Probing specific protein recognition by size-controlled glycosylated cyclodextrin nanoassemblies

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The balance between hydrophobic and hydrophilic components in amphiphilic β-cyclodextrins, targeted by receptor specific groups (SC6CDGlc, SC6CDGal, SC16CDGlc, SC16CDGal), sensitively influences the structural properties of these systems. The different amphiphilic features of single cyclodextrins generate micellar aggregates and vesicles with an internal aqueous compartment able to encapsulate guests, such as rhodamine 6G. Small-angle light scattering (SAXS), cryo-TEM and AFM investigations describe the size and shape of these self-organized glycoligands. Recognition of the nanoassemblies by a specific receptor has effectively been demonstrated by means of time resolved fluorescence and is addressed in water by the morphological properties of cyclodextrin aggregates. Exclusively galactosylated thiohexyl-cyclodextrin binds specifically lectin from *Pseudomonas aeruginosa*. β-D-Galactose competes with galactosylated cyclodextrin aggregates by inhibiting lectin binding but does not affect the mesoscopic environment of the protein. The better selectivity of the less hydrophobic cyclodextrins towards lectin should probably be ascribed to the morphology (size and shape) of these cyclodextrin aggregates. The recognition properties of this particular cyclodextrin (SC6CDGal) are probably due to the presence of small micelles which interact more efficiently with the lectin binding site. The modulation of the hydrophobic–hydrophilic balance of the macrocycle labelled with targeting groups allows the design of "active" nanosized carriers for drug delivery.

Introduction

Specific recognition of cell targeting systems (*e.g.* host carriers, self-aggregated species) is a major ambition in the application of supramolecular chemistry to medicine and life sciences. Synthetic glycoligands, which possess multivalent carbohydrates as binding sites, could act as selective vehicles for extracellular matrices and cell surface receptors. The specificity of the carbohydrate–protein interaction is essential in a wide variety of processes including cell–cell communication, differentiation, cell adhesion, inflammation, fertilization, immune defense, and metastasis.[1] In most biological events hierarchical aggregation of nano and mesoscopic colloidal species is a common process and concerns macromolecular association leading to *e.g.* viruses and cellular components up to cells and organs. In line with this concept various strategies have been adopted, *e.g.* self-assembling glycoclusters from amphiphiles, the "so called" glycoviruses,[2] anchoring glycolipids and neoglycoproteins in liposome membranes,[3] or constructing glycopolymers,[4] glycodendrimers[5] and glycocalixarenes.[6,7] One of the main challenges is to create self-optimizing cooperative systems[8] with a controlled number of components, shape, and size, able to target the complexed drug to its biological sites of action. Promising applications include the use of synthetic saccharide ligands with a high affinity for toxins,[7] viruses[4b] and pathogenic bacteria[9] which could reduce their binding activities with host cells. Also polyvalent systems based on gold nanoclusters functionalized with self-assembled monolayers of biologically significant oligosaccharides are powerful models with which to explore carbohydrate–carbohydrate interactions, which is an emerging mechanism for the comprehension of cell adhesion and recognition.[10]

Cyclodextrins (CDs) modified with multivalent mono- and oligosaccharides have been proposed as model systems to probe binding events involving receptors (lectins) and both a single or different types of sugar epitope expressed on heterogeneous cell surfaces.[11–13] The enhanced binding activity of these multi-glycosylated systems exploits the so called "cluster glycoside effect".[4,14]

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To date CDs have been used to increase the bioavailability of several drugs, even if in some cases they are not adequate for prolonged transport *in vivo*. Indeed the hosting ability of CDs, properly modified with receptor targeting groups, is of little practical significance *in vivo* since the host–drug binding constants are too small to enable actual transport of the drug. Recently it was demonstrated that specially designed amphiphilic β-CDs are capable of forming micellar aggregates[15] or vesicles[16,17] as potentially less immunogenic (due to their oligo-ethylene oxide exterior)[18] and more suitable drug encapsulators than a single CD molecule.[17,19] Differently from polymeric micelles based on reactive copolymers,[20] polymers[4b,c] and dendrimers,[5] these amphiphilic CDs display a relatively low but definite number of hydroxyl groups (seven terminal OH groups on the oligo-ethylene glycol chains) which are chemically versatile for easy access to new systems modified with receptor targeting groups.

