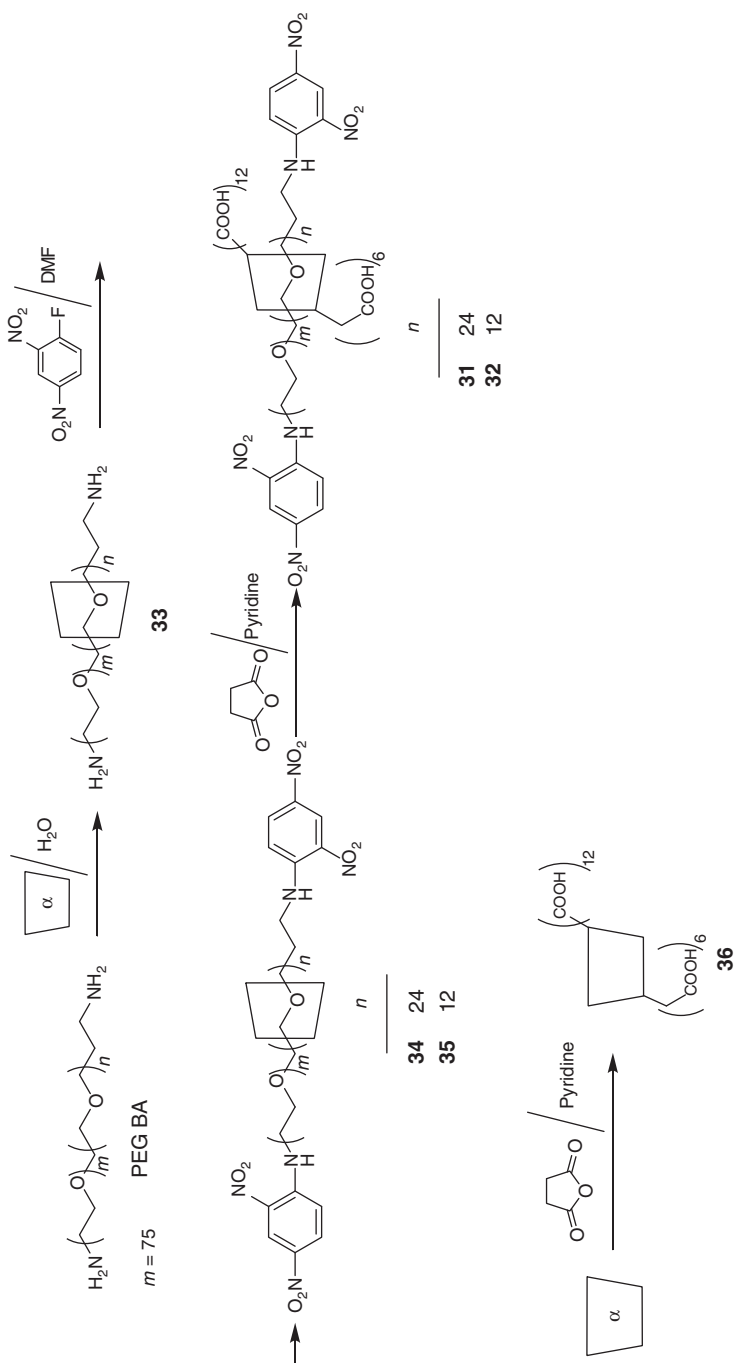
Figure 1.17 Synthesis of polyrotaxanes **31** and **32**, and the synthesis of carboxylated α-CD, used for comparison purposes.

containing **31** and **32.** In this process, the $CaCO_3$ thin films showing birefringence were obtained; the PRs **31** and **32** acted here as soluble crystal inducers. The results of Raman spectroscopy indicate that the thin films induced by **31** and **32** are mainly vaterite. Vaterite is the least stable polymorph of anhydrous $CaCO_3$; however, both PRs **31** and **32** stabilize vaterite thin films on the polyvinyl alcohol (PVA) matrices.

The scanning electron microscopic (SEM) images show different morphologies induced by **31** and **32** vaterite thin films formed on PVA matrices. The thin films induced by **31** have a relatively smooth surface, while on the surface of thin films induced by **32** a concentric pattern appears. These differences result from different densities of carboxylic acids on PRs, since the amount of carboxylic acids in **31** is about twice as that in **32.** Despite these differences, it was established that both thin films induced by **31** and **32** consist of nanocrystals. The PRs **31** and **32** containing accumulated carboxylic groups bind $CaCO_3$ with higher affinity than the carboxylated α-CD **36.** The PRs **31** and **32** probably stabilize the amorphous calcium carbonate precursor, which is important for the development of thin film morphologies of $CaCO_3$ [80].

The above results are promising for new applications of PRs in inorganic synthesis. The $CaCO_3$ thin films are of interest for biomedical purposes due to their high biocompatibilities [81]. It should be pointed out that the introduction of supramolecular chemistry in the construction of organic–inorganic hybrids is promising for the design of novel functional materials.

Macromolecular compounds which efficiently induce cellular internalization are promising for biomedical engineering. The efficiency of intermolecular uptake depends on the physicochemical properties of macromolecules, such as molecular weight, charge density, and hydrophobicity [82]. It is known that the introduction of cationic groups, such as derivatives of amines into macromolecules, improves their interaction with plasma membranes [83]; this approach was employed, for example, in linear polymers and in dendrimers.

PRs built from linear PEG threaded by α-CD units and capped with bulky end groups have a rigid structure, because steric hindrance and intermolecular hydrogen bonding between the CD units prevent the PEG chain from coiling [84]. In solution, PRs with higher number of CD units have a more rigid structure than PRs with less CD units. This property of PRs resulting from their supramolecular structure has been applied in biomedical engineering and in many other areas. It is known also that rigid PRs with larger number of threaded CDs showed higher electrostatic binding ability with small interfering RNA (siRNA) than flexible PRs [62]; this fact is of importance in achieving efficient cellular internalization of plasmid DNA and siRNA [85]. In this aspect, it was suggested that the supramolecular structure of PRs could influence their interaction with negatively charged cell membranes.

The influence of the supramolecular structure of CD-based aminated PRs on their cellular internalization was investigated. For this purpose, the AF-545-labeled aminated PRs containing different amounts of threaded α-CD units and amino groups were synthesized [86].

For the synthesis of the AF-545-labeled aminated PR, first the DMSO/water (1:1) solution of PEG-BA was treated with azido-α-CD, that is, Az-CD

[87, 88]. The formed azidated pseudopolyrotaxane Az PPR reacted with *N*-carbobenzyloxy-ʟ-tyrosine (Z-Tyr-OH) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride *n*-hydrate (DMT-MM) in methanol to give azidated polyrotaxane (Az-PR). The Az-PRs containing different numbers of threaded Az-CD units were obtained by varying the PEG-BA/Az-CD weight ratio.

The subsequent treatment of the DMSO solution of AzPR with aqueous solution of $CuSO_4 \cdot 5H_2O$, ascorbic acid and AF 545 afforded by click reaction the AF-545-labeled aminated PR (Figure 1.18). The modifications yielded aminated PRs containing approximately one AF-545 molecule per PR.

Then the influence of fluorescently labeled aminated PRs on cellular internalization was studied. It is known that cationic tertiary amine groups are effective in inducing macromolecule–cell interaction; therefore, in this investigation, the influence of cationic groups and the number of threaded CD units in the PR per molecule have been taken into account.

For comparison purposes, the linear macromolecules containing amino groups similar in number to that of the studied PRs have been included in investigations. To this end, methacrylate-based and polysaccharide-based macrocycles, namely, poly(2-(dimethylamino)ethyl methacrylate (PDMAEM) and aminated pullulan were chosen; they both have a random-coil structure (Figure 1.19). These macromolecules were synthesized and fluorescently labeled with AF-545.

The cellular internalization level for each sample was evaluated by flow cytometry; the cellular uptakes were analyzed using HeLa cells in serum. For comparison of flow cytometry results, all samples were synthesized with an average of one fluorescent molecule (AF-545) per sample molecule. The cellular uptake analysis has shown that in PRs the number of threaded CD units, rather than the number of amino groups, is a predominant factor in the interaction of PRs with cells. It is known that PR rigidity is influenced by the number of threaded CD units; therefore, the obtained results confirm that PR rigidity plays a crucial role in efficient cellular internalization.

It was found that the macromolecular conformation, such as a rigid or random-coil structure, is a critical factor for efficient cellular internalization; both PDMAEM and aminated pullulan had significantly lower fluorescence intensity than AF-545-labeled aminated PR. It is a confirmation that the internalization is easier for the rigid structure than for a random-coil structure.

It was established that the rigid PR structure, resulting from a large number of threaded CD units, facilitates a multivalent interaction with negatively charged cell membranes. The increase in the threaded CD units' number, rather than the amine content, improves the cellular uptake via endocytosis.

The cytotoxicity of investigated PRs is negligible; this is important in cellular internalization. The above results are promising for their application in the therapy and diagnosis fields.

