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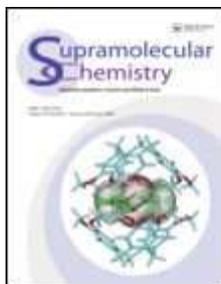
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CONFORMATIONALLY MOBILE GLUCOSYLTHIOUREIDO- CALIX[6]- AND CALIX[8]ARENES: SYNTHESIS, AGGREGATION AND LECTIN BINDING

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Abstract

Two new glucoclusters **2a** and **2b**, in which the sugar units are connected to the *upper rim* of methoxycalix[6]- and calix[8]arene derivatives *via* thiourea linkages, were synthesized and their aggregation properties in water studied by ¹H NMR, Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS). Small size aggregates (2-10 nm diameter) are formed by both macrocycles, which become much larger (200-300 nm) in the presence of a phosphate buffer, whereas other anions (Cl⁻, NO₃⁻, SO₄⁻) have no effect.

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4 The glycoclusters **2a** and **2b** interact with plasmid DNA but do not condense it, while in
5
6 the presence of a glucose specific lectin such as Concanavalin A (ConA) agglutination
7
8 occurs. The data obtained give useful insight into the mode of binding of calixarene-
9
10 based glycoclusters with lectins.
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16 **Keywords** glyco-calixarenes, multivalent systems, lectins, AFM, self-aggregation
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20
21 *Dedicated to David N. Reinhoudt for his outstanding contributions to Supramolecular*
22
23 *Chemistry and Nanotechnology*
24
25

26 27 28 **Introduction**

29
30 The fundamental role played by carbohydrates in many biological processes [1-8] and
31
32 the phenomenon of multivalency [9,10] frequently associated with the recognition of
33
34 these substrates have boosted the design and synthesis of a wide range of
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36 polyglycosylated compounds [11-17]. The major aim is to clarify the principles [10,18]
37
38 of the so-called glycoside cluster effect [19] and find new bioactive molecules for
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40 diagnostic [20] and therapy [21-23] based on the saccharide recognition.
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44 Since several years we have been engaged in the synthesis of calixarene-based
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46 multivalent glycoclusters [24] and proved their ability of binding to specific lectins [25]
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48 and toxins [26]. Although the multivalent presentation of the saccharide units seems to
49
50 play a positive role in the recognition process, the real mode of binding of these
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52 derivatives has not been clarified yet. Recent reports on calixarene glycoclusters having
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54 long alkyl chains at the *lower rim* have shown [27,28] that they can self-aggregate in
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4 water giving small micelle-like nanoparticles in which the real valency at work could be
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6 much higher compared to that of the monomeric species.
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9 We present here the synthesis and supramolecular properties of two new, water soluble
10 glycolcalixarenes having different size and characterised by the presence of glucose at
11 the *upper rim* and methyl groups at the *lower rim*. Among the neutral monosaccharides
12 glucose was chosen because of its high solubility in water and also for its biological
13 relevance in the perspective of using these molecules as selective molecular delivery
14 systems. Many glucose transporters are in fact present at the blood brain barrier which
15 are considered as possible targets for increasing brain access to drug molecules [29].
16 The thiourea unit, frequently used by us to link the carbohydrate unit to the calixarene
17 platform, can also act as hydrogen bonding donor group and influence the
18 supramolecular properties of the glycoclusters.
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35 **Results and discussion**

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37 Using the well established procedure involving amine and isothiocyanate units to form
38 thiourea groups, the condensation between the proper aminocalix[n]arenes [30] and
39 tetraacetyl- β -L-glucosylisothiocyanate (Scheme 1) afforded the protected
40 glucocalixarenes **1a** and **1b**, whose acetyl groups were subsequently removed by the
41 Zemplen method (NaOMe in methanol).
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52 [Insert Scheme 1]
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56 The ^1H NMR spectra of the protected compounds **1a** and **1b** in CDCl_3 , where hydrogen
57 bonding is possible, are characterised by broad signals which indicate a restricted
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4 mobility of the phenolic units and a slow exchange between the isomers of the thiourea
5 units typical for the disubstituted thioureas, including thioureidoglycosides [25]. In
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7 more polar solvents like CD₃OD and DMSO-*d*₆, which break hydrogen bonding, the
8
9 signals are fairly sharp and become even sharper by increasing the temperature, as
10
11 shown in Fig. 1 for the octamer **1b**.
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16 [Insert Figure 1]
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21 The deprotected glucoclusters **2a** and **2b** resulted soluble in water up to 5×10⁻³ M by
22
23 sonication, but the solutions of both become rapidly heterogeneous and a significant
24
25 precipitation of a white solid occurs after few minutes. More stable solutions are
26
27 obtained at a concentration of 10⁻⁴ and 10⁻³ M for the calix[6]- and calix[8]arene
28
29 derivatives, respectively. Also the ¹H NMR spectra of aqueous solutions of both
30
31 calixarenes show quite broad signals at room temperature (Fig. 2a) which are indeed
32
33 sharper at 363K (Fig. 2b), thus suggesting the formation of self-assembled aggregates in
34
35 equilibrium with the monomeric macrocycle.
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42 [Insert Figure 2]
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45 To shed more light on this phenomenon, we performed Atomic Force Microscopy
46
47 (AFM) experiments in tapping mode on freshly cleaved mica surfaces with 5×10⁻⁴ M
48
49 water solutions of the two glucoclusters. The results confirmed the tendency for the
50
51 calix[6]- **2a** and calix[8]arene **2b** to self-assemble (Fig. 3a) in small discoid-like
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53 particles (10-15 nm diameter). Most probably these have a spherical micelle-like shape
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55 in solution and then collapse upon deposition when the imaging in air is performed. For
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4 both compounds it is possible to observe some of these aggregates by dilution till to a
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7 10^{-6} M concentration.