In a preliminary investigation we reported the first evidence for multivalent effects of CD nanoaggregates modified with surface glycosyl groups in the binding with lectin proteins.[21] Analogous vesicles formed by amphiphilic cucurbit[6]uril noncovalentely modified with sugar[22] were prepared and their interactions with lectins were investigated. Syntheses of amphiphilic CDs substituted with one and seven bio-recognizable sugar residues on the primary face have been also reported.[23,24]

To date, biochemical, spectroscopic and thermodynamic studies have been addressed to analyze the binding affinity of saccharides to several lectins.[25] Lectins are known as useful probe/receptors for investigating the difference in carbohydrate expression of tumor cells and tissues. PA-I lectin is a protein extracted at high levels from *Pseudomonas aeruginosa* (PA) bacterium. It colonises patients with chronic lung desease by adhesion to cell surfaces and producing cytotoxic virulence factors. This lectin is specific for galactose containing molecules while it does not bind with analogues bearing glucose.[25a]

Recently high-throughput methods were developed by lectin chips[26] or carbohydrate microarrays.[27] Most of these techniques, in spite of their accuracy, perturb the investigated systems by immobilizing and/or labeling one of the interacting molecules. The binding of fluorescent ligands to proteins has been determined also by fluorescence correlation spectroscopy.[28] Steady-state, fluorescence anisotropy and time resolved fluorescence are powerful tools for studying function and conformation of proteins in vesicular membrane systems[29a] or to evaluate interaction with nucleic acids[29b] and lectins.[29c] Also hydrophobic nanocolloids, or domains of peptides were covered with receptors were prepared for the study of reversible interaction and adhesion processes by light scattering techniques.[30] It is well-known in the literature that the specificity of galactose is very different from glucose in terms of carbohydrate–protein interaction.[31] Our efforts were recently addressed to study, by means of spectroscopic techniques, the aggregation in water solution of galactose-specific lectin with CD nanostructures.[32] Despite the increasing number of reports which describe nanoaggregates having sugar epitopes for targeted drug delivery,[33] to the best of our knowledge, a structural control of this specificity has still not been deeply investigated. In this paper, we demonstrate how the morphological properties of CD host nanoassemblies, described through microscopy (cryo-TEM and AFM) and small-angle X-ray scattering (SAXS), can control the recognition of a receptor model (lectin from *Pseudomonas aeruginosa* (PA-I)) in aqueous solutions. These properties clearly depend on the molecular architecture of the individual constituent molecule (hydrophobic–hydrophilic balance of the single CD), and their fine tuning allows the design of "active" nanostructures able to encapsulate guests for targeted delivery. Competition experiments by inhibiting the lectin binding cleft with galactose molecules prove the high specificity of the galactosylated CD nanostructures.

Results and discussion

Controlling morphology of nanoassemblies

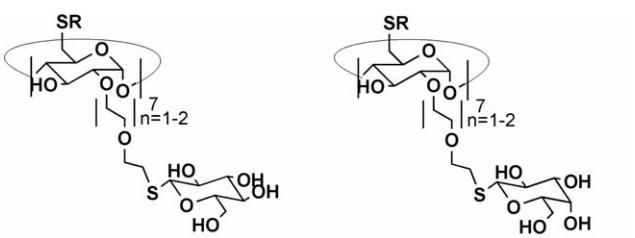
Glycosylated CDs (SC6CDGlc, SC16CDGlc, SC6CDGal and SC16CDGal, Chart 1) were synthesized according to the reported procedure.[21] The less hydrophobic thiohexyl-derivatives exhibit in water a low critical micellar concentration (8.1 μM), while the thiohexadecyl CDs form aggregates, even at the lowest examined concentration (1 μM).

CD nanoaggregate solutions were sonicated[34a] and characterized by means of cryo-TEM microscopy.