Conjugated polymers are intensively studied in view of their usefulness in optoelectronics [89]. Among them, the conjugated polyazomethines today receive growing attention due to their electronic, linear, and nonlinear optical properties [90]. However, a drawback in their application in polymer-based
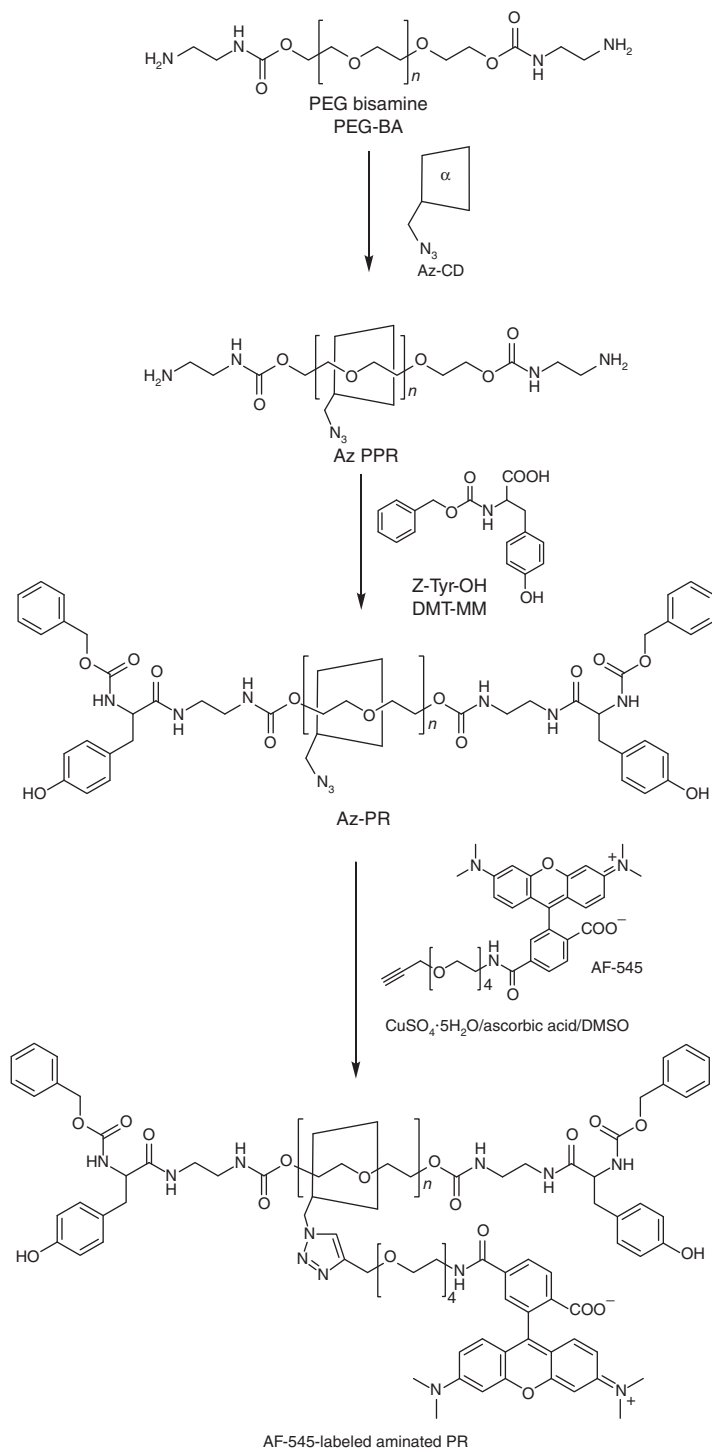
Figure 1.18 Synthesis of AF-545-labeled aminated polyrotaxane.
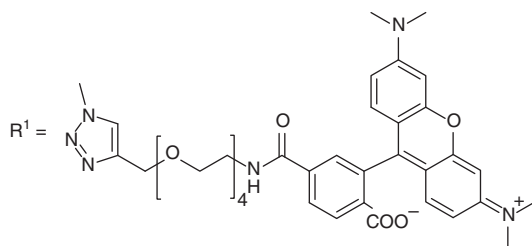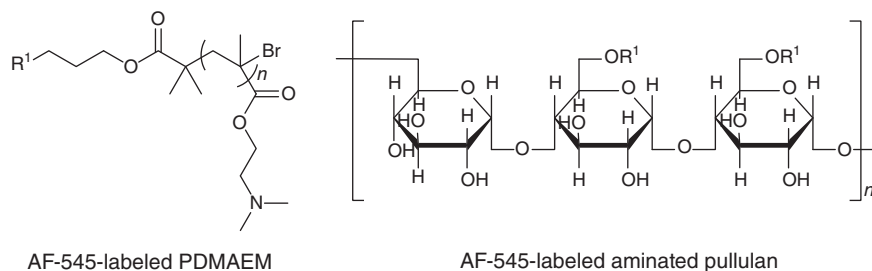
Figure 1.19 The AF-545-labeled PDMAEM and AF-545-labeled aminated pullulan.

devices is their rather low solubility in organic solvents and the high melting and glass-transition temperatures resulting from rigid macromolecular chains and strong intermolecular interactions.

It was found that the encapsulation of π-conjugated molecules into native CD cavities improves the solubility of conjugated polymers and their film-forming ability [91, 92]; here also functionalized CDs may be used [93, 94].

In the experiments, the oligoazomethine permethylated PRs have been synthesized and the influence on their threading by functionalized α-CD, that is, the permethylated α-CD, denoted as PMe-α-CD [95] on their solubility and morphology was investigated [96].

For the synthesis, first the inclusion complex of PMe-α-CD with terephthalaldehyde (TA), that is, TA/PMe-α-CD, was submitted to the solution polycondensation with 3,5-diamino-1,2,4-triazole (DT) in DMF, in the presence of p-toluenesulfonic acid as a catalyst, leading to PPR. In the final step of the reaction, DT was added in slight excess to introduce amino groups at both ends of the polymer. The formed PPR was then treated with 1-pyrenecarboxyaldehyde (1-PyrCHO) for the end capping by bulky pyrene moieties, affording oligoazomethine permethylated PR (Figure 1.20); the same procedure, but without PMe-α-CD yielded its dumbbell.

It was established that the PR film has a uniform and smooth surface. Due to the presence of PMe-αCD units, the PR has higher solubility than its dumbbell in DMF, DMSO, NMP, and even in CHCl$_3$; also, the thermal stability of PR is higher than that of its dumbbell.

Molecular imaging is an important tool for diagnosis of biological processes. It is known that multimodal imaging agents are more advantageous for complementary imaging than the use of only single agent.
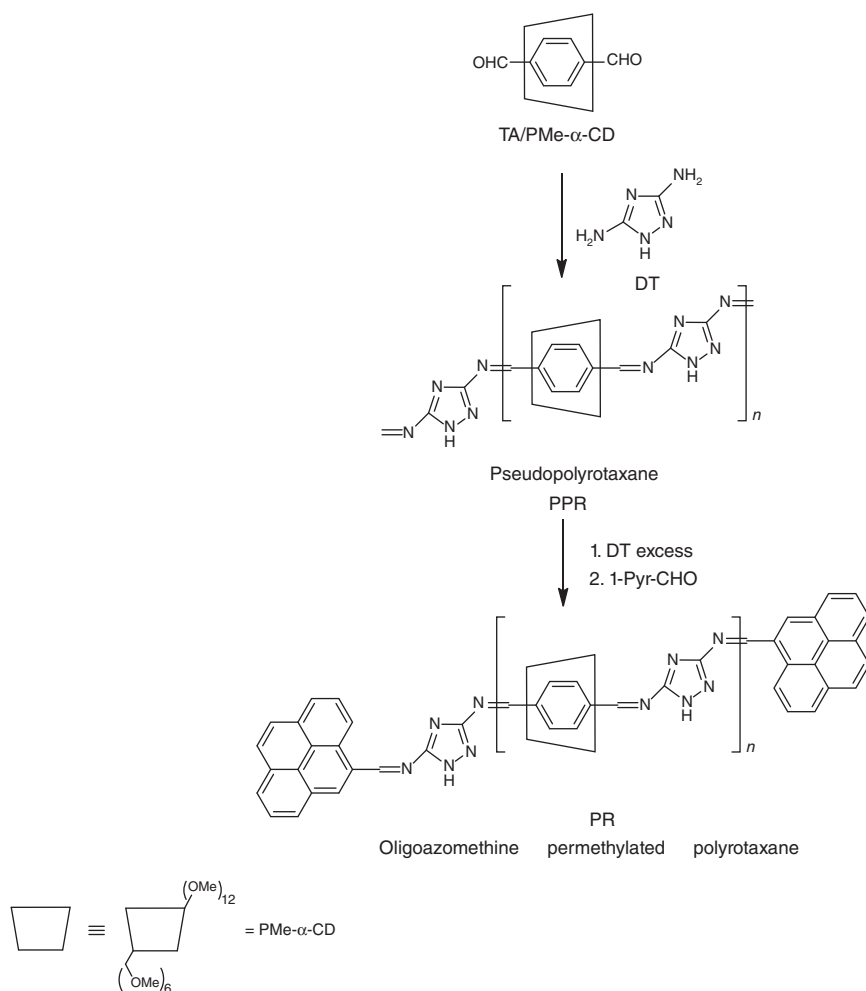
Figure 1.20 Synthesis of the oligoazomethine permethylated polyrotaxane PR.

Magnetic resonance imaging, that is, MRI is a nonradiative, noninvasive technique widely used clinically, advantageous for deep tissue penetration and high spatial resolution. In MRI, the intensity depends on the density of water protons and their longitudinal (T_1) and transverse (T_2) relaxation times. The relaxation of water protons can be enhanced by using contrast agents; in this way, the image contrast is increased.