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9 [Insert Figure 3]

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11 Dynamic Light Scattering (DLS) experiments on the same 5×10^{-4} M solution of **2b**
12 support the presence of small sized aggregates (3-5 nm diameter), [31] which do not
13
14 grow in the monitored time range of 24 h (Fig. 3b). The tendency of **2a** and **2b** to
15
16 aggregate in water was rather surprising to us, because the two macrocycles have short
17
18 methyl chains at the *lower rim* and should be characterised by sufficient conformational
19
20 freedom to prevent a sharp distinction between the lipophilic (methylated aromatic
21
22 nuclei) and the hydrophobic (sugar moieties) region in the molecule. Previously, we
23
24 actually observed that the tetrapropoxy-tetraglucosylthioureidocalix[4]arene gives self-
25
26 association in water solution, but it is fixed in the *cone* conformation, [25] and Micali *et*
27
28 *al* reported [28] on aggregation phenomena of octapropoxycalix[8]arene analogues
29
30 functionalised with *N*-acetylglucosylthioureido units. In both these examples the longer
31
32 propyl alkyl chains at the *lower rim* enhance the lipophilicity of the macrocycles. ESI-
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34 MS studies previously showed [25] that *cone* tetrapropoxy-glycocalixarenes are able to
35
36 bind anionic species, with some preference for phosphate/phosphonate containing
37
38 guests. Unfortunately, when we used the two calix[6]- and calix[8]arene derivatives **2a**
39
40 and **2b** in analogous, host-guest recognition MS experiments, no signal relative to any
41
42 host-anion complex was found in the spectra. This was probably due to the high cone
43
44 voltages necessary to detect the corresponding molecular peaks of these large
45
46 macrocycles. However, the ability of **2a** and **2b** to interact with phosphate anions is
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48 indicated by their behaviour in a phosphate buffer. In fact, in phosphate buffer
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50 solutions, the AFM images relative to octamer **2b** and hexamer **2a** show the presence of
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4 aggregates which progressively and rapidly increase their size (Fig. 4). For example, in
5
6 the case of **2b**, the aggregates appear larger (Fig. 4a) than in pure water after few
7
8 minutes, then become *ca* 100 nm sized after 90 min (Fig. 4b), and reach a diameter of
9
10 200-300 nm in 24 h, with the simultaneous disappearance of the smaller aggregates
11
12 (Fig. 4c). Higher concentrations of buffer and calixarene seem to speed up the rate of
13
14 formation of these larger aggregates (Fig. 4d) whose solubility is evidently ensured by
15
16 the presence of the charged phosphate anions included within the micelles. The particle
17
18 stability, however, does not appear very high since sonication or simple stirring of the
19
20 solution determine their dissociation in several small aggregates (Fig. 4e). A completely
21
22 similar behaviour is observed in the case of **2a** (*e.g. see* Fig. 4f). Upon standing for days
23
24 a slow precipitation for both glucocalixarenes is observed also in these conditions.
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33 [Insert Figure 4]
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37 DLS experiments (Fig. 5) performed on a 5×10^{-4} M solution of **2b** in 0.5 M phosphate
38
39 buffer (pH 7) show the same rapid formation and growth of the aggregates. Although
40
41 the aggregate diameter estimated by this technique after 90 min from the dissolution of
42
43 the calixarene in the buffer results slightly larger than that measured by AFM, these
44
45 DLS experiments confirm the phenomenon and its trend.
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51 [Insert Figure 5]
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56 Other anions such as chloride, sulfate and nitrate do not cause similar effects,
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58 suggesting a peculiar interaction of these glucoclusters with the phosphate anion. For
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4 this reason, we also studied by AFM [32,33] the possible interaction between our
5 glycoclusters and DNA. The images relative to mixtures of a 0.5 nM plasmid DNA with
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9 10^{-4} M glucocalixarenes **2**, registered after different, increasing incubation times,
10
11 indicate that a binding between the two species occurs. The DNA filaments deposited
12
13 on mica appear in the extended shape typical for supercoiled plasmids, while, in the
14
15 presence of the glucocalixarene, aggregates of the multivalent ligand result located on
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17 the DNA plectonemes which, at the same time, evidence a higher constrain. However,
18
19 we did not observe dramatic aggregation phenomena of DNA, changes in its folding or
20
21 filament condensation [34,35] which otherwise could suggest the use of these
22
23 derivatives as synthetic gene delivery systems [36-41]. In this respect the two
24
25 glucoclusters **2a** and **2b** behave quite differently from the glycoresorcarenes reported by
26
27 Aoyama and coworkers, which are able to condense DNA filaments and give cell
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29 transfection [42-44]. This quite different behaviour, could originate from the higher
30
31 lipophilicity and/or the higher number of monosaccharide units at the *upper rim* of the
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33 resorcarenes compared to glucocluster **2** [43].
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40 Although Concanavalin A (ConA) is selective for the α anomer of natural mannosides
41
42 and glucosides, the discrimination ability between the two anomers can be strongly
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44 affected by the group linked at the anomeric position of the sugar, [45] as previously
45
46 observed for glycosylthioureido cyclodextrins. [46] Therefore, we investigated the
47
48 ability of our β -glucocalixarenes **2a** and **2b** to bind this lectin. Turbidimetric studies
49
50 were performed using the glucoclusters at 5×10^{-5} M. In the presence of ConA *ca* 10^{-5} M,
51
52 both compounds are able to agglutinate the lectin (Fig. 6) with the formation of a
53
54 suspension which determines an increase in the absorbance of the solution and which is
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56 not detectable when another lectin like peanut agglutinin, selective for galactose, is used
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4 (results not shown). Surprisingly and for reasons at the moment not completely clear,
5
6 the efficiency of the octamer **2b** looks considerably higher than that of the hexamer **2a**.
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11 [Insert Figure 6]
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16 The agglutination of ConA in presence of **2b** was also followed by AFM as reported in
17
18 Fig. 8. Images of a *ca* 10^{-8} M solution of ConA show the mica covered by many
19
20 monomers of the lectin and some of its dimeric and tetrameric aggregates (Fig. 7a),
21
22 while the mixture of **2b** (10^{-4} M) and lectin (*ca* 10^{-8} M) shows, over time (Figs. 7b and
23
24 7c), the increase of the aggregate size and the simultaneous disappearance of the
25
26 monomeric lectin which is progressively agglutinated.
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32 [Insert Figure 7]
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37 All together, the results obtained in this study allow to draw a more accurate picture of
38
39 the aggregation properties by the multivalent glucoclusters **2a** and **2b** (Fig. 8). The
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41 ligands first aggregate in small particles (3-10 nm large) and these high valency species
42
43 then interact with ConA giving agglutination with the formation of large supramolecular
44
45 entities which progressively evolve toward precipitation because of the wide lectin
46
47 cross-linking. The phosphate buffer also gives rise to the formation of large aggregates
48
49 but, due to the fact that the alkyl chains at the calixarene *lower rim* are very short ($-\text{CH}_3$)
50
51 and the macrocycles are conformationally mobile, these particles formed in the presence
52
53 of phosphate buffer are not very stable in solution and a rather dynamic process exists
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55 which can be strongly affected by external factors. This makes the systems **2a** and **2b**
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4 different from other more lipophilic glycoclusters reported by others which give more
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6 robust aggregates [28,42].
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11 [Insert Figure 8]
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18 **Experimental procedures**