In the case of SC6CDGal, the images evidence aggregates with a diameter of 500–600 nm (Fig. 1A) composed of small nanoparticles of 20–30 nm (magnification in Fig. 1C). Also the presence of regions with a high density of bigger nanoparticles was detected (Fig. 1D).[34b] The morphologies of these amphiphiles resemble those of the unglycosylated precursors.[15b]

Light and small-angle X-ray scattering measurements indicate that glycosylated thiohexyl-derivatives can form in water both micellar aggregates[32] (in line with microscopy data) and micelles with a very small dimension ($R = 4 \pm 0.5$ nm) as detected by a SAXS intensity profile (Fig. 2) in agreement with the results reported for the SC6CDOH precursor.[15c]

Differently from the thiohexyl derivatives, it was possible to distinguish vesicles for SC16CDGal with a diameter of 100–200 nm (Fig. 1B), which become smaller upon prolonged sonication.[34a] The ability of the SC16CDOH precursor to form vesicles was preserved in the glycosylated derivatives.[16a,17b,34c] The vesicles possess a thin shell of 2.5–3.5 nm as shown in the image with increased magnification (Fig. 1E), suggesting different types of interdigitated bilayer membranes of glycosylated CD.[17b] The structure of these vesicles is



SC6CDGlc: R=C_6H_{13}
SC16CDGlc: R=$C_{16}H_{33}$

SC6CDGal: R=C_6H_{13}
SC16CDGal: R=$C_{16}H_{33}$

Chart 1

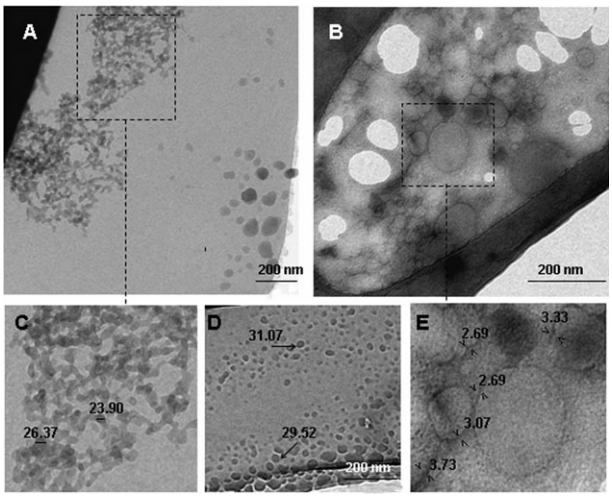


Fig. 1 Cryogenic transmission electron micrography: (A) micellar clusters of SC6CDGal; (B) vesicles of SC16CDGal; (C) magnification of A showing details of a micellar cluster (aggregates of micelles); (D) another topographic region of micellar aggregates of SC6CDGal; (E) magnification of B showing details of vesicles.

reminiscent of that of conventional liposomes[34a] which can be prepared in a similar manner (a decrease of particle size with sonication time has been proved in the present system, in line with other conventional phospholipid liposomes).

Still, AFM images obtained on areas 5 × 5 μm across revealed a smooth surface with high superficial density and the absence of microscopic inclusions generated by external impurities.

The images carried out on cast and dried glycosylated CDs displayed a less structured film of thiohexyl-derivatives (not shown), and vesicular aggregates of thiohexadecyl-derivatives with spherical and elongated shapes (Fig. 3), similar to immobilized lipid vesicles.[35] Nanoaggregates of different sizes have been found on the samples. In particular the topographic image in Fig. 3A shows structures with a medium roughness of 3.3 nm and an average dimension of approximately 200 nm.

The above mentioned results confirm that the aggregation behaviour of the investigated CDs is controlled by the balance

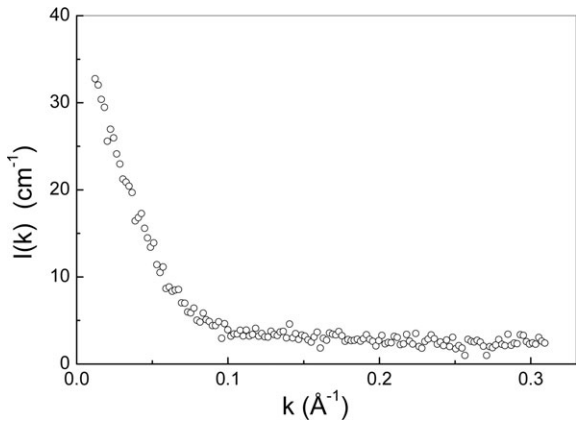


Fig. 2 X-Ray scattered intensity profile of SC6CDGal aqueous solution (9 mM).