Many methods for functionalization of CDs with lanthanide complexes [97] or with bodipy fluorescent tags [98] exist [99]. Among paramagnetic cations, Gd^{3+} showed to be the most efficient relaxation agent since it has high electronic spin ($S = 7/2$) and slow electronic relaxation. To prevent its toxicity, Gd^{3+} has to be chelated by multidentate ligands which form thermodynamically stable and kinetically inert biocompatible water-soluble complexes. The parameters important for contrast agent efficiency include the molecular rotational correlation

time (τ_r), the number of coordinated water molecules (q), and the lifetime of their water molecules in the inner coordination sphere (τ_m).

The commercial contrast agents are often based on Gd-DOTA (DOTA = 1,4,7, 10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or Gd-DTPA complexes (DTPA = diethylene triamine pentaacetic acid) [100]. It was observed that these small molecular weight agents "tumble" rapidly in solution. According to the Solomon–Bloembergen–Morgan relaxation theory, at relevant clinical imaging fields (0.5–3 T), slowing down the rotation by use of macromolecular structures increases relaxivity [101]. One should note that macromolecular structures, in addition to their higher relaxivity, are also advantageous since they increase blood pool retention and tumor permeability due to enhanced permeability and retention (EPR) effect and they can accumulate a large number of paramagnetic ions in a single molecule [102].

Fluorescent imaging has high sensitivity and enables the detection of cellular structures. Combining MRI with fluorescent imaging allows to ascertain *in vivo* results and to validate the MRI findings.

Today, the following two approaches for the design of MRI and fluorescent bimodal agents exist:

- *In a molecular approach,* large molecules containing paramagnetic and fluorescent moieties are synthesized [103].
- *In a nanochemical approach,* the paramagnetic species and organic fluorescent dyes are incorporated in liposomes [104] or grafted on nanoparticle (NP) platforms [105].

The performed investigations involve a supramolecular method combining the advantages of the above two approaches [106]. To this aim the PR containing functionalized α-CD units was synthesized to serve as a bimodal imaging probe. In the experiments, the α-CDs functionalized by a bodipy as a fluorescent tag or by Gd^{3+} complex as an MRI contrast agent have been synthesized and threaded onto a polyammonium chain to form PRs. The obtained PRs are a modular platform promising for bimodal (fluorescent and MRI) imaging applications.

PR consists of a water-soluble cationic polymer threaded by α-CD units which are functionalized by MRI contrast agents and by fluorescent tags. It should be pointed out that this approach allows tuning of the sensitivity difference between MRI and optical techniques by mixing appropriate amounts of two complementary imaging probes.

Advantages of this supramolecular approach are that the relaxation and fluorescence properties can be optimized separately on each CD unit; the same size of PR can be retained when various modifications of CD units are made. It is noteworthy that the system is biocompatible, since CD is biocompatible and biodegradable through the slow unthreading of the PR into easily excretable small units (Figure 1.21).

Syntheses of functionalized α-CDs involve reactions leading to **37** bearing bodipy followed by those leading to **38–41**, which bear lanthanide DOTA complexes. In these processes, the regioselective deprotection of CDs and click chemistry of azido-CD with alkyne-terminated imaging moieties were used.

Syntheses of functionalized α-CDs require azido-CDs; for this purpose, first the mono-azido-CD **42a** and bis-azido-CD **42b** were obtained. In this process,
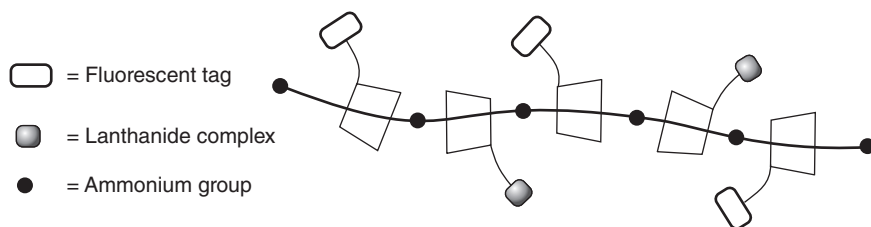
Figure 1.21 Schematic representation of polyrotaxane containing α-CD units functionalized by fluorescent tags and by lanthanide complexes.
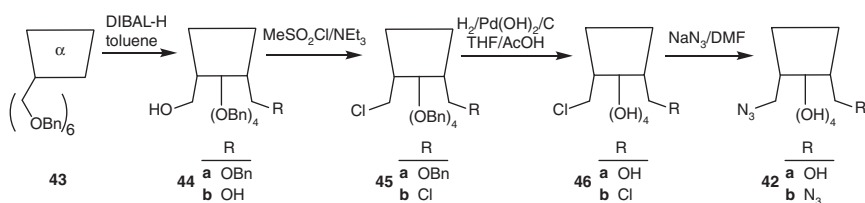
Figure 1.22 Synthesis of mono-azido- and bis-azido-α-CDs **42a,b.**

the perbenzylated α-CD **43** upon treatment with DIBAL-H was mono- or bis-debenzylated to give **44a** and **44b,** respectively. Compounds **44a,b** reacted with mesyl chloride to convert hydroxyl groups into chloride, affording chloro- and dichloro-CDs **45a,b.** The deprotection of the benzyl groups in **45a,b** by catalytic hydrogenolysis yielded **46a,b,** which upon nucleophilic substitution of chloride with sodium azide gave mono-azido-CD **42a** and bis-azido-CD **42b** (Figure 1.22).

1. *For the synthesis of 37,* the click reaction of **42a** with alkyne-terminated bodipy **47** was performed (Figure 1.23).
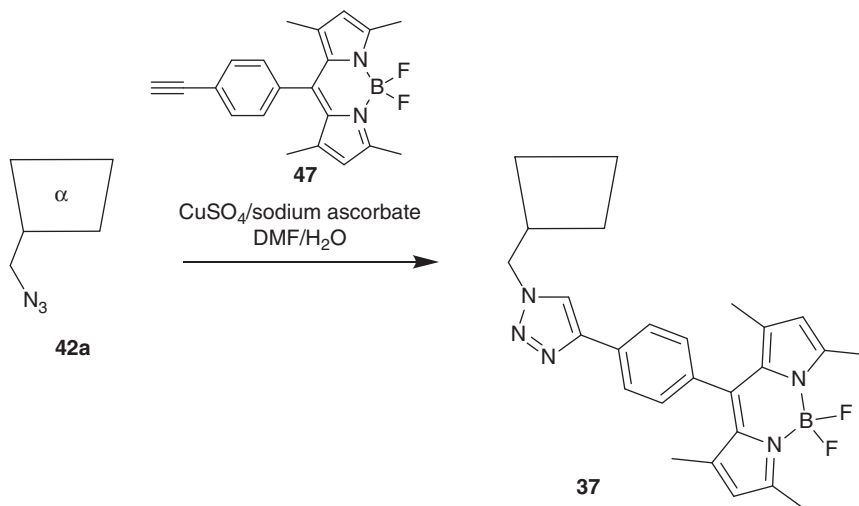
Figure 1.23 Synthesis of compound **37** functionalized by bodipy.

2. *For the synthesis of 38–41,* the click reactions of **42a,b** with DOTA lanthanide complexes **48** (Ln = Gd) and **49** (Ln = La) were used. The click reaction of mono-azido-CD **42a** with **48** and **49** affords CDs functionalized by one DOTA complex **38** and **39,** respectively. The click reaction of bis-azido-CD **42b** with **48** and **49** affords CDs functionalized by two DOTA complexes **40** and **41,** respectively (Figure 1.24).

Synthesis of Polyrotaxanes

After syntheses of **37** and **38–41**, their ability to form PRs was studied. PRs containing functionalized CDs are mostly obtained by the postfunctionalization of native CD units already present in CD PRs [107], and not by a direct threading of bulky functionalized CDs [108]. In this study, however, the direct threading of functionalized CDs was necessary.

It was shown that the affinity of these functionalized CDs for oligoethylene glycol serving as a thread is too weak to produce PRs; however, chains of polyammonium **50** are suitable backbones with high affinity and water solubility. This allowed to obtain PRs containing either CDs with fluorescent tags or CDs with lanthanide complexes, as well as PRs containing both functionalized CDs.

- Synthesis of polyammonium **50** involves the treatment of Nylon 11, that is, polymer A with $BH_3 \cdot Me_2S$ in THF, affording reduced polymer B which upon permethylation with MeI yields polyammonium **50** (Figure 1.25).
- Syntheses of PRs involve the threading of polyammonium **50** by CDs having fluorescent tags **37** and by CDs having lantanide complexes. The PRs were obtained by stirring polyammonium **50** with various functionalized CDs in water or in a mixture of water and MeOH at elevated temperatures. The formed PRs were purified by ultrafiltration.

Each bulky dimethylammonium unit acts as a pseudo stopper; the dethreading of the CD molecule through such a unit requires a high temperature. At room temperature the dethreading is very slow; therefore, the PRs are kinetically stable in aqueous solution over weeks and may be purified by dialysis.

Examples of PR syntheses are given below.

A solution of **50** and **37** in H_2O/MeOH (9 : 1) was stirred at 50 °C for 4 days to afford PR **51** (Figure 1.26).

A solution of **50** and **39** in water was stirred at 80 °C for 5 days to afford PR **52** (Figure 1.27).