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20 All moisture sensitive reactions were carried out under nitrogen atmosphere. All dry
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22 solvents were prepared according to standard procedures and stored over molecular
23
24 sieves. Melting points were determined on an Electrothermal apparatus in capillaries
25
26 sealed under nitrogen. ^1H and ^{13}C NMR spectra (300 and 75 MHz, respectively) were
27
28 recorded on a Bruker AV300 spectrometer (partially deuterated solvents were used as
29
30 internal standards). Mass spectra were recorded in ESI mode on a single quadrupole
31
32 Micromass ZMD instrument (capillary voltage = 3 KV, cone voltage = 30-160 V,
33
34 extractor voltage = 3 V, source block temperature = 80 °C, desolvation temperature =
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36 150 °C, cone and desolvation gas (N_2) flow-rates = 1.6 and 8 l/min, respectively). TLC
37
38 were performed on silica gel Merck 60 F₂₅₄, and flash chromatography using 32–63 μm ,
39
40 60 Å Merk silica gel. 5,11,17,23,29,35-Hexaamino-37,38,39,40,41,42-
41
42 hexamethoxycalix[6]arene and 5,11,17,23,29,35,41,47-octaamino-
43
44 49,50,51,52,53,54,55,56-octamethoxycalix[8]arene octahydrochloride were synthesised
45
46 according to the literature procedures [30]. Concanavalin A was purchased from Sigma-
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48 Aldrich
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4 **AFM sample preparation and imaging.** Calixarene samples were prepared by diluting
5 the compound to the desired concentration with milliQ water or with buffer or with the
6 salt solution whose effects were under investigation. DNA samples were prepared by
7 diluting the plasmid DNA to a final concentration of 0.5 nM in deposition buffer (4 mM
8 Hepes, 10 mM NaCl, 2 mM MgCl₂, pH 7.4) either in the presence or absence of
9 calixarenes. ConA samples were prepared by diluting the lectin to the concentration of
10 *ca* 10⁻⁸ M with milliQ water either in the presence or absence of calixarene. All the
11 solutions studied were incubated for a defined time at room temperature, then a 20 μl
12 droplet was deposited onto freshly-cleaved ruby mica (Ted Pella, Redding, CA) for one
13 minute. The mica disk was then rinsed with milliQ water and dried with a weak stream
14 of nitrogen. AFM imaging was performed in air on the dried sample with a Nanoscope
15 IIIA Microscope (Digital Instruments Inc.) operating in tapping mode. Commercial
16 diving board silicon cantilevers (NSC-15 Micromash Corp.) were used. Images of
17 512×512 pixels were collected with a scan size of 2 μm at a scan, rate of 3-4 lines per
18 second and were flattened after recording using Nanoscope software.
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43 **Turbidimetric Analysis.** 300 μl of an aqueous solution 10⁻⁴ M of glucocalix[n]arene
44 were quickly mixed with 300 μl of lectin aqueous solution (0.5 mg/ml). The turbidity
45 change of the solution was monitored by reading the absorbance at 350 nm at regular
46 time intervals until no noticeable changes could be observed, using a Perkin Elmer UV-
47 Vis Lambda BIO 20 spectrophotometer. The sample cell was thermostated by a Peltier
48 device at 25 °C. All experiments were performed in triplicate.
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4 **DLS analysis.** Calixarene solutions in milliQ water or phosphate buffer 0.5 M (pH 7)
5
6 were analyzed by using a Brookhaven ZetaPALS instrument. Measurements were
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8 performed at 25 °C, collecting scattered light at 90° for 8 min.
9
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13 **Synthesis of (tetraacetylglucosyl)thioureido calix[n]arenes.** Aminocalixarene and
14
15 tetraacetyl- β -glucosylisothiocyanate (1.5 equiv. for each NH₂ group) are reacted in
16
17 CH₂Cl₂ solution at room temperature for 24 h in the presence of NEt₃ (1 equiv. for each
18
19 NH₂ group). The reaction is quenched by evaporation of the organic solvent at the rotary
20
21 evaporator.
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28 **5,11,17,23,29,35-Hexakis[(2,3,4,6-tetra-O-acetyl- β -D-glucopyranosyl)thioureido]-**
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30 **37,38,39,40,41,42-hexamethoxycalix[6]arene (1a).** The compound is obtained by
31
32 purification of the crude by flash column chromatography on silica gel (eluent: from
33
34 hexane/AcOEt 3/2, v/v to hexane/AcOEt/MeOH 3/2/1, v/v/v). Yield: 60%; M.p.: 194-
35
36 195 °C. ¹H NMR (300 MHz, DMSO-d₆): δ 9.66 (bs, 6H, ArNH), 7.93 (bs, 6H,
37
38 CHNHCS), 7.14 (s, 12H, ArH), 5.87 (bs, 6H, H1), 5.35 (t, 6H, *J* = 9.3 Hz, H3), 4.95 (m,
39
40 12H, H2, H4), 4.18 (bs, 6H, H6), 3.97 (bs, 12H, H5, H6'), 3.84 (s, 12H, ArCH₂Ar), 3.20
41
42 (s, 18H, OCH₃), 1.96 (m, 72H, CH₃CO); ¹³C NMR (75 MHz, CDCl₃): δ 181.7 (s, CS),
43
44 170.9, 170.6, 169.7, 169.5 (CO), 155.6, 135.6, 135.1, 126.1, 124.9 (Ar), 86.3 (C1), 73.4,
45
46 72.7, 70.4, 68.1, 61.9 (C2-C6), 60.7 (OCH₃), 31.1 (ArCH₂Ar), 20.9, 20.7, 20.5, 20.3
47
48 (CH₃CO); ESI-MS: *m/z* 1595.9 [100%, (M+2Na)²⁺], 1071.6 [75%, (M+3Na)³⁺].
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57 **5,11,17,23,29,35,41,47-Octakis[(2,3,4,6-tetra-O-acetyl- β -D-**
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59 **glucopyranosyl)thioureido]-49,50,51,52,53,54,55,56-octamethoxycalix[8]arene (1b).**
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4 The compound is obtained by crystallization from methanol while analytically pure
5 samples by flash column chromatography on silica gel (eluent: hexane/AcOEt/MeOH
6
7 2.5/2.5/1, v/v/v). Yield: 72%. M.p.: 179-182 °C. ¹H NMR (300 MHz, CD₃OD, 343 K):
8
9 δ 6.96 (s, 16H, ArH), 5.89 (d, 8H, *J* = 9 Hz, H1), 5.35 (dd, 8H, *J* = 9.6, 9.3 Hz, H3),
10
11 5.02 (bs, 16H, H-2, H-4), 4.27 (dd, 8H, *J* = 8.1, 3.9 Hz, H6), 4.09 (dd, 8H, *J* = 8.1, 2.4
12
13 Hz, H6'), 4.02 (s, 16H, ArCH₂Ar), 3.96 (bs, 8H, H5), 3.51 (s, 24H, OCH₃), 1.99 (m,
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15 96H, CH₃CO); ¹³C NMR (75 MHz, CD₃OD): δ 181.7 (CS), 170.8, 170.5, 169.8, 169.6
16
17 (CO), 155.2, 135.1, 131.6, 125.8 (Ar), 82.7 (C1), 73.5, 73.0, 70.5, 68.2, 61.6 (C1-C6),
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19 60.9 (OCH₃), 29.8 (ArCH₂Ar), 20.4 (CH₃CO). ESI-MS: *m/z* 2120.3 [20%, (M+2Na)²⁺],
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21 1421.2 [30%, (M+3Na)³⁺].
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31 **General procedure for the deprotection of glucocalixarenes 1 from acetyl groups.**