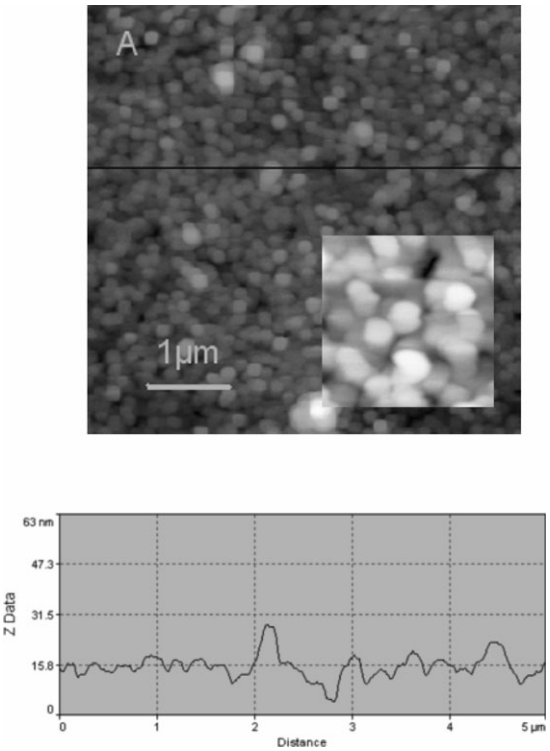


Fig. 3 AFM of SC16CDGal: (A) vesicular aggregates (sample cast from a solution of CD powder dissolved in MeOH, 200 μM); in the inset (1 × 1 μm) is reported a detail with higher resolution; (B) a line profile extracted along the marked black trace.

between hydrophilic ω-thioglycosyl-oligo(ethylene glycol) head groups and hydrophobic alkyl chains. In this respect this balance affects the exclusive formation of vesicles for thiohexyl CDs, while it drives the coexistence of micellar aggregates and small micelles, as evidenced by the combination of scattering and microscopy results. On the other hand the longer hydrophobic chains in thiohexadecyl conjugates favour mainly the formation of vesicles.

Guest encapsulation

The presence of an aqueous compartment both in micellar aggregates and in vesicles of glycosylated CDs was evidenced by encapsulation of the fluorescent dye Rhodamine 6G. The dye concentration was kept high enough to ensure self-quenching of the fluorescence. CD colloids were prepared in a solution of Rhodamine 6G, and the free dye was removed by gel filtration chromatography. As shown in Fig. 4, coincidence of encapsulated dye with the elution of nanoaggregates of SC6CDGal and SC16CDGal is in line with the carrier properties of these nanostructured systems. The elution volumes are in agreement with those previously reported for similar amphiphilic systems:[16,17b] vesicles of thiohexadecyl CDs < aggregates of smaller thiohexyl derivatives < free rhodamine.

Disruption of the nanoaggregates by adding Triton X-100 leads to an increase in fluorescence emission (data not shown), indicating relief of self-quenching upon release and dilution of the dye into the bulk solution. These results pointed out the encapsulation of the guest both in vesicles of SC16CDGal and

