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FULL PAPER

Mechanochemical preparation of stable sub-100 nm γ-cyclodextrin:Buckminsterfullerene (C60) nanoparticles by electrostatic or steric stabilization

Joachim F.R. Van Guyse, Victor R. de la Rosa and Richard Hoogenboom[a]*

Abstract: Buckminsterfullerene (C_{60})'s main hurdle to enter the field of biomedicine is its low bioavailability, which results from its extremely low water solubility. A well-known approach to increase the water solubility of C_{60} is by complexation with γ-cyclodextrins. However, the formed complexes are not stable in time as they rapidly aggregate and eventually precipitate due to attractive intermolecular forces, a common problem in inclusion complexes of cyclodextrins. In this study we attempt to overcome the attractive intermolecular forces between the complexes by designing custom γ-cyclodextrin (γCD)-based supramolecular hosts for C_{60} that inhibit the aggregation found in native γCD-C_{60} complexes. The approach entails the introduction of either repulsive electrostatic forces or increased steric hindrance to prevent aggregation, thus enhancing the biomedical application potential of C_{60}. These modifications have led to new sub-100 nm nanostructures that show long-term stability in solution.

Introduction

C_{60}, an allotropic form of carbon, was first discovered by Kroto *et al.*[1,2] in 1985, and its macroscopic production was first realized by Krätschmer in 1990.[3,4] Ever since, this molecule has been the subject of extensive research, ranging from astrochemistry to, more recently, materials science and biomedicine, fields where C_{60} has an enormous application potential.[5–7] In the biomedical context, C_{60} stands out as a powerful antioxidant, *i.e.* a scavenger of cytotoxic reactive oxygen species (ROS). Moussa *et al.* demonstrated this by administering a C_{60}-olive oil suspension to rats, which resulted in a pronounced increase in their life span, implying their potential use as a powerful anti-ageing agent and a preventative measure for cancer and neurodegenerative diseases, such as Alzheimer's.[8] In addition, C_{60} could also be used as a photosensitizer, as it generates ROS upon light irradiation.[9,10] Therefore, C_{60} could be used in photodynamic therapy to treat several pathologies, including cancer and bacterial infections.

Despite C_{60}'s large application potential in biomedicine, the realization thereof is hampered by its negligible water solubility of 10^{-8} ng L^{-1}.[11] To increase C_{60}'s water solubility, several procedures can be applied, one of which is the covalent modification of C_{60}. However, this can induce toxicity, the loss of C_{60}'s beneficial intrinsic properties and it is not straightforward due to lack of stereoselectivity.[12,13] An alternative procedure is based on the dispersion of C_{60} in water through co-solvent evaporation, creating meta-stable C_{60} clusters. This method has been used in the past to determine the toxicity of C_{60}, mistakenly being identified as toxic due to the remaining organic solvents.[14] Another drawback of this method is the decrease of biological activity with the increasing size of the C_{60} clusters.[15,16]

Another approach is the exploitation of host-guest chemistry for the complexation of a hydrophobic guest with a water-soluble host. Here, cyclodextrins are popular building blocks for the preparation of supramolecular assemblies and nanoparticles by exploiting dynamic host-guest interactions.[17–19] The utilization of these water-soluble hosts, such as calixarenes and cyclodextrins, has led to the successful solubilization of C_{60}, forming 2:1 water-soluble host-guest complexes with C_{60}.[20,21] These complexes retain the intrinsic properties of C_{60} while allowing the incorporation of additional functionalities through the modification of the host molecule. Cyclodextrins are especially suited for the complexation of C_{60}, as demonstrated by multiple research groups, based on the hydrophilicity of these cyclic oligosaccharides.[20,22–24] Despite their excellent complexation abilities with hydrophobic guests, native CDs and their inclusion complexes with C_{60} are known to rapidly aggregate and often precipitate in aqueous solution.[25] This tendency to rapidly aggregate greatly limits their biological application potential, as biological relevance drops with increasing size of the aggregates by limiting cellular uptake.[26,27] This unwanted aggregation behavior can be mainly attributed to attractive forces between individual complexes, as it is well known that cyclodextrins establish efficient intermolecular hydrogen bonds (see **Figure 1**).[26,28,29]

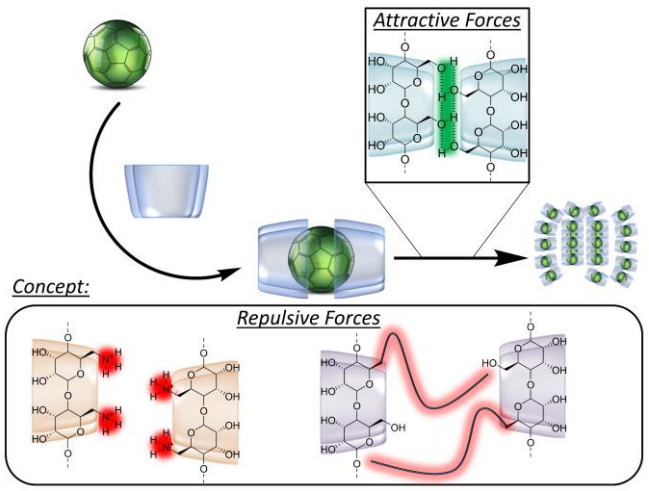

Figure 1. Schematic representation of the proposed concept used to prevent aggregation between individual 2:1 γCD-C_{60} complexes based on electrostatic (bottom left) and steric (bottom right) stabilization.

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In addition, the complexed C_{60} units are still partially exposed to the aqueous environment in the γCD-C_{60} complexes, potentially inducing hydrophobic interactions that further induce unwanted aggregation. In an attempt to enhance the stability of γCD-C_{60} complexes and suppress their natural tendency to aggregate, we have designed functional γCD hosts aiming to minimize the attractive intermolecular forces between individual CDs and their complexes, by the incorporation of repulsive charges or steric hindrance by polymer chains (**Figure 1**). The incorporation of these Coulombic or steric factors is hypothesized to hinder or slow down the aggregation of the individual CDs and complexes, leading to more stable complexes.

Results and Discussion

The Coulombic and steric factors anticipated to stabilize the γCD-C_{60} copmlexes were incorporated in γCD by modification of the primary hydroxyl groups, which are the most straigthforward to chemically modify due to their higher nucleophilicity and accessability compared to their secondary counterparts.[30] Moreover, performing the modifications on the primary hydroxyl groups –cyclodextrin's narrow rim- will have the least impact on inclusion complex formation, as this occurs at the cyclodextrin's wide rim side, where the secondary hydroxyl groups, are located.[31] Additionally, the secondary hydroxyl groups are important for the structural integrity of cyclodextrins, as they 'lock' the structure into its shape through intramolecular hydrogen bonding, thus having a major influence on the molecule's flexibility, solubility and complexation ability.[30,31]

Starting from native γCD, novel γCD hosts for C_{60} were synthesized through either selective tosylation of one of the primary alcohols or iodination of all the primary alcohols. Next, the azide was easily and almost quantitatively obtained *via* an S_N2 substitution with sodium azide. The polymer-bearing γCDs were then obtained by copper(I)-catalyzed-azide-alkyne cycloaddition (CuAAC) click chemistry between an alkyne-bearing polymer chain and mono-(6-azido-6-deoxy)- or octakis(6-azido-6-deoxy)-γCD, yielding products **1** and **3** respectively (**Figure 2**).