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33 The protected glucocalixarene **1** is suspended in methanol and a solution in the same
34 solvent of NaOMe is added till pH = 9. After 30 min the reaction is quenched by
35
36 neutralization with Amberlite IR-120 (H⁺), then resin beads are filtered off and the
37
38 desired product is recovered pure by evaporation to dryness of the organic solvent.
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45 **5,11,17,23,29,35-Hexakis(β-D-glucopyranosylthioureido)-37,38,39,40,41,42-**

46
47 **hexamethoxycalix[6]arene (2a).** Yield: 90%; ¹H NMR (300 MHz, DMSO-*d*₆, 353 K):
48
49 δ 9.36 (bs, 6H, ArNH), 7.76 (bs, 6H, NH), 7.2 (s, 12H, ArH), 5.24 (bs, 6H, H1), 4.73
50
51 (bs, 24H, OH), 3.86 (s, 12H, ArCH₂Ar), 3.67 (bd, 6H, *J* = 11.1 Hz, H6), 3.49 (bd, 6H, *J*
52
53 = 11.6 Hz, H6'), 3.33-2.95 (overlapped broad multiplets, 24H, H2, H3, H4, H5), 3.19 (s,
54
55 18H, OCH₃); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 182.3 (CS), 153.1, 134.8, 134.3, 126.0
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(Ar), 83.5 (C1), 78.6, 77.9, 73.1, 70.2, 61.1 (C2-C6), 60.5 (OCH₃), 30.9 (ArCH₂Ar);

ESI-MS: m/z 2160.0 [100%, (M+Na)⁺].

5,11,17,23,29,35,41,47-Octakis(β -D-glucopyranosylthioureido)-

49,50,51,52,53,54,55,56-octamethoxycalix[8]arene (2b). If necessary, crystallization from methanol may result useful. Yield: 95%; ¹H NMR (300 MHz, D₂O, 363 K): δ 6.95 (s, 16H, ArH), 5.35 (bd, 8H, $J = 8.4$ Hz, H1), 3.88 (s, 16H, ArCH₂Ar), 3.76 (bd, 8H, $J = 12.0$ Hz, H6), 3.64 (bdd, 8H, $J = 12.0, 3.6$ Hz, H6'), 3.60-3.30 (overlapped broad multiplets, 32H, H2, H3, H4, H5), 3.37 (s, 24H, OCH₃); ¹³C NMR (75 MHz, D₂O, 363 K): δ 183.0 (CS), 155.3, 135.3, 133.8 and 126.9 (Ar) 84.5 (C1), 77.9, 77.3, 73.1, 70.2, 61.6 (C2-C6), 61.4 (OCH₃) 30.5 (ArCH₂Ar); ESI-MS: m/z 2873.0 [10%, (M+Na)⁺], m/z 1448.1 [100%, (M+2Na)²⁺].

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Captions to Figures

Figure 1. ^1H NMR spectra (300 MHz) of compound **1b** in a) CDCl_3 at 298K, b) CD_3OD at 298K, c) CD_3OD at 343K.

Figure 2. ^1H NMR of **2b** 10^{-3} M in D_2O at a) 298 K and b) 363 K.

Figure 3. a) AFM image ($2 \times 2 \mu\text{m}$) in tapping mode of a 5×10^{-4} M solution of **2b** and b) relative abundance vs diameter size of its aggregates detected by DLS.