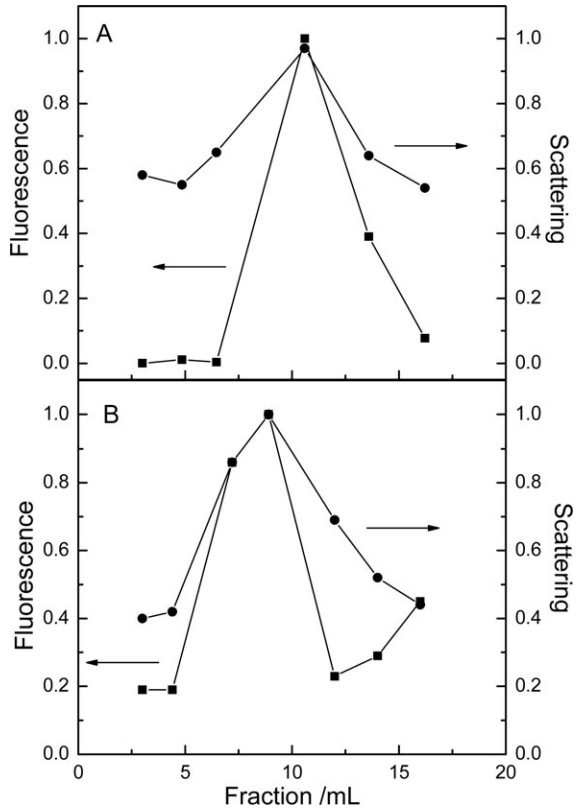


Fig. 4 Encapsulation of Rhodamine 6G in micellar aggregates of SC6CDGal (A) and in vesicles of SC16CDGal (B): elution profile on a G-25 Sephadex column. (●) Normalized light scattering at 400 nm. (■) Normalized fluorescence intensity of Rhodamine 6G at 550 nm.

in micellar aggregates of SC6CDGal which, similarly to other micellar systems,[36] could contain in their interior up to 20% v/v of water.

Galactose and galactosylated CD nanoaggregates competitive binding to a receptor protein

Interaction between PA-I lectin and SC6CDGal aggregates is evident from time-resolved fluorescence experiments. This technique is very sensitive for evaluating subtle changes in the chemical environment of protein fluorophores.[37,38] The time-resolved fluorescence decays for all the investigated systems are constituted by two relaxation times (see Table 1), tentatively assigned to the emission of tryptophan residues. Fig. 5 shows an example of fluorescence decays of lectin and self assembled lectin/CD systems at 1 : 16 molar ratio.

The longer lifetime (τ_2) remains constant and equal to that of free lectin (3.1 ns) in the case of self-assembled PA-I/SC6CDGlc system for any investigated concentration value, whereas it decreases on increasing SC6CDGal.

Also the short-living fluorescent species of lectin exhibit different lifetimes and different energy percentages in the presence of SC6CDGal (τ_1 ~0.8 ns, 56%). However, because of the different changes in the energy percentage of the two components, the mean fluorescence lifetime τ_{av} should be better considered, and as shown in Table 1, its trend reproduces that of single components.

Table 1 Fluorescence lifetimes (τ_1, τ_2, τ_{av})[a] and relative energy percentages (A_1 and A_2) of PA-I and PA-I/CD: competitive evaluation in presence of β-D-Galactose (Gal) at different PA-I/CD/Gal molar ratios

Sample	τ_1/ns	τ_2/ns	A_1 (%)	A_2 (%)	τ_{av}/ns
PA-I[b]	0.4	3.10	19	81	2.6
PA-I/SC6CDGlc[c]	0.4	3.15	33	67	2.2
PA-I/SC6CDGal[c]	0.8	2.60	56	44	1.6
PA-I/SC16CDGlc[c]	0.4	2.90	23	77	2.3
PA-I/SC16CDGal[c]	0.5	2.80	24	76	2.2
PA-I/Gal[d]	0.6	3.10	19	81	2.6
PA-I/SC6CDGal/Gal[e]	0.5	2.30	29	71	1.8
PA-I/SC6CDGal /Gal[f]	0.5	2.65	25	75	2.1
PA-I/SC6CDGal/Gal[g]	0.4	2.80	23	77	2.2

[a] The reproducibility of lifetimes is within ±0.05 ns and χ^2 is between 0.98 and 1.02; λ_{exc} = 290 nm, λ_{em} = 340 nm.[41] [b] [PA-I] = 10 μM. [c] PA-I/CD at 1 : 16. [d] PA-I/Gal at 1 : 112 [e] PA-I/CD/Gal at 1 : 16 : 56 (defect of Gal). [f] PA-I/CD/Gal at 1 : 16 : 112. [g] PA-I/CD/Gal at 1 : 16 : 120 (excess of Gal).

The selectivity of glycosylated CD systems with different hydrophobic chains (SC16CDGlc and SC16CDGal) was also tested, even if selective interactions with lectin were not ascertained. Fluorescence lifetimes of both PA-I/SC16CDGlc and PA-I/SC16CDGal systems (Fig. 5B) are not actually distinguishable one from another and are close to that of PA-I. The reasons for the lack of selectivity of SC16CDGal is not clear. However the vesicular form of SC16CDGal aggregates, evidenced by cryo-TEM images, could hinder the binding. This result is not comparable with a ligand CD–lectin interaction as investigated by surface plasmon resonance (SPR). In this latter case the binding response of CD nanostructures towards lectin could be influenced by immobilization of the protein on the gold surface.[21]