In addition, this synthetic approach grants easy access to a charged γCD through Staudinger reduction of the azide groups, yielding octakis(6-amino-6-deoxy)-γCD, which can then be converted to its respective water-soluble salt **2** by addition of hydrochloric acid.
As a stabilizing polymer we chose poly(2-ethyl-2-oxazoline), which is a biocompatible and water-soluble polymer, easily functionalized by initiating the cationic ring opening polymerization with an alkyne bearing initiator,[32] such as propargyl benzenesulfonate.[33–36] Additionally, these polymers where chosen as they exhibit excellent chemical and physical stability, anticipated to lead to good compatibility with the high speed vibration milling (HSVM) treatment.[37,38]

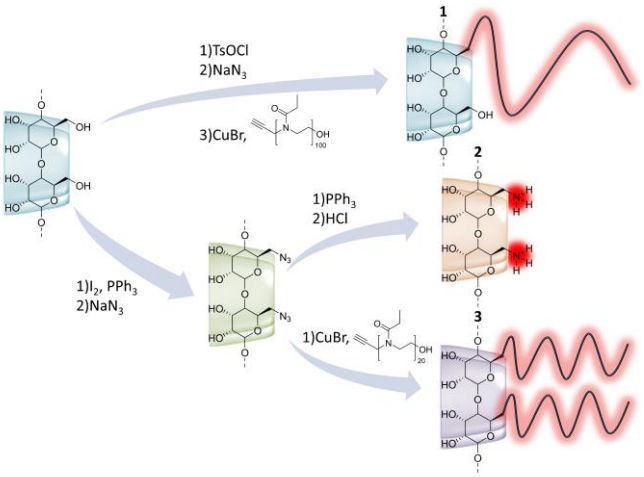

Figure 2. Synthesis scheme of the different modified cyclodextrin hosts, by incorporation of either one or multiple polymer chains or multiple charges.

Once the new γCD hosts were synthesized, the formation of supramolecular host-guest complexes with C_{60} was investigated. In literature two methods are mainly used to form native-γCD-C_{60} complexes: the first utilizes a two-phase toluene:water system, which exploits the lower solubility of C_{60} at higher temperatures in toluene to displace it into the γCD-containing aqueous phase, where it then forms complexes with γCD.[20] The second method circumvents the use of solvents by applying HSVM, using mechanical forces in a ball mill to yield the γCD-C_{60} complexes from the solid reagents, followed by their dispersion in water.[39–41] For our purposes, the second method allows a better comparison between the native and modified γCDs, disregarding the difference in solubility of the γCD hosts, which could have an influence on the complexation with C_{60}. Additionally, the HSVM method is faster, more effective, and does not involve the use of organic solvents, which is beneficial when considering biomedical applications. In fact, this method applies some principles of green chemistry as it avoids the use of solvents, is more energy efficient and is overall safer.[42–44]

Following the synthesis of the modified γCD hosts, the stability of these hosts in aqueous solution was evaluated and compared to native γCD. As can be seen from **Figure 3 (top),** the freshly prepared native γCD solution shows a size distribution of less than 5 nm in the volume plot, which corresponds to the expected molecularly dissolved CD. However, 5 hours later, the size distribution looks significantly different, showing micron sized aggregates in both the intensity and volume plots. This is in agreement with previous reports stating that native CDs rapidly aggregate in solution due to the establishment of intermolecular hydrogen bonds.[26,28,29] The modified γCDs **1-3** show a different behavior as the size distributions for both intensity and volume remain relatively unchanged over the course of 24 hours. The volume plot shows a size distribution around the expected values for molecularly dissolved hosts, which is larger for the polymer bearing γCDs.

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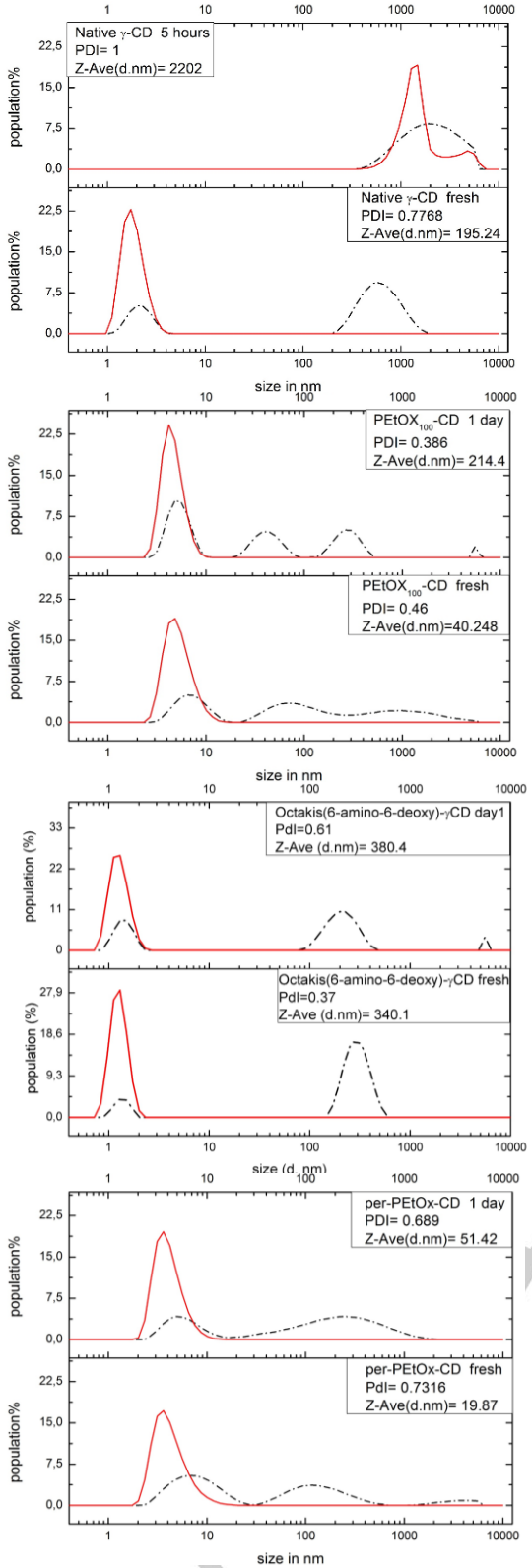

Figure 3. DLS spectra of γ-CD, mono-P(EtOx)$_{100}$-γCD **1**, octakis(6-amino-6-deoxy)-γCD **2** and octakis-P(EtOx)$_{20}$-γCD **3** (1mg/mL) from top to bottom. DLS spectra show the particle size distributions both by intensity (.-.) and volume (—).