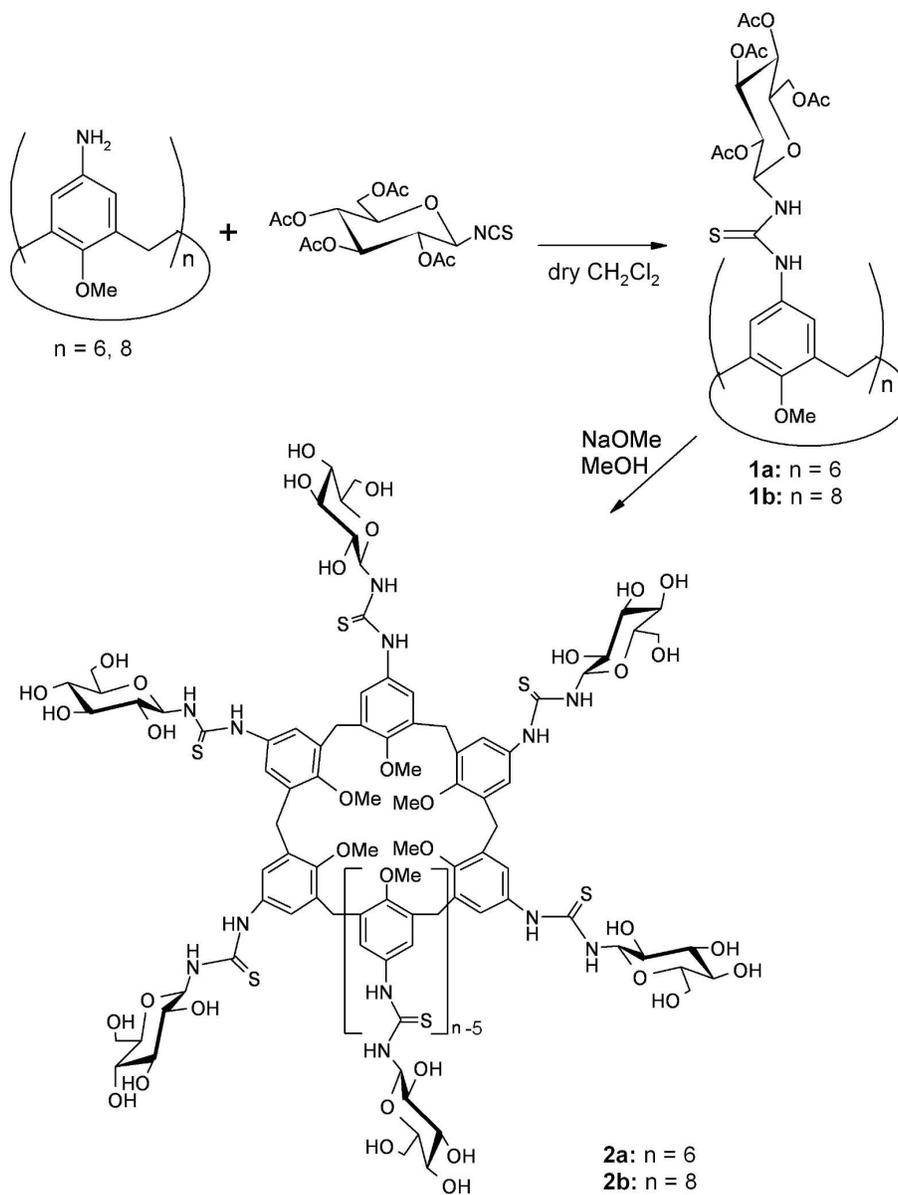
Figure 4. AFM images ($2 \times 2 \mu\text{m}$) on mica of a 10^{-4} M solution of **2b** in phosphate buffer 0.1 M (pH 7) after a) 8 min, b) 90 min and c) 20 h from the preparation of the solution; d) of a 5×10^{-4} M solution of **2b** in phosphate buffer 0.5 M (pH 7) after 90 min from the preparation; e) the same of d) but after sonication for 1 h before deposition on mica; f) of a 5×10^{-5} M solution of **2a** in phosphate buffer 0.1 M (pH 7) after 24 h from the preparation.

Figure 5. Relative abundance vs diameter size of the aggregates detected by DLS for a 5×10^{-4} M solution of **2b** in phosphate buffer 0.5 M (pH 7) after 5 min (top), 30 min (middle) and 18 h (bottom).

Figure 6. Optical density values (350 nm) vs time relative to a solution of **2a** (○) and its mixture with ConA (△) and a solution of **2b** (●) and its mixture with ConA (■).

Figure 7. AFM images ($2 \times 2 \mu\text{m}$) on mica of a) a $ca 10^{-8}$ M aqueous solution of Con A, b) a mixture of **2b** (10^{-4} M) and ConA ($ca 10^{-8}$ M) after 15 min and c) after 3 h of incubation.

Fig. 8. Schematic representation of the aggregation processes involving glucocalixarenes **2** and ConA or phosphate anions.



Scheme
136x176mm (600 x 600 DPI)