On the contrary a strong support for the specificity of SC6CDGal in aqueous solution towards the binding cleft of PA-I lectin came from competition experiments in the presence of β-D-galactose (Gal). Time resolved fluorescence measurements have been carried out adding SC6CDGal to PA-I/Gal solutions at different concentration of Gal. Mean fluorescence lifetime values of PA-I/Gal system are similar to that of free PA-I, and larger than in the ternary PA-I/ SC6CDGal/Gal systems (see Table 1). By increasing the amount of Gal (from a defect to an excess in molar concentration with respect to molar Gal content in SC6CDGal, respectively traces f, g, and h in Fig. 5C), τ_{av} tend to assume the same values as in the free lectin. This spectroscopic evidence indicates that the presence of Gal, by directly interacting with the protein binding site, inhibits the interaction with CD nanoaggregates.

The effectiveness of this class of amphiphilic CDs as drug delivery systems has already been demonstrated by studying the entanglement of porphyrins in cationic CD nanoassemblies, their intracellular localization, and cellular death upon irradiation.[19a,c] Also the effect of amphiphilic CDs on the phototoxicity of anti-inflammatory drugs was investigated.[19b] The cationic nanoaggregates have been proposed as molecular templates for the development of a new series of gene delivery vector.[19d] In this context the selectivity of the glycosylated carrier for a receptor-model such as PA-I lectin can be exploited in targeted drug delivery. In perspective the

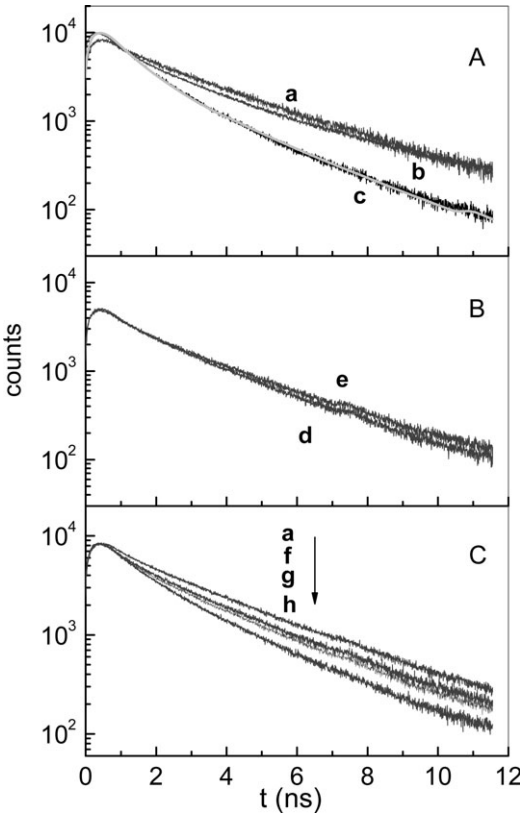


Fig. 5 Time-resolved fluorescence decays (at 340 nm) of PA-I/CD systems at 1 : 16 molar ratio, [PA-I] = 10 μM: (A) PA-I, PA-I/SC6CDGlc and PA-I/SC6CDGal (traces a, b, and c, respectively); (B) PA-I/SC16CDGal, PA-I/SC16CDGlc (traces d and e, respectively); (C) PA-I, PA-I/SC6CDGal/Gal (1 : 16 : 120, excess of Gal), PA-I/SC6CDGal/Gal (1 : 16 : 112), PA-I/SC6CDGal/Gal (1 : 16 : 56, defect of Gal) (traces a, f, g and h respectively). The continuous line for curve c is an example of the fit according to a two exponential decay model (λ_{exc} = 290 nm).

specificity of self-assembled CD carriers could be further increased by combining the drug-internalization efficacy of cationic amphiphilic CDs and the properties of glycosylated CDs, in terms of modulation of the morphological features, and functionalization with targeting groups. These galactosylated mixed systems could find applications in the selective recognition of cell-receptors overexpressed in different disease (*i.e.* proteins on hepatocyte cell surfaces) and drug internalization. These findings are under observation in our laboratory and will be the object of further reports.