The fact that the size distributions (both volume and intensity) remain unaltered over 24 hours, already indicates the inhibition of hydrogen bonding induced aggregation between the γCDs by the presence of either Coulombic or steric repulsion. Next, the γCD hosts were complexated to C_{60} to evaluate their ability to complexate C_{60} and assess the stability of the formed complexes. The complexation of C_{60} with the newly synthesized γCD hosts **1-3** was performed by HSVM and compared with γCD as a control. Subsequently, water was added to extract the complexes after which the solution was filtered to remove any excess of uncomplexed C_{60}. Next, the formed complexes were analyzed to quantify the C_{60}-content and assess their size and stability in solution. C_{60}-content was determined by UV-Vis spectroscopy, using the Lambert-Beer law to calculate the concentration from the characteristic absorption band of C_{60}, using the known molar extinction coefficient of ε(335nm)= 51900 $_M^{-1}$ cm^{-1}.[45] From the C_{60} concentration in a 1 mg/mL solution of each complex, we were able to calculate the weight percentage of C_{60}, the yield of C_{60} incorporation in each of the formulations and the ratio of CD to C_{60} in the obtained nanoparticle formulations (**Table 1**). All hosts display the ability to complexate C_{60} as the UV-vis spectra showed the presence of absorbance bands related to C_{60}. **Table 1** shows that both the native γCD and the per-amino-γCD **2** have a similar weight percentage of C_{60}, while the polymer-bearing γCDs **1** and **3** show a lower weight percentage. This is a result of the larger molecular weight of the polymer-bearing hosts compared to the native and per-amino-γCDs allowing the addition of more CD host to the milling process. Importantly, when the yields are compared, it can be seen that the modified hosts lead to more efficient incorporation of C_{60} than the native γCD. An increased host/guest ratio is obtained for all hosts after milling and filtering, which is expected as the non-water soluble C_{60} aggregates were removed from solution by filtration. At this stage it is not yet clear whether the excess of CD is present in the nanoparticles or is free in solution, but the observed increase in molar ratio corresponds well to the observed loss of C_{60} from the calculated yields.

Table 1. Weight and mol percentages of C_{60} in the nanoparticles that are formed with the different hosts.

Host	Molecular weight Host (Da)	Wt%	Yield in %	Molar ratio CD/C_{60} in formulation
γCD	1297	5.4	43	9.75
1	11291	2.6	79	2.38
2	1490	5.8	50	7.85
3	17804	1.4	54	2.85

Next, the stability of the C_{60} complexes with the new γCD hosts was investigated by measuring the evolution of their particle-size distribution in time by dynamic light scattering (DLS). The

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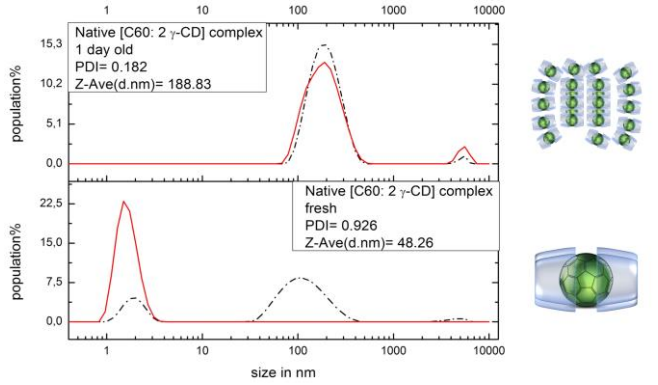

Figure 4. DLS spectra showing the particle size distribution for native C60-γCD complexes (1mg/mL) both by intensity (.-.) and volume (___). Bottom plot shows the size distribution of the time zero sample, the top plot shows the size distribution for t = 24 hours.

nanoparticles were filtered prior to the first measurement, and remeasured over the course of two weeks. The results are shown in **Figures 4** and **5**.

Figure 4 shows the fast aggregation of the native γCD-C_{60} complexes: initially, small nanoparticles were observed with a size of *ca*. 3 nm, corresponding to the expected 2:1 γCD-C_{60} complex together with free CD in solution. However, the spectrum also shows the presence of larger aggregates in the intensity plot, but the volume plot shows the majority of the complexes are molecularly dissolved. However 24 hours later, the particle-size distribution shows a large shift with the mean particle-size evolving from 3 nm to *ca*. 200 nm, indicating aggregation due to attractive intermolecular forces, as was also seen in the solution of native γCD, this is in agreement with previous reports.[25,26] Within three days after sample preparation, the formation of a macroscopic precipitate was observed. Sonication and refiltration proved ineffective to break the aggregates and the defined complexes could not be regenerated.

In contrast to these results with native γCD, the modified γCD hosts showed an entirely different behavior as observed in the DLS measurements. Instead of obtaining small sub 10 nm particles, which would correspond to a molecularly dissolved bicapped 2:1 complex, rather defined nanoparticles with a size of *ca*. 50 nm were observed which was later confirmed by transmission electron microscopy (TEM).

These results together with the UV-vis results indicate the successful complexation of C_{60} with the modified hosts. However, the presence of defined nanoparticles rather than discrete 2:1 complexes indicates that additional intermolecular forces are present and the the absence of a population with small size in DLS indicates that the excess of γCD is incorporated in the nanoparticles. Since self-assembly of the modified γCDs does not occur in absence of the guest, the hydrophobic C_{60} is hypothesized to play a major role in the controlled self-assembly, which is in agreement with a report from Uekama *et al*.[46,47] As hypothetically visualized in **Figure 5**, the further agglomeration of the γCD-C_{60} complexes is ascribed to the establishment of hydrophobic interactions and Van der Waals forces between the partially exposed C_{60} guests within the complexes. Additional