A solution of **50** and **41** in D_2O was stirred at 80 °C for 7 days, and methanol was added to afford PR **53** (Figure 1.28).

A solution of **50** and **39** in water was stirred at 80 °C for 24 h, then MeOH and **37** was added and stirring was continued at 60 °C for 3 days to afford PR **54** (Figure 1.29).

Bimodal PRs **55** and **56** containing CDs functionalized identically by bodipy **37** and by lanthanide **38,** that is, CD containing two identical functional groups, but at their different ratio, were synthesized. For **55** the mixture CD-bodipy **37**:CD-Gd **38** equal to 1 : 4 was used and for **56** the mixture CD-bodipy **37**:CD-Gd **38** equal to 1 : 1. It was found that **55** was more soluble than **56** in
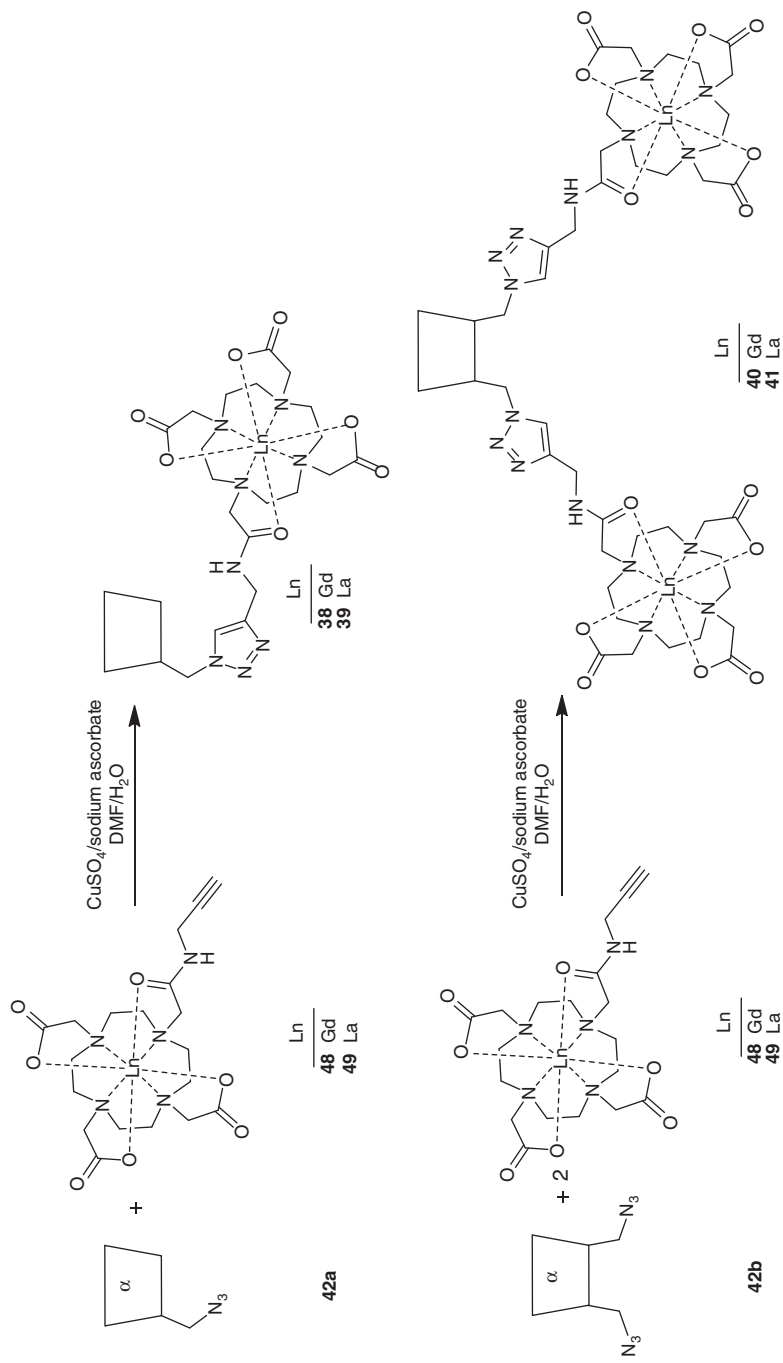
Figure 1.24 Synthesis of compounds **38, 39** monofunctionalized and of compounds **40, 41** bisfunctionalized by DOTA lanthanide complexes.
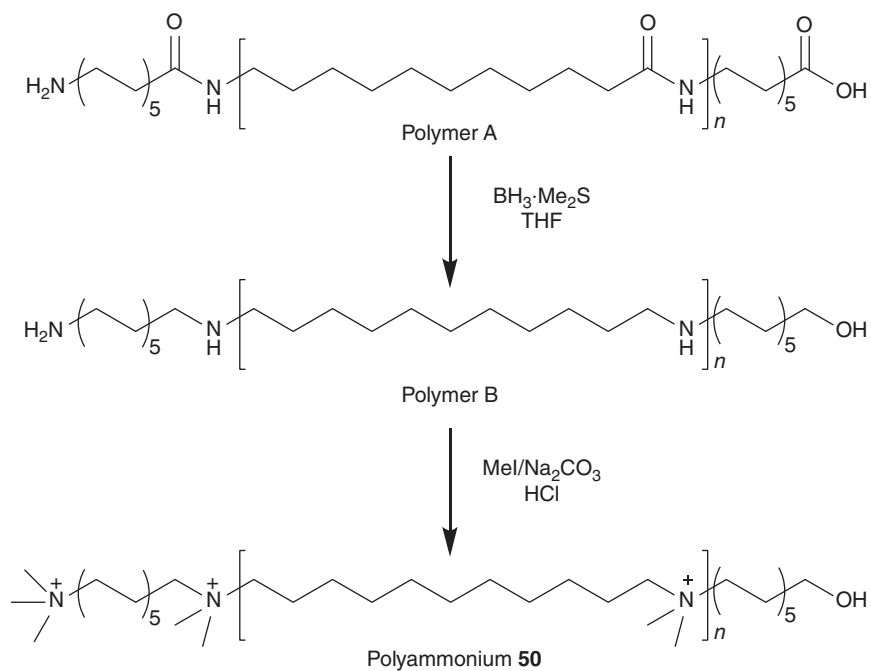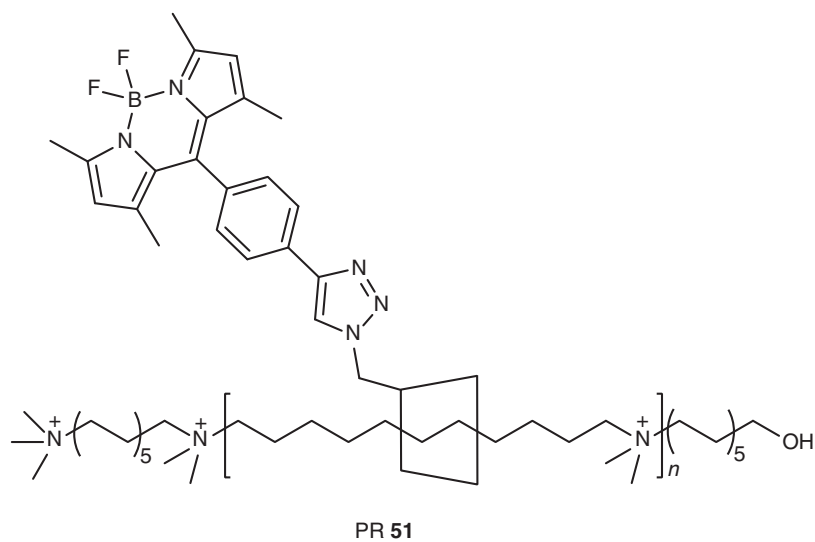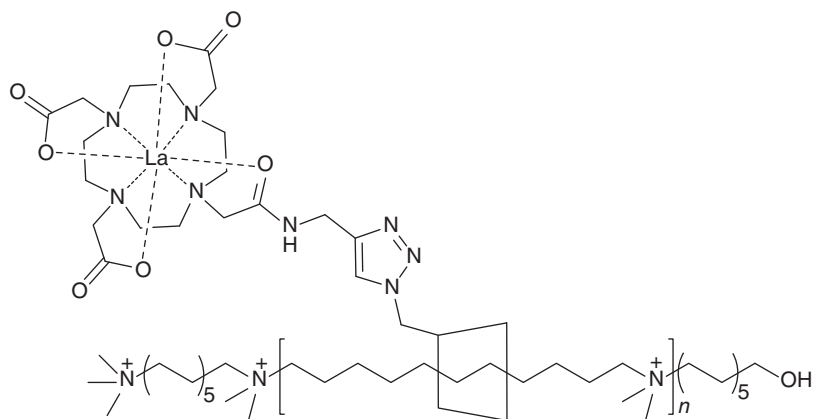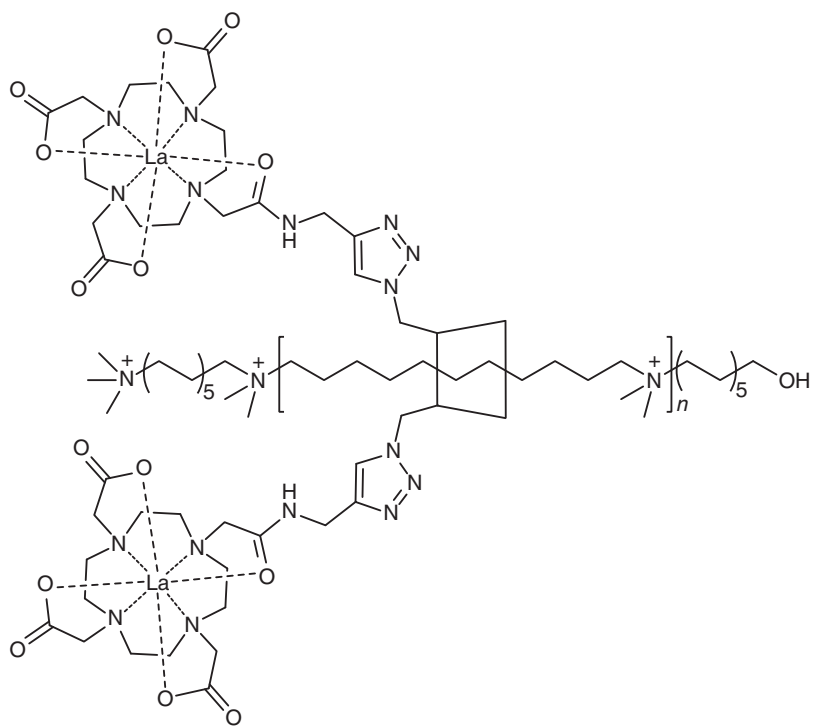
Figure 1.25 Synthesis of polyammonium **50**.