Conclusion

This study has provided the first example of recognition addressed by morphological features of CD nanostructures. The aggregation properties of glycosylated amphiphilic CDs with different molecular architectures depend on the hydrophobic–hydrophilic balance (length of alkyl chains). The CDs bearing shorter hydrophobic tails tend to form micellar aggregates. On increasing the hydrophobic character, modified glycosylated CDs lead to vesicles. These nanoaggregates are able to encapsulate fluorescent guests. More significantly, their recognition capability *versus* PA-I lectin was investigated in aqueous solutions by means of time resolved fluorescence which does not perturb the investigated self-assembled system. The rearrangement of peculiar lifetimes points to the specificity of this particular CD towards the protein. The recognition of lectin by CDs is driven by their morphology and the specificity can be probably ascribed to the presence of micellar aggregates able to disassemble and interact more efficiently with the lectin binding site.

This property together with the encapsulation of guest molecules opens the way to the potential design of cell targeting carriers for drug delivery.

Experimental

Materials

β-Cyclodextrin (Wacker) was crystallized from distilled water and dried under vacuum (0.1 mmHg, 80 °C) for 4 hours. Amphiphilic glycosylated CDs (heptakis{2,6-dideoxy-2-[S-ω-1-D-glycopyranosyl-ω-thio-oligo(ethylene oxide)]-6-alkylthio}-β-CD; glycopyranosyl = β-D-galactose, alkyl = hexyl SC6CDGal; glycopyranosyl = β-D-glucose, alkyl = hexyl SC6CDGlc; glycopyranosyl = β-D-galactose, alkyl = hexadecyl SC16CDGal; glycopyranosyl = β-D-glucose, alkyl = hexadecyl SC16CDGlc) were synthesized according to the reported procedure.[21]

PA-I lectin lyophilized powder purchased from Aldrich contains $CaCl_2$ (about 2%) and other salts and was purified according to the Gilboa-Garber method.[31b] The solvents used were purified and dried by standard techniques. All the other reagents were of the highest commercial grade available and were used as received or were purified by distillation or recrystallization when necessary.

Sample preparation

CD colloids were prepared through conventional experimental procedures.[32] CD stock solutions (180 μM) in $CHCl_3$ were slowly evaporated overnight to form a thin film. Films of the thiohexyl derivatives were easily dissolved by hydration and slight sonication (20 min at 50 °C), whereas those of the thiohexadecyl conjugates were sonicated for 30 min (3 cycles of 10 min and after each cycle were cooled at 4 °C). All the diluted solutions were prepared in pure microfiltered water and were not filtered differently from the described procedure.[16] CD nanoaggregates were investigated by Cryo-TEM and, after casting on the surface, by AFM.

Micellar aggregates of SC6CDGal (1 mM) and vesicles of SC16CDGal (1 mM) were prepared in water solutions of Rhodamine 6G (5 mM, pH 7). Encapsulated Rhodamine 6G was separated from the free dye by gel filtration on a 25 × 1.5 cm column (Sephadex G-25). Addition of Triton X-100 (0.1%) was carried out on the eluted vesicles (7–8 mL) and micellar aggregates (10–11 mL). Fluorescence and light scattering spectra (at 400 nm) of the solutions eluted from the column before and after Triton X additions were registered on a Jasco model FP-750 spectrofluorimeter by using a synchronous scan protocol for scattering techniques.

PA-I lectin stock solutions (80 μM) were prepared in water, stored at 4 °C overnight and equilibrated at room temperature.[42] Different aqueous colloidal solutions containing respectively SC6CDGal, SC6CDGlc, SC16CDGal and SC16CDGlc were added to an aqueous solution of PA-I lectin. The lectin/CD solutions were stirred and equilibrated for 10 min. The self assembled systems were studied at 1 : 16 lectin : CD molar ratios ([PA-I] = 10 μM, pH 7). β-D-Galactose was used as competitor molecule and was added to the PA-I solutions. Afterwards a colloidal solution of SC6CDGal was injected in to three sets of PA-I lectin/Gal solution. The ternary system PA-I/SC6CDGal/ Gal was investigated at 1 : 16 : 56, 1 : 16 : 112, and 1 : 16 : 120 molar ratios, respectively.