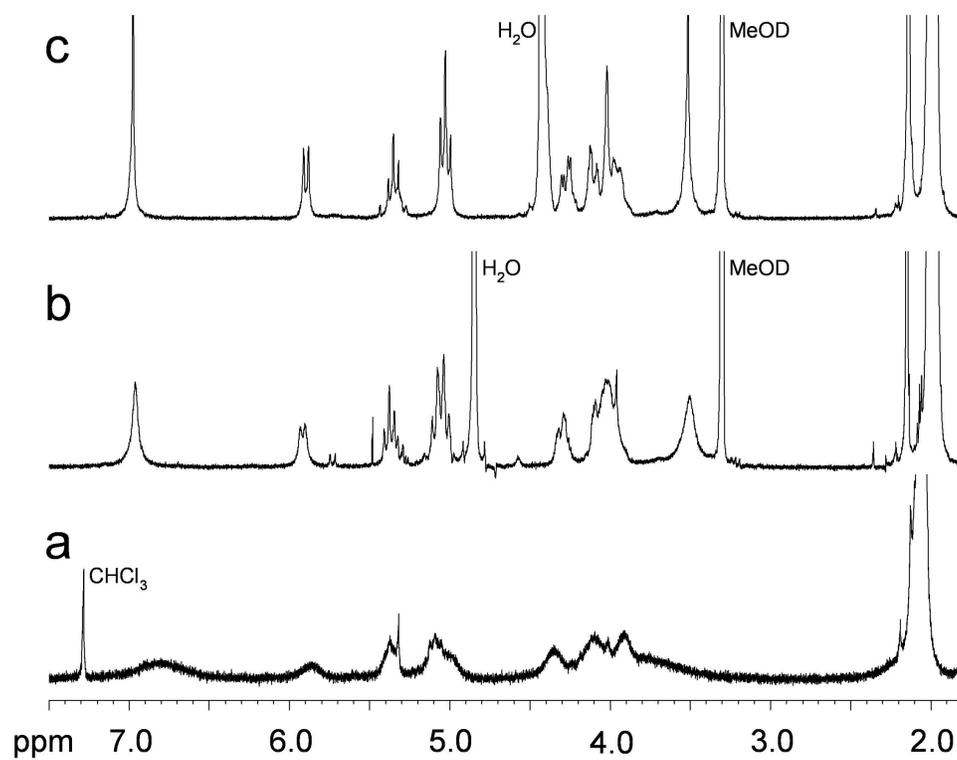


Figure 1
99x77mm (600 x 600 DPI)

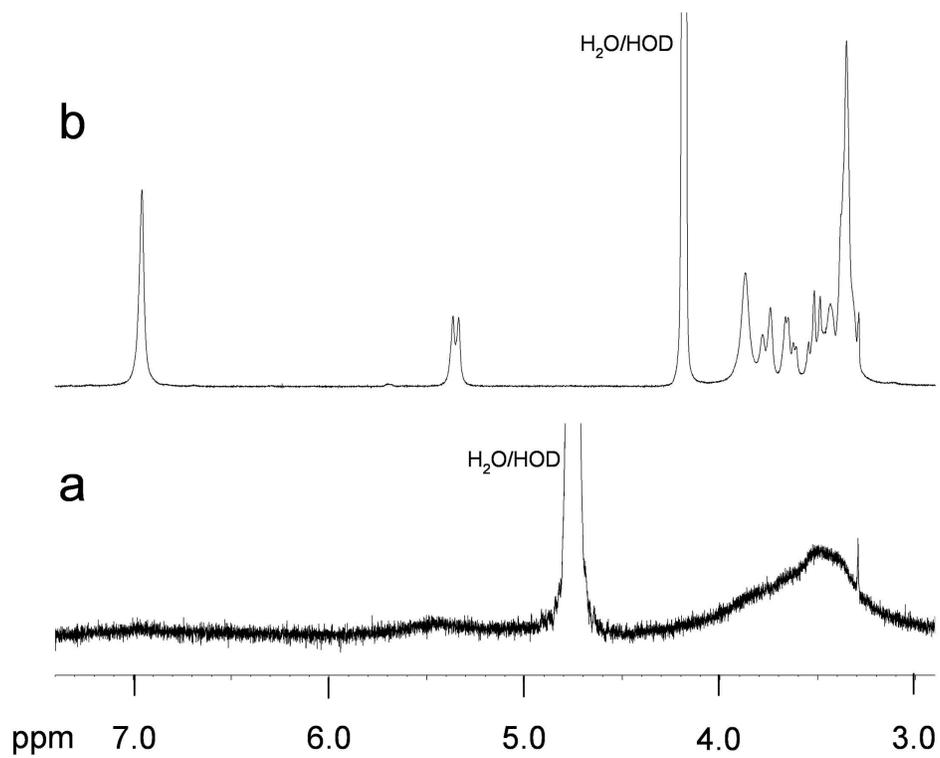


Figure 2
98x75mm (600 x 600 DPI)

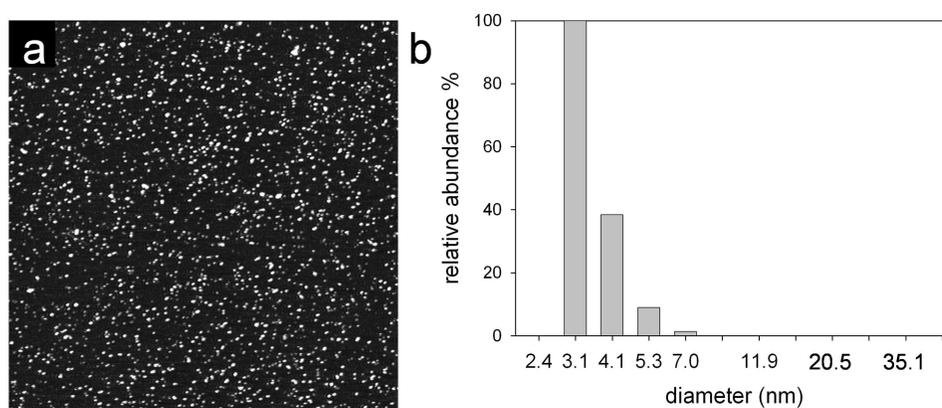


Figure 3
127x52mm (600 x 600 DPI)