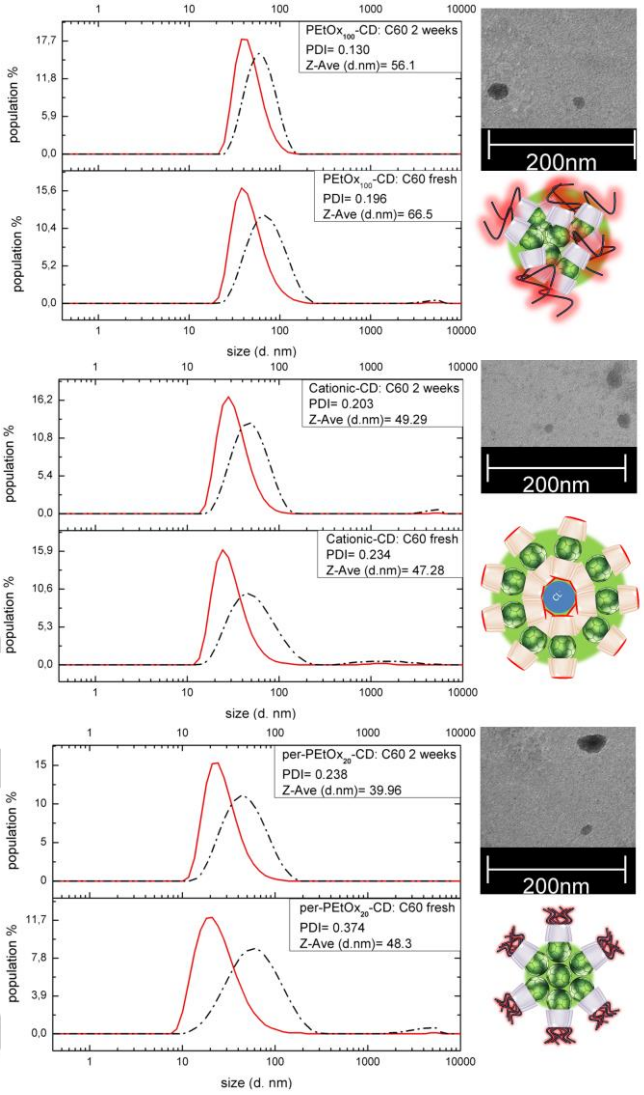

Figure 5. DLS spectra and TEM pictures of mono-P(EtOx)$_{100}$-γCD-C_{60} complexes **1** (top), octakis(6-amino-6-deoxy)-γCD-C_{60} **2** (center) and octakis-P(EtOx)$_{20}$-γCD-C_{60} complexes **3** (bottom) (1mg/mL) with their proposed structures. DLS spectra show the particle size distributions both by intensity (.-.) and volume (—). Bottom plots show the size distribution of the t_0 sample, while the top plots show the size distribution for t = 2 weeks.

interactions that might play a role are Coulombic interactions between the cationic per-amino-γCD 2 and their respective counterions, or poly(2-oxazoline)-C_{60} donor and acceptor interactions with C_{60} as reported by Kabanov *et al*.[21]

This controlled assembly towards this nanoparticle structure seems to be based on a fine balance of forces during the HSVM process. In the native γCD-C_{60} complexes, a bicapped molecularly dissolved complex is initially observed, which is favoured as the secondary hydroxyl groups 'lock' the sandwich-like structure via intermolecular hydrogen bonding. The further aggregation in time is then caused by the hydrogen bonding via CD's primary hydroxyl groups. In the modified hosts, the

introduction of a repulsive moiety both sterically or Coulombic, is hypothesized to partially disrupt or weaken the interlocking mechanism between the 2:1 complexes, resulting in a more exposed C_{60}. This could make the formed complexes prone to assemble into the observed nanoparticle structure mainly through hydrophobic interactions from C_{60}. Once assembled, the assembly remains stable in solution, which is hypothesized to be caused by Coulombic or steric stabilization. Both these repulsive factors will be mainly projected onto the surface of the assembly in order to minimize the overall charge or maximize the free volume that the polymer chains can occupy. The contribution of the repulsive factors to the stability of the assembly could already be observed from the stability of the modified hosts themselves and will also inhibit further aggregation between the separate nanoparticle assemblies, as could be evidenced from the absence of change in the DLS size distribution plots over the course of 2 weeks. Therefore, the stability and assembly of this system relies on the subtle balance of attractive host-guest and hydrophobic interactions and repulsive Coulombic or steric factors, as was achieved before in other dynamic supramolecular cyclodextrin assemblies.[46–49]

Even though the modified hosts did not lead to the formation of a stable molecularly dissolved bicapped C_{60} complex, they were successful at complexating C_{60} into nanoparticles and capable of inhibiting aggregation of the separate assemblies. Furthermore, the size of the obtained particles is sub-100 nm, which will influence their cellular uptake pathways and kinetics. Recent studies on cell internalization show that particles ≤ 200 nm are internalized via pinocytosis as opposed to unwanted phagocytosis and ≤100 nm particles show faster uptake than their larger counterparts.[16,27] Therefore, the developed stable C_{60} nanoparticles will be interesting candidates for further research with regard to their activity as an antioxidant or photosensitizer, especially since a recent study suggests that the photosensitizing properties of C_{60} depend on its aggregate structure, where aggregated C_{60} has a shorter-lived triplet state, leading to diminished ROS production.[10]

Conclusions

In summary, we have explored the inhibition of intermolecular hydrogen bond driven association/aggregation between cyclodextrins *via* the incorporation of charges or steric hindrance. Both strategies proved to be successful in improving the stability of γCDs in aqueous solution, as observed from the DLS data. Next, we studied the influence of these modifications on the complexation behavior with C_{60}. Here UV-vis data showed the ability of the modified hosts to interact and solubilize C_{60} in water. However, upon characterization of the complexes via DLS and TEM, well defined nanoparticles were observed rather than molecularly dissolved 2:1 CD-C_{60} complexes. Surprisingly, both the steric and Coulombic systems displayed a similar behavior, indicating a common driving force in the formation of the supramolecular assemblies, *viz.* C_{60}, during HSVM. The formation towards the observed kinetically trapped supramolecular assemblies was rationalized and attributed to a balance of attractive and repulsive forces. We hypothesize that the introduction of repulsive forces affects the formation of sandwich-like complexes, thus leaving C_{60} more exposed and allowing the strong hydrophobic character of C_{60} to steer the supramolecular assembly. Further aggregation of the obtained supramolecular assembly seems to be inhibited by these repulsive forces, therefore successfully counteracting the natural tendency of native cyclodextrins to aggregate, as was confirmed before. The enhanced stability could be rationalized by a nanoparticle conformation where the charges and polymers are directed away from one another, thus minimizing the electrostatic repulsion or steric hindrance, *i.e.* maximizing the volume that the polymer chain(s) can occupy. Therefore, the combination of supramolecular interactions led to the synthesis of highly stable nanoparticle solutions that, unlike their native precursors, remained unchanged for weeks, while maintaining a relatively high C_{60}-content.

The proposed concept could lead to the design of modified C_{60} complexes, which are more suitable for biological applications as they are more stable in solution. In addition, the larger hydrodynamic volume of these C_{60} nanoparticles could possibly result in a beneficial longer retention time in the body and enhanced uptake in tumors by the EPR effect. Furthermore, additional functionalities may be added by varying the side chains and termini of the polymer(s), allowing the incorporation of specific targeting groups for selective delivery to aid in the treatment of cancers or neurodegenerative disorders.