Figure 1.26 Polyrotaxane **51**.

PR **52**

Figure 1.27 Polyrotaxane **52.**

PR **53**
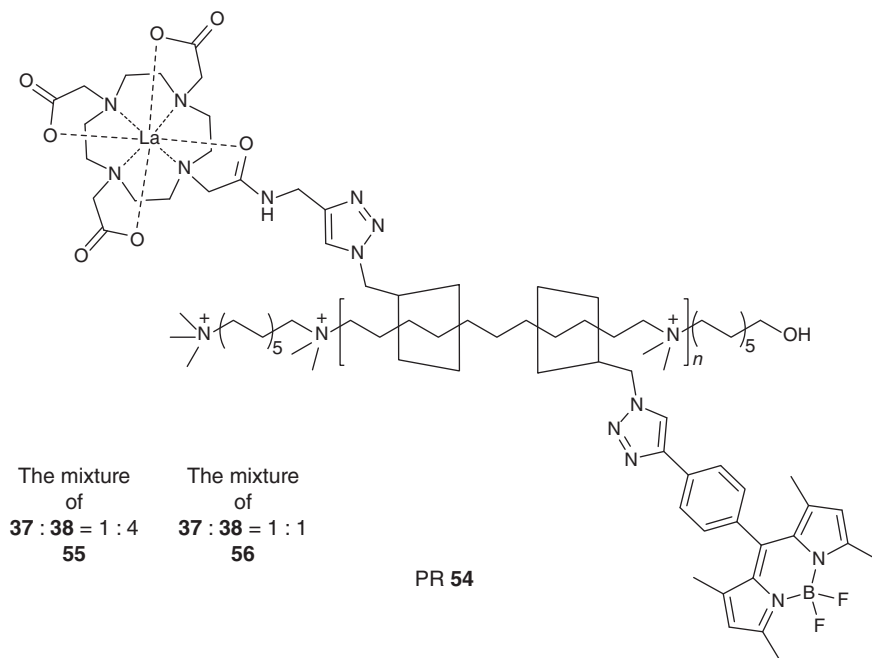
Figure 1.28 Polyrotaxane **53.**

Figure 1.29 Polyrotaxane **54.**

aqueous solution and better adapted to the sensitivity difference between optical and MRI detection. Being more convenient for imaging applications, **55** was further studied.

The ^{1}H NMRD (nuclear magnetic relaxation dispersion) profile of the PRs shows a bump at high field (20–80 MHz), characteristic of macromolecular contrast agents [101]. This observation indicates that by threading the functionalized CDs on the polymer, the motion of the CD-Gd **38** complex is strongly reduced, which results in higher relaxivity.

The relaxivities at 60 MHz are slightly lower at 25 °C than at 37 °C; this fact can be explained by a relative increase in the water exchange rate compared to the tumbling at high temperature. Therefore, the obtained system behaves as a typical macromolecular contrast agent with improved properties at body temperature.

The very high relaxivity observed at high frequencies (up to 120 MHz; 3 T) is especially promising since today the clinical practice tends to use such high magnetic fields to increase sensitivity. Thus, the above supramolecular approach provides an improvement in the relaxivity properties of the Gd^{3+} complex as compared to small molecules, and offers a great modularity that is not available in the case of other macromolecular structures.

The α-CDs functionalized with a bodipy fluorescent tag or a Gd^{3+} complex were synthesized in high yield with a total control of the number of subunits. The threading of the CDs is statistical, but each threaded CD is molecularly defined; this fact is advantageous as compared to a statistical postfunctionalization of CD PRs.

The bodipy tag fully maintains its fluorescent properties in the obtained system. The relaxivities of the Gd-bearing PRs are five times higher than that of the widely used commercial Gd-DOTA. This makes the synthesized PRs especially promising for bimodal (fluorescent and MRI) imaging applications. One should point out that the modularity and versatility of the developed supramolecular approach are its important advantages over conventional covalent assemblies.

It is known that the applications of polyurethanes (PUs) result from properties of starting materials and preparation conditions. The type of cross-link point of PUs has also an influence on their elastic properties; therefore, the PUs cross-linked by PRs are promising for designing PU elastomers.

There exist many advanced polymers cross-linked by rotaxanes [109, 110]; it seemed of interest to incorporate CD-based PR molecules as cross-link points of PUs. Although PUs cross-linked by CDs are known [111, 112], investigations of PU properties containing PRs as the cross-link points are rare [113].

PUs cross-linked by PRs, built from PEGs with different chain lengths and half-methylated α-CDs, have been synthesized to investigate the influence of PR structures on their properties [114]. The performed reactions involve the synthesis of three PUs cross-linked by PRs which consist of different filling ratios of half-methylated α-CDs and of PEGs (with chain lengths 1500, 4000, and 6000); they are PR 1500, PR 4000, and PR 6000. The hydroxyl groups of CDs were half methylated to enhance the solubility of PRs. For comparison purposes, the PU without the PR, that is, CD Me-PU was synthesized. In the experiments, first the PRs were partially methylated by MeI to give PR 1500 Me, PR 4000 Me, and PR 6000 Me.

- *For the methylation of PR 1500*, the DMSO solution of PR 1500 was treated with sodium hydride (dispersion in oil) under a nitrogen atmosphere. After the generation of hydrogen gas finished, the iodomethane was added. Then the reaction mixture was neutralized with aqueous HCl to precipitate a solid, that is, PR 1500 Me.
- *For the methylation of PR 4000*, the process was performed as for PR 1500 Me, but after neutralization with aqueous HCl the solvent was evaporated *in vacuo.* The residue was treated with methanol to precipitate a solid, that is, PR 4000 Me. Methylation of PR 6000 proceeded as for PR 4000 Me, affording PR 6000 Me. The same procedure was used for the half methylation of α-CD, affording CDMe.