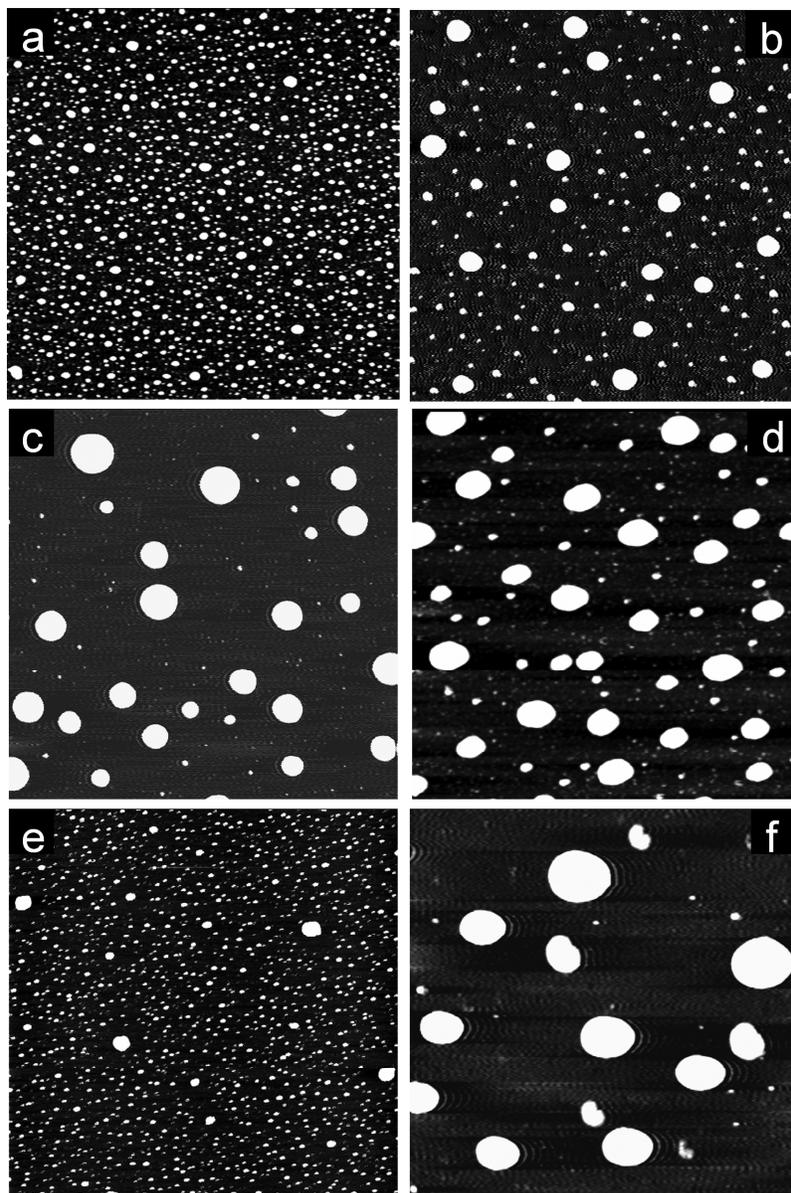


Figure 4
101x153mm (600 x 600 DPI)

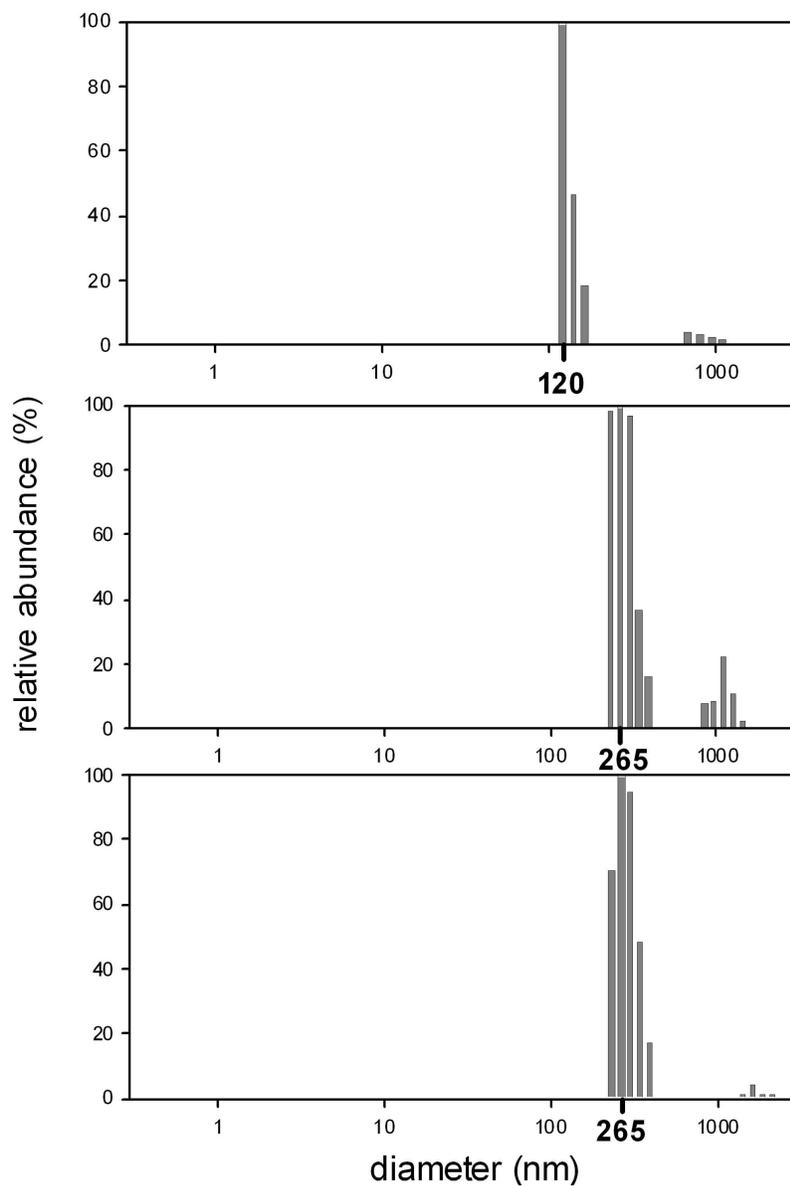


Figure 5
90x127mm (600 x 600 DPI)