Experimental Section

Materials and methods:

Size-exclusion chromatography (SEC) was performed on an Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostatted column compartment (TCC) at 50°C equipped with two PLgel 5 µm mixed-D columns and a guard column in series, a 1260 diode array detector (DAD) and a 1260 refractive index detector (RID). The used eluent was *N,N*-dimethylacetamide DMA containing 50 mM of LiCl at a flow rate of 0.593 mL/min. The spectra were analyzed using the Agilent Chemstation software with the GPC add-on. Molar mass and Đ values were calculated against PMMA standards from PSS. UV-VIS spectra were recorded on a Varian Cary 100 Bio UV-VIS spectrophotometer equipped with a Cary temperature and stir control. Samples were measured in either quartz or disposable cuvettes with a pathlength of 1.0 cm in the wavelength range of 200 to 700 nm. The concentration of each sample was 1.0 mg/mL in milliQ water. Dynamic light scattering (DLS) and Zeta potential measurements were executed on a Zetasizer Nano-ZS Malvern apparatus (Malvern Instruments Ltd.) using disposable cuvettes. The excitation light source was a He−Ne laser at 633 nm and the intensity of the scattered light was measured at an angle of 173°. The concentration of each sample was 1.0 mg/mL in milliQ water. All samples were filtered with a 0.2 µm pore sized filter prior to measurement. Matrix assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) was performed on an Applied Biosystems Voyager De STR MALDI-TOF mass spectrometer equipped with 2 m linear and 3 m reflector flight tubes, and a 355 nm Blue Lion Biotech Marathon solid state laser (3.5 ns pulse). All mass spectra were obtained with an accelerating potential of 20 kV in positive ion mode and in either reflectron or linear mode. The

polymerizations were performed in capped vials in a single mode microwave Biotage initiator sixty (IR temperature sensor) (Biotage, Uppsala, Sweden) following a previously reported protocol.[34] Infrared spectra were measured on a Perkin-Elmer 1600 series FTIR spectrometer and are reported in wavenumber (cm^{-1}). ESI-MS spectra were acquired on a quadrupole ion trap LC mass spectrometer (Thermo Finnigan MAT LCQ mass spectrometer) equipped with electrospray ionization. Lyophilisation was performed on a Martin Christ freeze-dryer, model Alpha 2-4 LSC plus. High Speed Vibration Milling (HSVM) was performed in a Fritsch Mini-Mill Pulverisette 23 in a 10 mL stainless steel grinding bowl with 15 mm diameter grinding ball(s). Preparative size exclusion chromatography was performed with Disposable PD-10 Desalting Columns from GE Healthcare. Nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were recorded on a Bruker Avance 300 or 400 MHz spectrometer at room temperature. The chemical shifts are given in parts per million (δ) relative to TMS. The compounds were dissolved in either $CDCl_3$, D_2O or DMSO-d6 from Eurisotop.

Unless otherwise stated, all chemicals were used as received. All HPLC grade solvents were purchased from Sigma-Aldrich (acetone, diethylether, DMA, dichloromethane, methanol, acetonitrile), from Fischer Scientific (Toluene) or from Acros (dry DMF, DMSO). All reagents were purchased from Sigma-Aldrich, with the exception of triphenyl phosphine and sodium-L-ascorbate, which were bought from Acros. γ-Cyclodextrin was kindly provided by Wacker Chemie. 2-Ethyl-2-oxazoline was kindly provided by PCI, and was further purified by distilling over BaO. Propargylbenzenesulfonate was purchased from Sigma-Aldrich and was further purified by vacuum distillation. Acetonitrille was dried through a custom-built solvent purification system whereby the solvents pass an Alumina oxide column for drying. Further characterization details (NMR spectra, MALDI-TOF-MS spectra, *etc.*) on the synthesis of the described compounds can be found in the supporting information.

Synthesis of propargyl-poly(2-ethyl-2-oxazoline)s

The polymerization mixture was prepared in accordance with the method of Hoogenboom *et al.*[34] 10 mL microwave vials were dried in a high temperature oven (180°C) for at least 2 hours, after which they were allowed to cool under vacuum, in the vacuum chamber of a glovebox. Inside the glovebox two mixtures were prepared, with a monomer:initiator (2-ethyl-2-oxazoline:propargyl benzenesulfonate) ratio of 20 and 100. An appropriate amount of dry acetonitrile was added, to obtain a 4M monomer solution. Subsequently, the vials were capped and polymerized in a microwave synthesizer at 140°C until a conversion of roughly 100% was reached (3.2 min for DP20 and 16 minutes for DP100). The reaction was terminated with a methanolic solution of tetramethylammonium hydroxide. Afterwards, the polymers were isolated by precipitation in cold diethyl ether from dichloromethane. This precipitation cycle was repeated three times, after which the solvent traces were removed by placing the obtained solid in a vacuum oven at 50°C. ^{1}H NMR(300 MHz, $CDCl_3$): δ 4.4-4.15 (2H,m , C-CH_2-N), 3.65-3.00 (80H, m, N-CH_2-CH_2-N),2.50-2.14 (40H, m, OC-CH_2-CH_3), 1.18-0.89 (60H, m, CH_3); SEC: $PEtOx_{20}$: Mn:6140 Da Đ: 1.15, $PEtOx_{100}$: Mn=16400 Đ= 1.03; MALDI-TOF-MS: $PEtOx_{20}$: 2061.84Da = $[M+Na]^+$ $PEtOx_{83}$: 8310.9 Da = $[M+Na]^+$

Synthesis of Mono(6-O-p-toluenesulfonyl))-γ-cyclodextrin

This procedure was adapted from Stadermann *et al.*[50] γ -Cyclodextrin (20 g, 15 mmol, 1 eq.) was dried overnight in a vacuum oven at 50°C, after which it was weighed and dissolved in 200 mL of a 0.4M NaOH aqueous solution at 0°C. Once dissolved, *p*- toluenesulfonyl chloride (13 g, 67 mmol, 4.5 eq.) was added in small portions over the course of 5 minutes under vigorous stirring. The mixture was stirred for half an hour at -5°C. Next, the mixture was filtered and the filtrate was neutralized with hydrochloric acid (pH range from 5-6). Following the neutralization, the solution was precipitated in 1L of acetone. This resulted in the co-precipitation of the starting material and the product. The presence of the starting material did not entail any issues for the following steps and the product was used as such, correcting for its purity. Yield= 54%, purity= 28% (calculated from ^{1}H NMR). ^{1}H NMR (300 MHz, D_2O): δ 7.61 (0.54H, d, SO_2Cl-C-CH-CH), 7.29 (0.58H, d, R-C-CH-CH)), 5.03 (8H, d, 2(O)-CH-CH), 3.90-3.70 (32H, m, 16H (CH-OH)), 8H (CH-CH-OCH),8H (CH-CH-CH-OH), 3.60-3.45 (16H, CH-CH_2-OH).;^{13}C NMR (75 MHz, D_2O): δ 129.27 (CH), 125.33 (CH), 101.63 (CH), 80.37 (CH), 72.82(CH), 72.16 (CH), 71.65 (CH), 60.02 (CH_2); LC-MS: Mass Theoretical =1451.31 Da, Found= 725.55 Da = $[M^{2-}]$