The PUs were synthesized by a prepolymer method. The prepolymer was prepared from diphenylmethanediisocyanate (MDI) and poly(tetrahydrofuran) 2000, that is, PTHF 2000 with a ratio $[NCO]_{MDI}/OH_{PTHF} = 3.0$ in DMF at 95 °C under an argon atmosphere. Then the prepolymer reacted with PR 1500 Me, PR 4000 Me, PR 6000 Me, and CDMe to give PR 1500 Me-PU, PR 4000 Me-PU, PR 6000 Me-PU, and CDMe-PU. For this purpose, the DMF solution of prepolymer was added dropwise into the DMF solutions of PRs 1500 Me, 4000 Me, or 6000 Me and into the DMF solution of CDMe. The reaction mixture was stirred at 80 °C overnight, and then methanol was added to deactivate the excess isocyanates. Then the reaction mixture was concentrated, poured into a
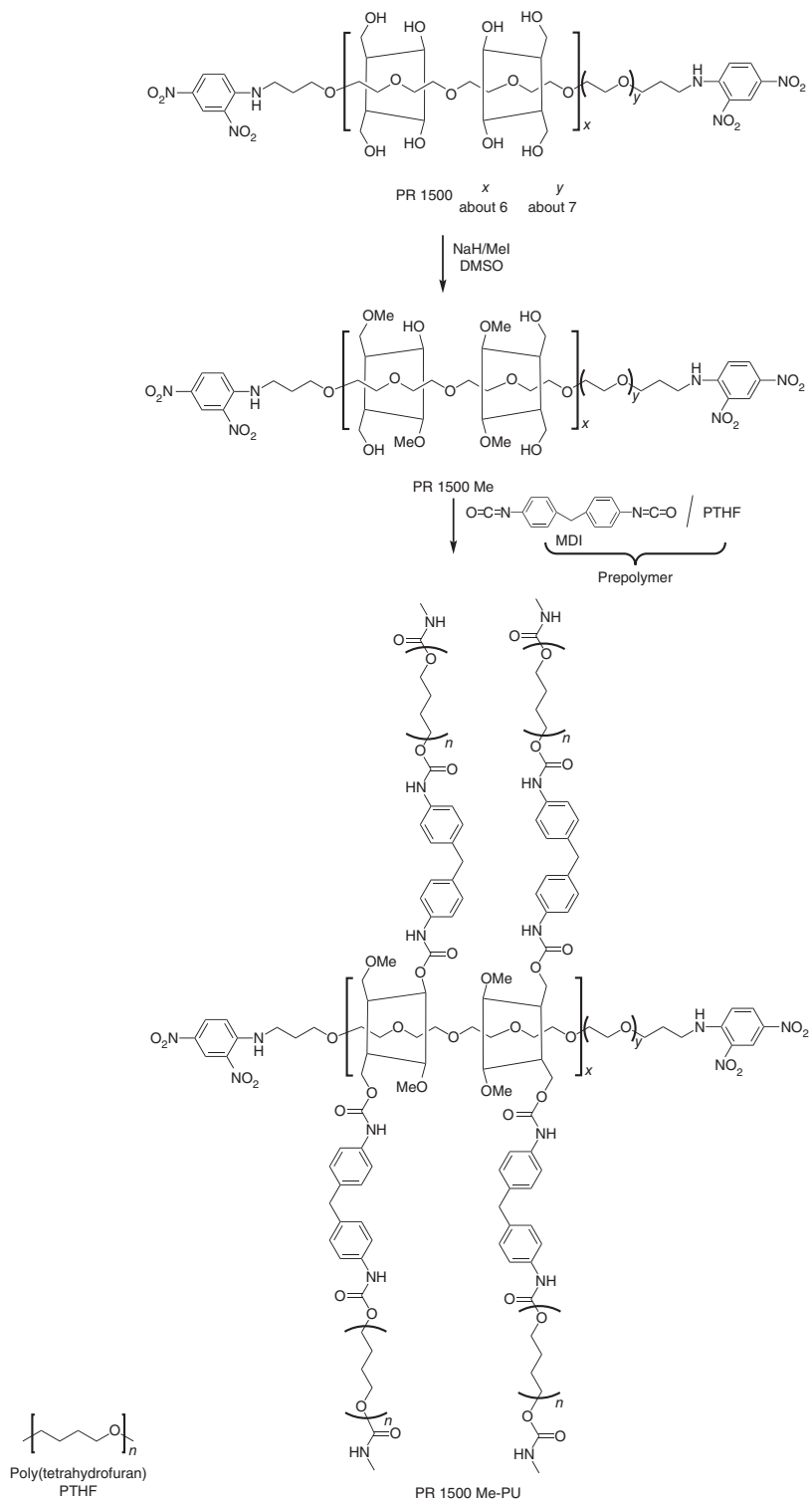
Figure 1.30 Synthesis of PR 1500 Me-PU.

Teflon vessel, and dried at 40 °C. The obtained films were washed with toluene, methanol, and then water, and pressed at 100 °C under 1.0 MPa; they are PR 1500 Me-PU (Figure 1.30), PR 4000 Me-PU, PR 6000 Me-PU, and CDMe-PU (Figure 1.31).

The results of the study have shown that PR 1500 Me-PU has the highest filling ratio of CDs, and the mobility of CDs is inhibited. The degrees of swelling for PR 4000 Me-PU and PR 6000 Me-PU are higher than those of PR 1500 Me-PU and of CD Me-PU. The PUs having the PR structure in which a sufficient sliding space for CDs exists, that is, PRs with longer PEG chains have higher degree of swelling. The measurements of mechanical properties of synthesized PUs have shown that their tensile strength decreases in the order PR 4000 MePU > PR 6000 MePU > CDMePU > PR 1500 MePU.

It was found that PR 1500 Me-PU, with the highest filling ratio of CDs and the shortest chain, enhances the reorganized crystallization of the soft segment chains because of the formation of the pure domain for the soft segment chains. In PR 6000 Me-PU, with the lowest filling ratio of CDs and the longest chain, the CDs act as individually dispersed cross-link points. The PR 4000 Me-PU, with the moderate filling ratio of CDs and middle length chain, shows slow reorganized crystallization of the soft segment chains in the PU and has the highest tensile strength among the studied PUs.

1.3 CD-Based Pseudopolyrotaxanes

The polymeric micelles (PMs) are promising for use as carriers for antitumor drug delivery; therefore, their preparation by the self-assembly of amphiphilic copolymers, graft copolymers, and dendrimers is today intensively studied [115]. A driving force involving $\pi-\pi$ stacking interaction has been developed for preparation of micelles, namely, the hydrophobic polymer chains in polymeric amphiphiles were replaced by small molecules with $\pi-\pi$ conjugated structure, and it was found that the $\pi-\pi$ stacking interaction between micelles and antitumor drugs strongly influences the drug release [116].

It is known that the CD-based PRs can self-assemble into NPs by the regular stacking of CD units along the polymeric chains. The PR NPs composed of PEG and α-CDs are convenient carriers for antitumor drug delivery; however, due to the compact structure of PR micelles, they are only seldom used in this area.

In experiments performed with the aim of developing antitumor drug delivery carriers, first the PPR **57** was obtained and then its self-assembly afforded micelles. The synthesis of **57** involves the preparation of PEG/cou and its threading with α-CD; the subsequent self-assembly of **57** affords micelles [117–119].

For the synthesis of PEG/cou, the CH_2Cl_2 solution of PEG, 7-carboxy-methoxycoumarin, and DMAP was treated with CH_2Cl_2 solution of DCC and stirred at room temperature for 48 h. The solid dicyclohexylurea was removed by filtration, the filtrate was concentrated, and upon addition of Et_2O the PEG/cou precipitated.
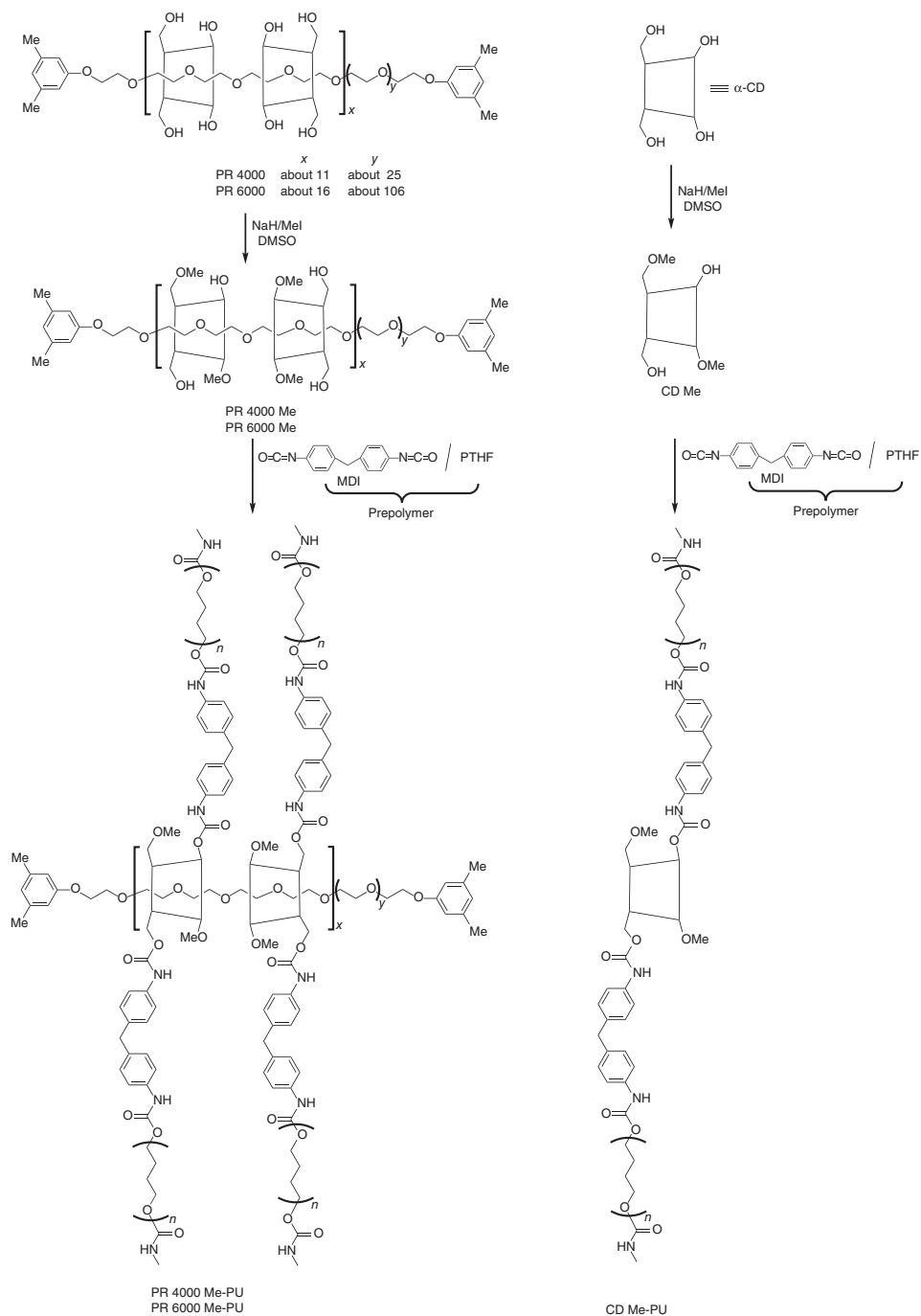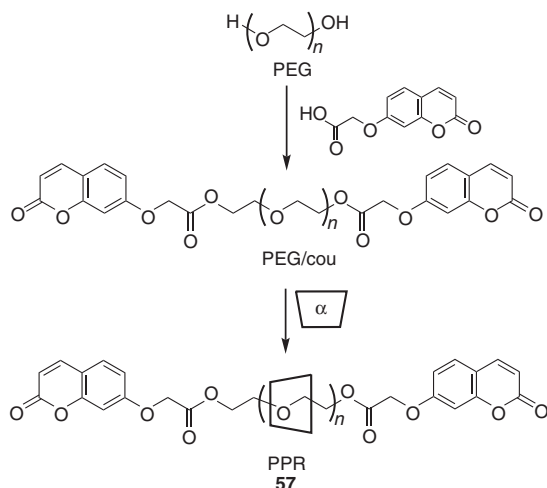
Figure 1.31 Synthesis of PR 4000 Me-PU, PR 6000Me-PU, and CDMe-PU.