PA-I lectin and self assembled PA-I/CD samples were investigated at $T = 298 \pm 0.01$ K by fluorescence spectroscopy (steady-state and time resolved). To check the reproducibility, all spectroscopic titrations were run at least five times. The time resolved fluorescence experiments evidence a good reproducibility for the same protein stock.

Measurements

Cryogenic transmission electron microscopy. Solutions of galactosylated CDs (160 μM, 5 μl) were placed on a holey carbon film grid and vitrified by rapid immersion in liquid ethane (EM CPC Leica). The grid was insert into a cryo-holder by a cryo-transfer (Gatan). All devices were kept at −170 °C by means of liquid N_2. TEM observations were carried out on a Leo 912ab (Zeiss) under low-electron dose irradiation by focusing in an adjacent area to the selected specimen area at 120 KV by inserting the Omega filter.

Small angle X-ray scattering. The SAXS patterns have been recorded by laboratory instrumentation consisting of a Philips PW X-ray generator (providing Cu-Kα, Ni-filtered X-ray radiation of wavelength = 1.5418 Å) with a Kratky-type small angle camera in the "finite slit height geometry" equipped with step scanning motor and scintillator counter as detector. The range of scattering vector covered is $0.005\ \text{Å}^{-1} < k < 0.6\ \text{Å}^{-1}$. All measurements were carried out at a temperature of T = 25 °C. The scattering data were normalized with respect to transmission, and were corrected by the empty cell and solvent contribution. The data were fitted according to the Ornstein–Zernike relation:

$$|I(k)|^{-1} \propto 1 + \xi^2 k^2 \qquad (1)$$

The obtained correlation length ξ is about 25 Å. Size (R) was obtained as $\sqrt{3}\xi$.

Atomic force microscopy. Solutions of SC6CDGal and SC16CDGal were both prepared by dissolving CD powder in MeOH (200 μM) according to the conventional procedure. Silica substrates used in the experiments were cleaned in piranha solution (70% concentrated ammonia solution and 30% hydrogen peroxide) for 15 min and rinsed with large amounts of high-purity water, ethanol and subsequently dried. CD solutions (5 μL) were cast onto substrates, evaporated overnight and analysed by AFM. The AFM measurements were performed in air at room temperature using a commercial setup based on a Veeco Instruments Explorer scanning probe microscope in contact mode. We have found practically artifact-free images by using AFM probes made by Veeco Inst. with a length of approximately 3 μm and a nominal tip radius of 30 nm.

Time-resolved fluorescence. A pulsed mode-locked Ar^+ laser (λ = 514 nm) is used as excitation source for the fluorescence lifetime measurements, with a repetition rate of 82 MHz and pulse duration of 150 ps. A synchronous dye (Rhodamine 6 G) laser converts the incoming beam to $\lambda \sim 580$ nm and to a pulse duration of 2 ps. In order to excite protein molecules at ~290 nm, a BBO duplicator is inserted in the laser path; before impinging on the sample, the beam passes through a Glan–Taylor polarizer prism to eliminate the partial spurious depolarization from the mirrors and then is focused. The power of the resulting UV laser beam on the sample is about 20 mW.

The experimental data were obtained by time-correlated single-photon counting (TCSPC). The fluorescence emission is collected at 90°, through a monochromator, by a "microchannel plate" photomultiplier (MCP Hamamatsu R1645U-01) having a response of about 200 ps. The signal, properly discriminated (constant fraction), is then correlated with the excitation pulse by means of a TAC (EG & G Ortec 567); the measured delay times are analysed by a multi-channel (EG & G Ortec Trump-8K/2K) which furnishes the fluorescence decay curve. The total fluorescence intensity, from a general point of view, can be constituted by a multiexponential decay:[37]

$$I(t) = I_0 \sum_i A_i \exp(-t/\tau_i) \qquad (2)$$

where I_0 is the intensity at t = 0,

A_i (under the condition that $\sum_i A_i = 1$) the relative amplitude of the i-th component and τ_i the correspondent lifetime. The energy contribution of each component is

$$E_i = \frac{A_i \tau_i}{\sum_i A_i \tau_i}. \qquad (3)$$

The measured fluorescence decays, therefore, are fitted according to eqn (3) and using the nonlinear least-squares iterative reconvolution procedures based on the Marquardt algorithm.[43] The goodness of fit was assessed by using the plots of weighted residuals, reduced χ^2 values.

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