Synthesis of Mono(6-azido-6-deoxy)-γ-cyclodextrin

Following the procedure of Stadermann *et al.*[50], mono(6-O-p-toluenesulfonyl))-γ-cyclodextrin was weighed (3.57 g, 700 µmol, 1 eq., 28% pure) and dissolved in 20 mL of water, which was then heated to 80°C. Subsequently, sodium azide (225 mg, 3.5 mmol, 5 eq.) was added to the solution. The reaction was left overnight, followed by the precipitation of the solution in 500 mL of acetone. The resulting precipitate (co-precipitated with γ-cyclodextrin) was filtered off, washed with milliQ water and dried. Yield= 77% purity = 21%. ^{1}H NMR (300 MHz, DMSO-d6): δ 6.9-6.7 (16 OH, m, CH-OH), 4.88 (8 H, d, 2(O)-CH-CH), 4.52 (8 OH, t, CH_2-OH), 3.70-3.50 (32 H, m,16H (CH-OH), 8H (CH-CH-O-CH),8H (CH-CH-CH-OH)), 3.40-3.25 (8H, m, CH-CH_2-OH + H_2O); ^{13}C NMR (75 MHz, DMSO-d6)**:** δ 101.40 (CH), 80.37 (CH), 72.91 (CH), 72.17 (CH),71.69 (CH), 60.20 (CH_2); LC-MS:Mass Theoretical =1322.14 Da, Found= 659.7 Da = [(M-$2H^+$)/2]

Synthesis of Octakis-6-iodo-6-deoxy-γ-cyclodextrin

The synthesis of Octakis-6-iodo-6-deoxy-γ-cyclodextrin was adjusted from the procedure reported by Ashton *et al*.[51] γ -Cyclodextrin (13 g, 10.2 mmol, 1 eq.) was dried overnight in a vacuum oven at 50°C. A 1L round bottom flask was filled with 250 mL of dry dimethylformamide (DMF). Next, triphenylphosphine (40 g, 153 mmol, 15 eq.) was added to the dry DMF under an argon flow. The following step required the addition of iodine (40.5 g, 160 mmol, 15.7 eq.) over the course of 15 minutes under vigorous stirring and an argon flow. Lastly, γ- cyclodextrin was added under an argon flow and the mixture was stirred for 24 hours at a temperature of 70°C. Afterwards, the reaction was left to cool to room temperature and then concentrated under reduced pressure until it reached one third of its original volume. The next day, a 3 M sodium methoxide solution in methanol was added and left to stir for one hour, after which the solution was precipitated in 1L of methanol, resulting in a white precipitate. The suspension was then filtered and the yellow solid obtained was extracted with methanol for 5 days with a Soxhlet apparatus. The white solid was then dried in a vacuum oven at 50°C. Yield= 48% purity= 95%. ^{1}H NMR (300 MHz, DMSO-d6): δ 6.2-5.9 (16 OH, m, CH-OH), 5.03 (8H, d, 2(O)-CH-CH), 3.82 (8 H, d, O-CH-$(CH)_2$), 3.70-3.50 (16 H, m, CH-OH), 3.45-3.2 (24 H, m, (16CH2,8 CH-CH2 + H_2O); ^{13}C NMR (75 MHz, DMSO-d6): δ 101.92 (CH), 85.16 (CH), 72.32 (CH),71.74 (CH), 71.00(CH), 35.74(CH_3,DMF), 30.77(CH_3,DMF), 9.22(CH_2); LC-MS: Mass Theoretical =2176.31 Da Found= 2174.85 Da = $[M-1H^+]^-$

Synthesis of Octakis-6-azido-6-deoxy-γ-cyclodextrin

The synthesis of Octakis-6-iodo-6-deoxy-γ-cyclodextrin was adjusted from the procedure reported by Ashton *et al.*[51] Octakis-6-iodo-6-deoxy-γ-cyclodextrin (5 g, 2.3 mmol, 1 eq.) was added under a nitrogen flow to a

250 mL flask filled with 100 mL of dry DMF. Subsequently, NaN_3 (1.67 g, 26 mmol, 11 eq.) was added. Next, the solution was heated to 60°C and left to react for 24 hours, after which the solution was concentrated with a rotary evaporator. The remaining liquid was precipitated in a large excess (1L) of H_2O to obtain a white powder. Yield= 88% Purity > 95%. ^{1}H NMR (300 MHz, DMSO-d6): δ 6.1-5.6 (16 OH, m), 4.94 (8 H, d,2(O)-CH-CH),3.8-3.67 (16 H, m, O-CH-CH, CH-CH_2), 3.65-3.50 (16 H, m, CH-OH), 3.45-3.2 (16H,m, CH_2+H_2O); ^{13}C NMR (75 MHz, DMSO-d6): δ 101.95 (CH), 82.56 (CH), 72.39 (CH), 72.15 (CH),51.07 (CH_2); IR: 3300 (OH-str), 2979 (CH_2-str), 2200 (N≡C-str); LC-MS: Mass Theoretical =1496.47 Da, Found= 1495.9 Da = $[M-H^+]^-$, 747.2 Da = $[M-2H^+]^-$

Synthesis of Octakis-6-amino-6-deoxy-γ-cyclodextrin (Host 2)

The synthesis of Octakis-6-amino-6-deoxy-γ-cyclodextrin was adjusted from the procedure reported by Ashton *et al*.[51] Octakis-6-azido-6-deoxy-γ-cyclodextrin (0.4 g, 267 µmol, 1 eq.) was dissolved in 8 mL of DMF and triphenylphosphine (1.261 g, 4.81 mmol, 18 eq.) was added. The development of nitrogen gas was observed by the formation of bubbles in the reaction vessel. When the development of nitrogen gas ceased, concentrated aqueous NH_3 (1.35 mL, approximately 35%) was added in a drop-wise manner to the solution. After the addition was complete, the reaction mixture turned into an off-white suspension. This suspension was stirred for 18 hours before it was concentrated under reduced pressure to approximately 10 mL. The product was precipitated by the addition of 100 mL EtOH. The precipitate was washed with EtOH and dried under high vacuum to yield a white solid. This solid was then converted into the HCl salt by suspending the product in a small volume of water followed by the addition of a dilute solution of HCl until a pH of 6 was reached. At this pH, a clear solution formed which became yellowish when evaporated under reduced pressure. The obtained product was then dialyzed and freeze-dried. ^{1}H NMR (300 MHz, D_2O): δ 5.15 (8H, d,2(O)-CH-CH), 4.25-4.15(8H, m, O-CH-CH),3.98 (8H, m, CH-CH_2), 3.66 (8H, m, CH-OH), 3,57 (8H, m, CH-OH), 3.44 (8H ,m,CH_2), 3.26 (8H,m,CH_2); LC-MS: Mass Theoretical =1490 Da, Found= 645 Da $[M + 2H^+ - 8\ HCl]^{2+}$

Synthesis of mono-PEtOx$_{100}$-γ-cyclodextrin (Host 1)