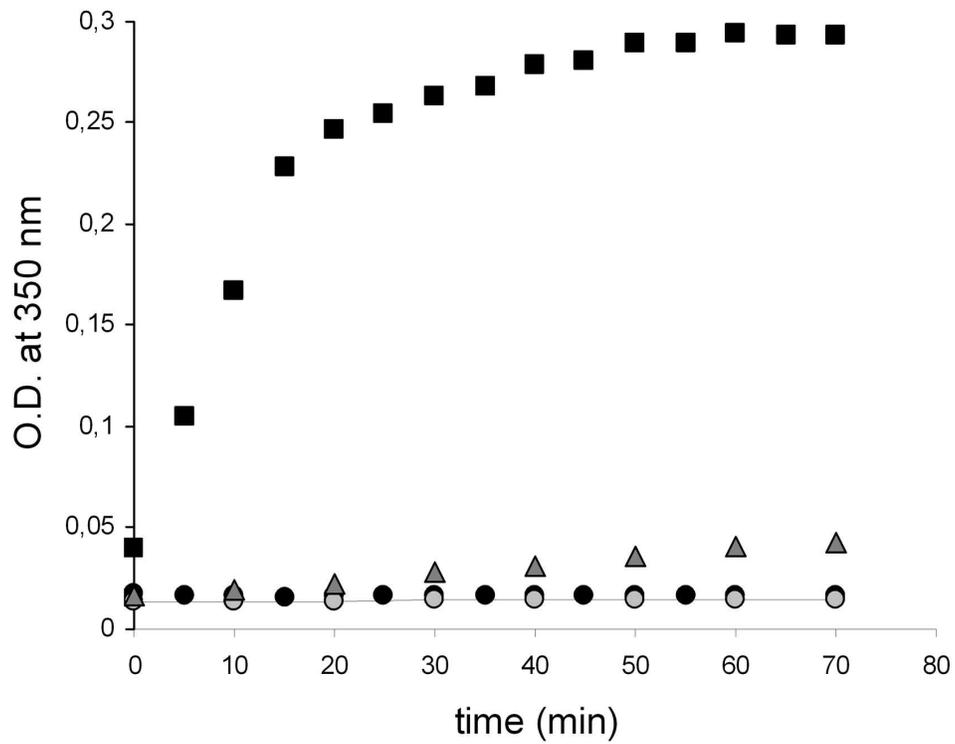


Figure 6
75x59mm (600 x 600 DPI)

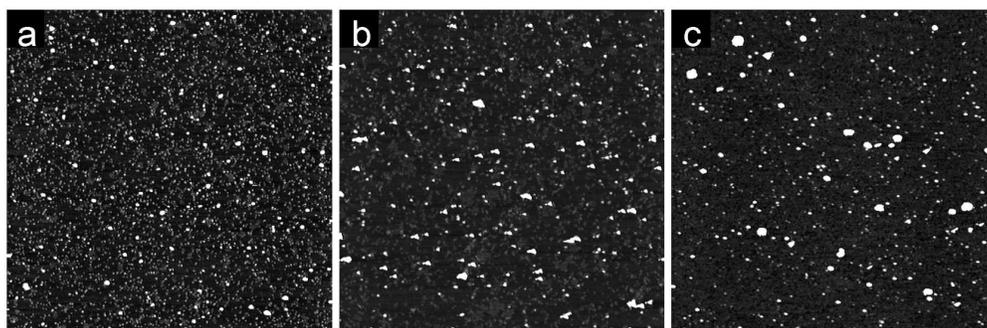


Figure 7
141x47mm (600 x 600 DPI)

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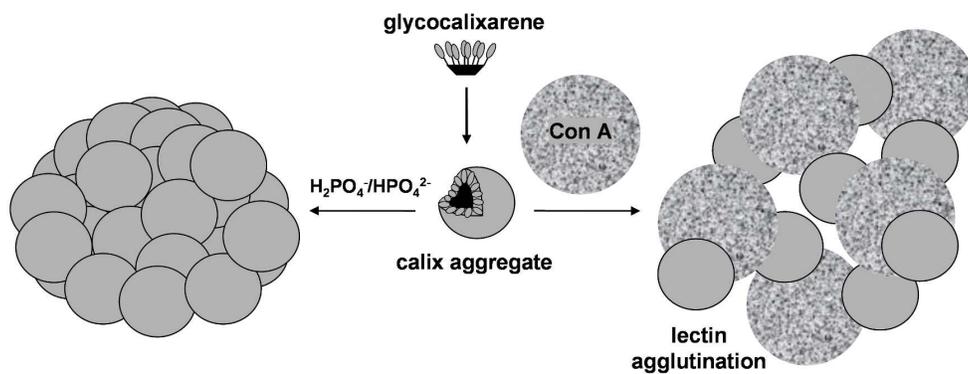
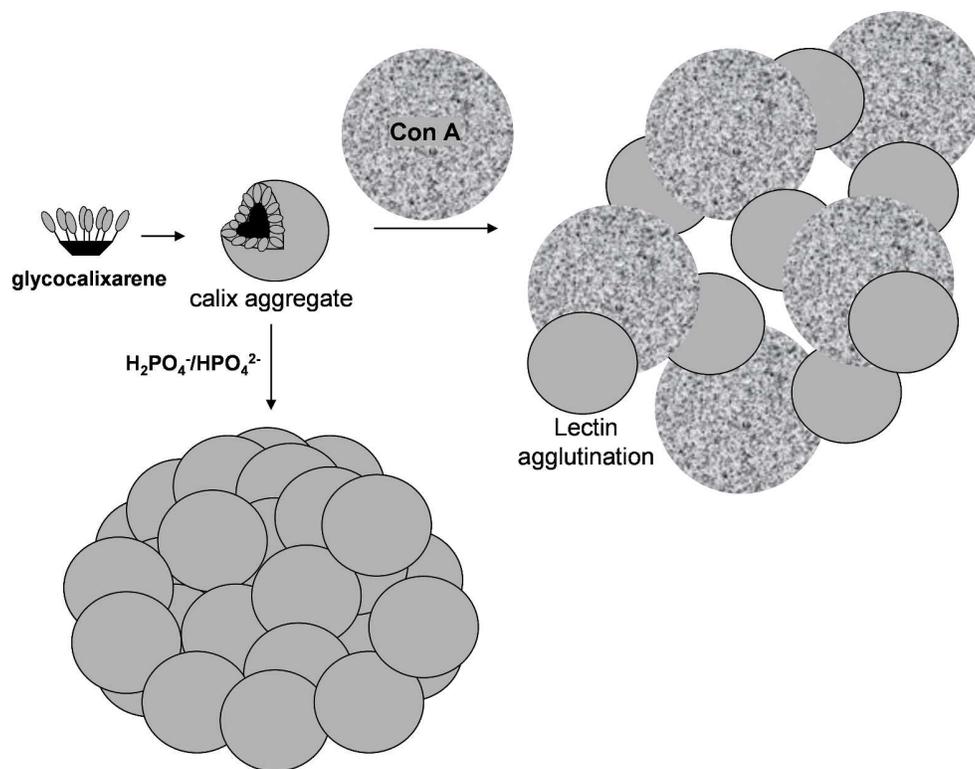


Figure 8
227x83mm (600 x 600 DPI)



Picture for the graphical abstract
204x156mm (600 x 600 DPI)