Figure 1.32 Synthesis of pseudopolyrotaxane **57.**

*For the synthesis of PPR **57***, the aqueous solution of PEG/cou was treated with aqueous solution of α-CD at room temperature. Upon ultrasonication the white solid precipitated and was vacuum dried to give **57** (Figure 1.32).

The hydrophobic interaction between coumarin moieties and the crystallization of PPRs leads to the self-assembly of PPRs into micelles. In this process, the coumarin segments aggregate as the hydrophobic core, and PPR chains form the hydrophilic micellar shell. The created core–shell structure of PPR micelles is loose and is convenient for trapping hydrophobic antitumor DOX.

The PPRs are amphiphiles, since coumarin moieties are hydrophobic and PEG is hydrophilic. Upon the DOX trapping, the amphiphiles self-assemble into micelles to load DOX in the hydrophobic core, and the folded necklace-like PPR chains crystallize in the hydrophilic shell. Therefore, the driving forces for the formation of drug-loaded micelles are both the hydrophobic interaction and the crystallization. The PPRs self-assembled into spherical micelles of a mean diameter of 30 nm; and after the drug was loaded, the size increased to a mean diameter of about 80 nm. It was observed that the drug-loading content of the micelles was lower when the PEG chain was longer.

One should note that the PPR micelles are nontoxic to cells. The DOX-loaded micelles were incubated with mice TC1 lung cancer cells and B16 melanoma cells for cellular uptake and *in vitro* study of antitumor activity.

It was established that the drug-loaded micelles were internalized efficiently; the sustaining release of DOX could last for 32 h. The above results show advantageous properties of PPR micelles as carriers for antitumor drug delivery.

The self-assembly of hydrogel systems is driven by weak noncovalent interactions such as hydrogen bonding, van der Waals forces, or hydrophobic effect [120]. It is known that α-CD and poly(ethylene oxide), that is, PEG, or PEG-based block copolymers, such as Pluronic polymers form self-assembled

PPR hydrogels in which α-CD units are threaded onto the PEG chain of the polymer [121]. These PPRs self-assemble into larger structures forming a hydrogel. The α-CD/Pluronic systems are able to perform tunable gelation and self-healing. Moreover, CDs can be functionalized, and this ability makes PPR hydrogels suitable for biomedical applications.

Self-assembly is a gelation mechanism promising for materials useful in tissue engineering applications since it avoids the use of chemicals or external stimuli (e.g., UV) to cross-link the hydrogels; the chemicals or external stimuli could be harmful to cells. However, a drawback is that the self-assembled systems are reversible, that is, their dissociation occurs. Such reversibility is of interest in some applications; however it is undesirable when the hydrogel is to be used for tissue engineering. If the hydrogels are to be inserted in the body, they will be exposed to interstitial fluid flow that will lead to their degradation. Moreover, the rapid degradation of the self-assembled PPR hydrogels would release a large amount of α-CD. Large amounts of free α-CD are cytotoxic to cells, because they can solubilize membrane lipids (e.g., cholesterol), and as a result they will disrupt the cell membrane. Therefore, the rapid localized degradation of these types of hydrogels is undesirable from a tissue engineering viewpoint.

To improve the stability of PPR hydrogels for future tissue engineering applications, the introduction of a *covalent* cross-linking into the gels was investigated. It is known that the peroxidase-catalyzed oxidation enables the coupling of two phenolic moieties under mild conditions. In the presence of hydrogen peroxide and of horseradish peroxidase (HRP), the C—O and C—C bonds can be formed between two phenol groups, allowing the synthesis of *hydrogels* from polymers functionalized with phenolic groups (Figure 1.33). Such a mechanism of cross-linking has been employed for various synthetic polymers (Tetronic [122] or eight-arm PEG [123]) and biopolymers (hyaluronic acid [124, 125], gelatin [126], and chitosan [127]).

H_2O_2 at high concentration is toxic to cells, but it is consumed by conversion to H_2O during the cross-linking, and its consumption rate can be controlled by changing the reaction kinetics with the use of various H_2O_2/HRP ratios. In this way, the exposure time of H_2O_2 to the cells can be minimized. It was established that the H_2O_2-/HRP-mediated cross-linking has been successfully applied for cell culture in 2D using mouse myoblasts [128] and human umbilical vein endothelia cells (HUVECs) [122]. Also, in 3D, the human mesenchymal stem cells (hMSCs) have been successfully encapsulated within hydrogels cross-linked with H_2O_2/HRP.

In the experiments, the hydrolytically degradable PR hydrogels have been prepared from α-CD and Pluronic polymers [129]. An enzymatically mediated cross-linking function was introduced onto the Pluronic end groups on the PPRs via the use of a phenolic moiety (here, tyramine was used) in order to create *covalent* cross-links between the PPR components of these hydrogels. Moreover, a tyramine-functionalized eight-arm PEG, that is, PEG-Tyr was added into the hydrogels to generate a branched network and improve the stability of hydrogels. The obtained hydrogels were assessed for possible tissue engineering applications relying on drug and cell delivery.
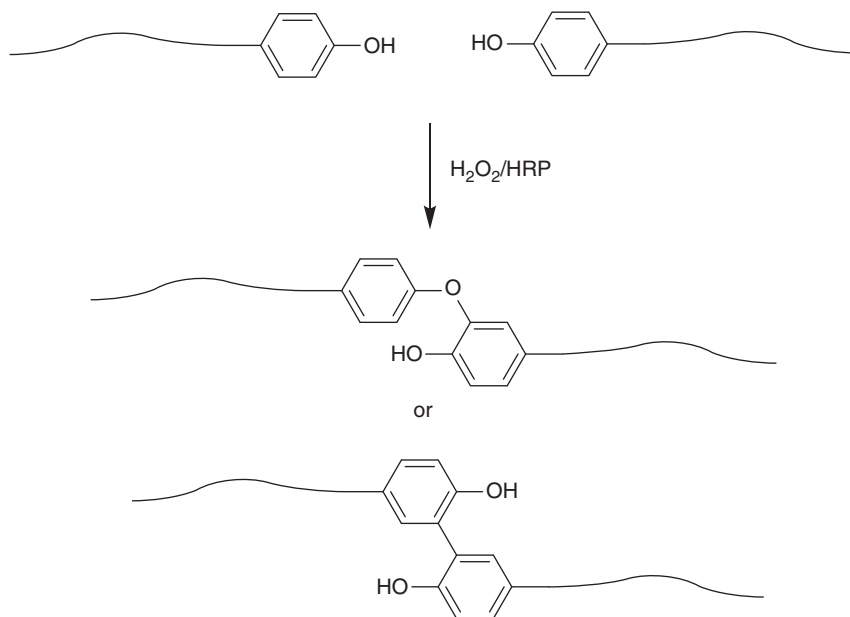
**Figure 1.33** Formation of the C—O and C—C bonds between two phenyl groups.

Gels containing α-CD, threaded onto Pluronic F68 or F127 with tyramine end groups, have been investigated. All gels have been *covalently* cross-linked through the phenolic end groups using H_2O_2/HRP.

For the synthesis of tyramine-functionalized Pluronic (i.e., F68/Tyr or F127/Tyr), first Pluronic functionalized by carboxyl groups Pluronic/COOH had to be obtained. To this end, the 1,4-dioxane solution of polymer was treated with succinic anhydride in the presence of DMAP and TEA. After stirring at room temperature for 24 h, 1,4-dioxane was evaporated and the addition of Et_2O precipitated Pluronic/COOH.

The THF solution of Pluronic/COOH was treated with tyramine, DCC, and *N*-hydroxysuccinimide, and stirred at room temperature for 24 h. After evaporation of THF, the addition of Et_2O precipitated the tyramine-functionalized Pluronic, that is, F68/Tyr or F127/Tyr (Figure 1.34).