The protocol for this reaction was adjusted from the protocol reported by Hoogenboom *et al*.[33] Propargyl-PEtOx (1 g, 0.1 mmol, 1 eq.), 100 repeating units, was weighed in a microwave vial. Next mono(6-azido-6-deoxy)-γ-cyclodextrin (1.260 g, 0.2 mmol, 2 eq., 21 % pure) was added to the microwave vial, followed by the addition of a 10 mL DMF solution of copper(I) bromide (22 mg, 0.15 mmol, 1.5 eq.), pentamethyldiethylenetriamine (PMDTA) (26 mg, 0.15 mmol, 1.5 eq.) and sodium-*L*-ascorbate (2 mg, 0.01 mmol, 0.1 eq.). The microwave vial was then filled with argon and capped. 10 mL of dry DMF was added, the reaction mixture became slightly blue and the reaction mixture reacted for 15 minutes at 100°C in the microwave, after which it turned yellow. The mixture was allowed to cool to room temperature, after which the DMF was removed by freeze-drying. The remaining polymer film was redissolved in water and extracted with CH_2Cl_2 3 times to isolate the desired product. The product was then concentrated under reduced pressure and precipitated in cold diethylether. This precipitation protocol was repeated 3 times. The excess solvent was then removed in the vacuum oven at 50°C. Yield= 49.26%. ^{1}H NMR (400 MHz, DMSO): δ 3.57 (s, 7H, CD), 3.51 (s, 50H, CD), 3.40 (m, 427H, N-CH_2-CH_2-N + H_2O), 2.37 (m, 207H, OC-CH_2-CH_3), 1.23 – 0.78 (m, 300H, CH_3); SEC: mono-PEtOx$_{100}$-CD: Mw=19900 Da Đ=1.098; MALDI-TOF-MS: mono-PEtOx$_{100}$-CD: 8142.24 Da = $[M+Na]^+$

Synthesis of per-PEtOx-γ-cyclodextrin (Host 3)

The protocol for this reaction is similar to the protocol used for the synthesis of mono-PEtOx100-γ-cyclodextrin. Propargyl-PEtOx (188 mg, 140 µmol, 10.5 eq.), 20 repeating units, was weighed in a microwave vial. Next Octakis-6-azido-6-deoxy-γ- -cyclodextrin (20 mg, 13 µmol, 1 eq.) was added to the microwave vial, followed by the addition of CuBr (3 mg, 22 µmol, 1.6 eq.), PMDETA (3.7 mg, 22 µmol, 1.6 eq.) and Sodium-L-ascorbate (0.26 mg, 1.3 µmol, 0.1 eq.). The microwave vial was then filled with argon and capped. Subsequently, 4 mL of dry DMF were added, the reaction mixture became slightly blue and the reaction mixture reacted for 15 minutes at 100°C in the microwave, after which it turned yellow. The isolation of the star polymer is identical to the method described for mono-PEtOx100-γ-cyclodextrin. To separate the excess of polymer from the product, a PD-10 column was used, after which the obtained fractions were freeze-dried. Yield= 56 %. ^{1}H NMR (300 MHz, CDCl3): δ 6.00-3.96 (72 H, m, CD), 3.90-3.00 (738 H, m, N-CH2-CH2-N), 2.62-1.85 (416 H, m, OC-CH2-CH3), 1.24-0.60 (607 H, m, CH3); SEC:per-PEtOx20-γ-cyclodextrin: Mw= 29420 Da, Đ= 2.02; MALDI-TOF-MS (linear mode): per-PEtOx20-γ-cyclodextrin: ±17000 Da

Complexation of CDs with C_{60} via HSVM

The complexation of the CDs with C_{60} was carried out in a Fritsch Mini-Mill Pulverisette 23 in a 10 mL stainless steel grinding bowl equipped with one 15 mm diameter grinding ball. The solid reagents were added and the mixture was agitated for 10 min at 50 Hz. Next 1 mL of milliQ water was added and the mixture was agitated again for 2 min at 50 Hz, in order to solubilize the solids. The resulting solution was then filtered over a 0.2µm PTFE pore filter. The following molar equivalents of the respective γCDs to C_{60} were used: 3.88; 1.87; 3.44 and 1.53 for γCD, host 1, host 2 and host 3, respectively. The total mass of the powders was kept constant at 50 mg.

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Keywords: Cyclodextrins • Fullerenes • C_{60} • Aggregation • poly(2-oxazoline)s

[1] A. G. Avent, P. R. Birkett, C. Christides, J. D. Crane, A. D. Darwish, P. B. Hitchcock, H. W. Kroto, M. F. Meidine, K. Prassides, R. Taylor, et al., *J. Mol. Struct. THEOCHEM* **1994**, *325*, 1–11.

[2] H. W. Kroto, J. R. Heath, S. C. O'Brien, R. F. Curl, R. E. Smalley, *Nature* **1985**, *318*, 162–163.

[3] W. Krätschmer, K. Fostiropoulos, D. R. Huffman, *Chem. Phys. Lett.* **1990**, *170*, 167–170.

[4] W. Krätschmer, L. D. Lamb, K. Fostiropoulos, D. R. Huffman, *Nature* **1990**, *347*, 354–358.

[5] I. V. Bagrov, I. M. Belousova, a. V. Ermakov, V. M. Kiselev, I. M. Kislyakov, T. K. Kris'ko, T. D. Murav'eva, *Opt. Spectrosc.* **2008**, *105*, 720–725.

[6] A. G. Avent, P. R. Birkett, C. Christides, J. D. Crane, A. D. Darwish, *Pure Appl. Chem.* **1994**, *66*, 1389–1396.

[7] J. C. Barnes, E. J. Dale, A. Prokofjevs, A. Narayanan, I. C. Gibbs-Hall, M. Juríček, C. L. Stern, A. A. Sarjeant, Y. Y. Botros, S. I.

Stupp, et al., *J. Am. Chem. Soc.* **2015**, *137*, 2392–2399.

[8] T. Baati, F. Bourasset, N. Gharbi, L. Njim, M. Abderrabba, A. Kerkeni, H. Szwarc, F. Moussa, *Biomaterials* **2012**, *33*, 4936–46.

[9] M. Hurtgen, A. Debuigne, M. Hoebeke, C. Passirani, N. Lautram, A. Mouithys-Mickalad, P. H. Guelluy, C. Jérôme, C. Detrembleur, *Macromol. Biosci.* **2013**, *13*, 106–115.

[10] E. M. Hotze, J. Labille, P. Alvarez, M. R. Wiesner, *Environ. Sci. Technol.* **2008**, *42*, 4175–80.

[11] D. Heymann, *Lunar Planet. Sci.* **1996**, *27*, 543–544.

[12] J. Kolosnjaj, H. Szwarc, F. Moussa, *Adv. Exp. Med. Biol.* **2007**, *620*, 168–180.

[13] P. Rajagopalan, F. Wudl, R. F. Schinazi, F. D. Boudinot, *Antimicrob. Agents Chemother.* **1996**, *40*, 2262–2265.

[14] T. B. Henry, E. J. Petersen, R. N. Compton, *Curr. Opin. Biotechnol.* **2011**, *22*, 533–7.

[15] D. Y. Lyon, L. K. Adams, J. C. Falkner, P. J. J. Alvarezt, *Environ. Sci. Technol.* **2006**, *40*, 4360–6.

[16] C. He, Y. Hu, L. Yin, C. Tang, C. Yin, *Biomaterials* **2010**, *31*, 3657–3666.

[17] R. Mejia-Ariza, L. Graña-Suárez, W. Verboom, J. Huskens, R. Kojima, D. Aubel, M. Fussenegger, E. Salvati, F. Stellacci, S. Krol, et al., *J. Mater. Chem. B* **2017**, *5*, 36–52.

[18] S. Tan, K. Ladewig, Q. Fu, A. Blencowe, G. G. Qiao, *Macromol. Rapid Commun.* **2014**, *35*, 1166–84.

[19] Q. Hu, G. Tang, P. K. Chu, *Acc. Chem. Res.* **2014**, *47*, 2017–2025.

[20] Z. Yoshida, H. Takekuma, S. Takekuma, Y. Matsubara, *Angew. Chemie Int. Ed. English* **1994**, *33*, 1597–1599.

[21] J. Tong, M. C. Zimmerman, S. Li, X. Yi, R. Luxenhofer, R. Jordan, A. V. Kabanov, *Biomaterials* **2011**, *32*, 3654–3665.

[22] C. N. Murthy, K. E. Geckeler, *Chem. Commun.* **2001**, *60*, 1194–1195.

[23] T. Anderson, K. Nilsson, M. Sunda, G. Westman, O. Wennerstrom, *J. Chem. Soc. Chem. Commun.* **1992**, 604–606.

[24] K. Komatsu, K. Fujiwara, Y. Murata, T. Braun, *J. Chem. Soc.* **1999**, 2963–2966.

[25] D. Iohara, F. Hirayama, K. Higashi, K. Yamamoto, K. Uekama, *Mol. Pharm.* **2011**, *8*, 1276–84.

[26] Y. He, P. Fu, X. Shen, H. Gao, *Micron* **2008**, *39*, 495–516.

[27] J. Rejman, V. Oberle, I. S. Zuhorn, D. Hoekstra, *Biochem. J.* **2004**, *377*, 159–69.

[28] M. Messner, S. V. Kurkov, P. Jansook, T. Loftsson, *Int. J. Pharm.* **2010**, *387*, 199–208.

[29] T. Loftsson, M. Másson, M. E. Brewster, *J. Pharm. Sci.* **2004**, *93*, 1091–1099.

[30] A. R. Khan, P. Forgo, K. J. Stine, V. T. D'Souza, *Chem. Rev.* **1998**, *98*, 1977–1996.

[31] J. Szejtli, *Chem. Rev.* **1998**, *98*, 1743–1753.

[32] O. Sedlacek, B. D. Monnery, S. K. Filippov, R. Hoogenboom, M. Hruby, *Macromol. Rapid Commun.* **2012**, *33*, 1648–62.

[33] M. W. M. Fijten, C. Haensch, B. M. van Lankvelt, R. Hoogenboom, U. S. Schubert, *Macromol. Chem. Phys.* **2008**, *209*, 1887–1895.

[34] R. Chapman, P. J. M. Bouten, R. Hoogenboom, K. a Jolliffe, S. Perrier, *Chem. Commun.* **2013**, *49*, 6522–6524.

[35] M. J. Isaacman, K. A. Barron, L. S. Theogarajan, *J. Polym. Sci. Part A Polym. Chem.* **2012**, *50*, 2319–2329.

[36] B. Verbraeken, B. D. Monnery, K. Lava, R. Hoogenboom, *Eur. Polym. J.* **2016**, DOI 10.1016/j.eurpolymj.2016.11.016.

[37] F. Wiesbrock, R. Hoogenboom, M. Leenen, S. F. G. M. Van Nispen, M. Van Der Loop, C. H. Abeln, A. M. J. Van Den Berg, U. S. Schubert, *Macromolecules* **2005**, *38*, 7957–7966.

[38] T. X. Viegas, M. D. Bentley, J. M. Harris, Z. Fang, K. Yoon, B. Dizman, R. Weimer, A. Mero, G. Pasut, F. M. Veronese, *Bioconjug. Chem.* **2011**, *22*, 976–986.

[39] T. Braun, A. Buv, L. Barcza, I. Konkoly-thege, M. Fodor, B. Migali, *Solid State Ionics* **1994**, *74*, 47–51.

[40] Z. Hu, Y. Huang, C. Zhang, L. Liu, J. Li, Y. Wang, *J. Mater. Chem. B* **2014**, *2*, 8587–8597.

[41] S.-E. Zhu, F. Li, G.-W. Wang, *Chem. Soc. Rev.* **2013**, *42*, 7535–70.

[42] P. T. Anastas, M. M. Kirchhoff, *Acc. Chem. Res.* **2002**, *35*, 686–694.

[43] K. Wieczorek-Ciurowa, K. Gamrat, *J. Therm. Anal. Calorim.* **2007**, *88*, 213–217.

[44] S. L. James, C. J. Adams, C. Bolm, D. Braga, P. Collier, T. Friščić, F. Grepioni, K. D. M. Harris, G. Hyett, W. Jones, et al., *Chem. Soc. Rev.* **2012**, *41*, 413–47.

[45] H. M. Wang, G. Wenz, *Beilstein J. Org. Chem.* **2012**, *8*, 1644–1651.

[46] A. Altaf, H. Aldawsari, Z. M. Banjar, D. Iohara, M. Anraku, K. Uekama, F. Hirayama, **2014**, *2014*.

[47] D. Iohara, F. Hirayama, H. Kansui, H. Aoshima, S. Yamana, M. Yano, J. Kitaguchi, S. Takashima, K. Uekama, *Chem. Lett.* **2009**, *38*, 1104–1105.

[48] L. Graña Suárez, W. Verboom, J. Huskens, *Chem. Commun.* **2014**, *50*, 7280–7282.

[49] Y.-Z. Zhuang, W.-X. Gu, J.-J. Yang, X. Chen, H. Gao, *RSC Adv.* **2015**, *5*, 96464–96471.

[50] J. Stadermann, H. Komber, M. Erber, D. Frank, H. Ritter, B. Voit, *Macromolecules* **2011**, 3250–3259.

[51] P. R. Ashton, R. Koniger, J. F. Stoddart, D. Alker, V. D. Harding, *J. Org. Chem.* **1996**, *61*, 903–908.

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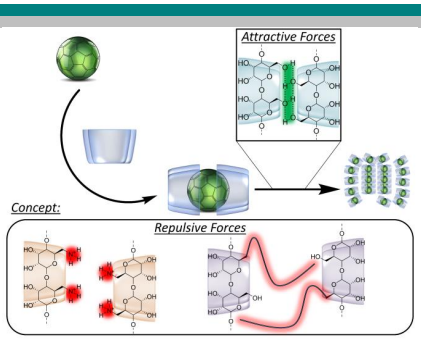
FULL PAPER

Overcoming attraction: A well-known problem of cyclodextrin inclusion complexes is their aggregation. In this study we attempt to overcome the attractive intermolecular forces between the complexes, by designing custom γ-cyclodextrin(γCD)-based supramolecular hosts for C_{60} to inhibit the aggregation found in native-γCD:C_{60} complexes, *via* Coulombic repulsion or steric hindrance. This led to partial inhibition of the aggregation, yielding stable sub-100 nm nanostructures.



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Mechanochemical preparation of stable sub-100 nm γ-cyclodextrin:Buckminsterfullerene (C60) nanoparticles by electrostatic or steric stabilization