The optimization of H_2O_2 and HRP amounts is necessary to achieve the optimal cross-linking time and the highest mechanical properties:

- The optimal cross-linking time should allow manipulation of the gels (e.g., cell encapsulation) and therefore it cannot be less than a few seconds.
- The highest mechanical properties are desired in order to decrease the loss of mechanical properties resulting from the shearing of the PPR hydrogels preceding the enzymatic cross-linking. The ratio of H_2O_2 to HRP also had to be optimized to avoid depletion of the H_2O_2, manifested as a sudden plateau in the mechanical properties. The decrease in mechanical properties and the increase in the gelation time upon increase of the H_2O_2 concentration may result from inactivation of HRP by H_2O_2 [130] and has been observed for other hydrogels [123].
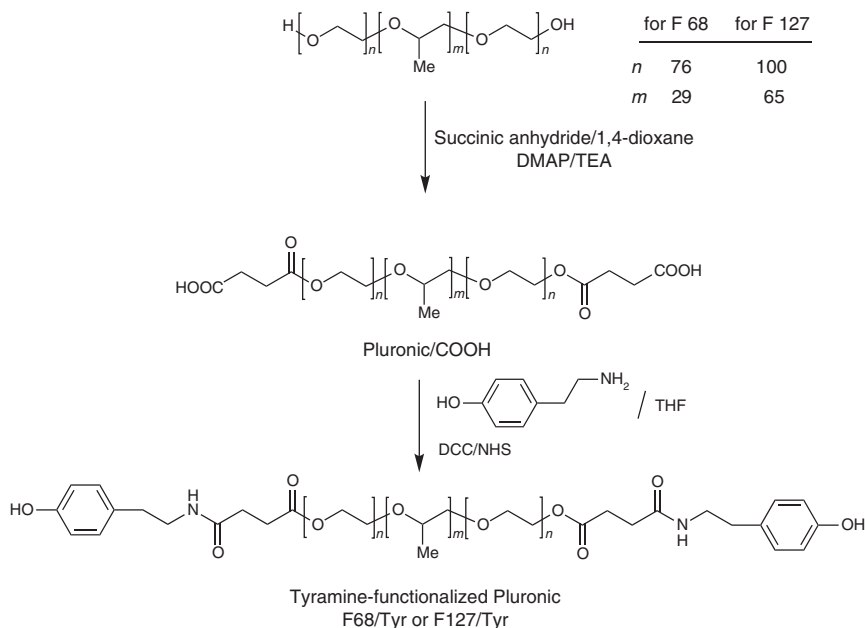
Figure 1.34 Synthesis of tyramine-functionalized Pluronic F68/Tyr or F127/Tyr.

In the study, PEG-Tyr was subsequently added in various ratios in order to create a branched network. PPRs should have sufficient time to form by threading of the α-CD units onto the Pluronic polymers before covalent cross-linking of gels with H_2O_2 and HRP occurs. Enzymatic cross-linking using HRP is a relatively fast process (a few minutes) compared to the formation of PPRs (a few hours). In order to accommodate for these two very different time scales, the self-healing ability of the PPR gels was employed [121]. The PPR gels were allowed to form for 4 h before the shearing to allow the addition of PEG-Tyr, HRP, and H_2O_2 (Figure 1.35).

- To assess whether the tyramine-based hydrogels could offer a support for the sustained delivery of poorly water-soluble drugs, the 6-aminofluorescein serving as a model drug was encapsulated within the hydrogels of various compositions. In the absence of PEG-Tyr, the release of 6-aminofluorescein was rapid (60 h). The addition of PEG-Tyr led to a prolonged and sustained release that lasted up to 15 days for F68-Tyr-based gels and 17 days for F127-Tyr-based gels. It was found that a similar steady release was obtained both with and without the presence of α-CD. One can conclude that the covalently cross-linked tyramine-based hydrogels under study are a convenient platform for the sustained delivery of poorly water-soluble molecules over a period of 14 days.

It is known that delivery systems of longer drug release are advantageous, especially for poorly water-soluble drugs. Hydrogels showed to be an interesting class of delivery systems due to their possibility to tune the release rate by varying the cross-linking density. The release of a poorly water-soluble drug from the *covalently cross-linked* PR hydrogels lasts for about 14 days.
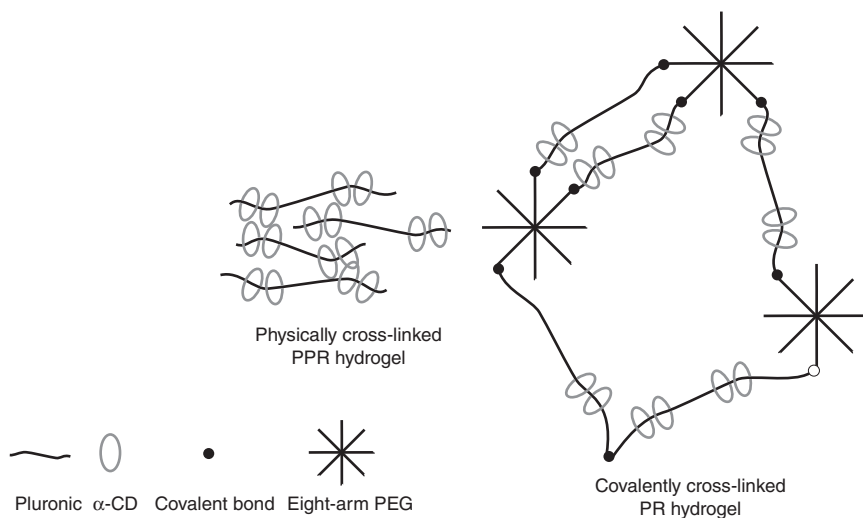
Figure 1.35 Schematic representation of physically cross-linked PPR hydrogel and of covalently cross-linked PR hydrogel.

- To evaluate the usefulness of the covalently cross-linked hydrogels containing PEG-Tyr for short-term cell encapsulation, the mouse 3T3 fibroblasts were encapsulated within the hydrogels. It was established that the rate of H_2O_2 consumption (and conversion to H_2O) by HRP was high enough to ensure that the H_2O_2 level was always below a toxic threshold.

It is known that the self-assembled PPR hydrogels have many advantageous properties, including tunable gelation and self-healing abilities. However, due to their self-assembled nature, they are more sensitive to environmental conditions than covalently cross-linked systems and can dissociate rapidly when their equilibrium state is disrupted. Rapid dissolution of these metastable hydrogels can lead to the release of toxic products. The PPR-based hydrogel previously obtained [121] required a stabilization, which was performed in the present work by introduction of a *covalent* cross-linking function.

Although some of the advantages of the self-assembly, for example, the ability to form a hydrogel purely via physical cross-linking, without chemicals or other stimuli are lost, the presented method used for preparation of *covalently linked* PRs is necessary for creation of PR hydrogels useful for drug delivery and tissue engineering.

The developed cross-linking method involves the coupling of two phenolic moieties by peroxidase-catalyzed oxidation. The phenol moieties were introduced into Pluronic and into branched PEG by reaction of the hydrogel end groups of each chain with succinic anhydride, and the subsequent addition of a tyramine end groups with the use of DCC/NHS coupling reaction.

The cross-linking of the phenolic groups introduced at the Pluronic end groups that constitute the backbone of the PPR affords long multiunit chains spanning multiple PPR aggregates, while the introduction of the phenol-functionalized eight-arm PEG enables the formation of a branched, *covalently cross-linked*

network, increasing the stability of gels. The eight-arm PEG serves also as an end-capping group for the α-CD/Pluronic PPRs, converting them into PRs and preventing dethreading.

The hydrogels under investigation have many tunable mechanical properties; they can be tuned by varying the type of Pluronic, the coverage of α-CD, and the amount of PEG-Tyr (eight-arm PEG) units. It was found that for the same α-CD coverage, the elastic modulus of covalently cross-linked PPR hydrogels is higher than that of physical PPR hydrogels, and increases with higher α-CD concentration.

The hydrogels covalently cross-linked have also tunable degradation properties. The introduction of the covalent cross-linking considerably increases the stability of PPR hydrogels. When immersed in PBS, the enzymatically cross-linked hydrogels take a longer time to degrade than the physical hydrogels. The functionalization, involving the coupling of tyramine to polymers, introduced an ester bond between the polymer backbone and the end group; the presence of this ester bond creates a hydrolytically degradable hydrogel network. The rate of the degradation of an ester-containing hydrogel depends on the number of ester groups present. In the performed study, the rate of degradation was tuned by changing the number of eight-arm PEG molecules, which is equivalent to increasing the number of ester groups.

The self-assembled PPR hydrogels cannot be handled and weighed due to their sensitivity to shear, especially during the dissolution process. The *covalently cross-linked* hydrogels show a lower sensitivity to shear than physical purely self-assembled PPR hydrogels, and therefore are easier to handle. The covalently cross-linked hydrogels have dual properties, namely, the covalent network created through the enzymatic cross-linking of the tyramine end groups is reinforced by the interactions of CD units in the initial formation of PPRs. This property allows generating interesting cellular responses. This behavior is important for tissue engineering, since cells can modulate their local environment via migration, and the covalent network offers a more durable scaffold.

It was shown that remodeling of the cellular microenvironment in hydrogels could influence hMSC behavior [131]. The enzymatically cross-linked PR hydrogels, with the possibility for remodeling of the physically associated components while maintaining the covalent network structure, can offer a new route to explore this effect in stem cell biology.

The above-described hydrogels are hydrolytically degradable in 2–8 days due to the presence of ester bonds, making them suitable for short-term cell encapsulation and for sustained release of poorly water-soluble drugs.

The mechanical properties of the studied hydrogels show features of both the cross-linked network and of the self-assembled network, since they may be tuned by changing the amounts of eight-arm PEG and of α-CD, participating in the network. This dynamic double network, in which covalently bound PRs are still able to interact via physical bonds at the molecular level, is promising for applications in tissue engineering.