Exploring the Assembly of Resorc[4]arenes for the Construction of Supramolecular Nano-Aggregates

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Abstract: Many biologically active compounds feature low solubility in aqueous media and, thus, poor bioavailability. The formation of the host-guest complex by using calixarene-based macrocycles (i.e., resorcinol-derived cyclic oligomers) with a good solubility profile can improve solubilization of hydrophobic drugs. Herein, we explore the ability of resorc[4]arenes to self-assemble in polar solutions, to form supramolecular aggregates, and to promote water-solubility of an isoflavone endowed with anti-cancer activity, namely Glabrescione B (GlaB). Accordingly, we synthesized several architectures featuring a different pattern of substitution on the upper rim including functional groups able to undergo acid dissociation (i.e., carboxyl and hydroxyl groups). The aggregation phenomenon of the amphiphilic resorc[4]arenes has been investigated in a THF/water solution by UV–visible spectroscopy, at different pH values. Based on their ionization properties, we demonstrated that the supramolecular assembly of resorc[4]arene-based systems can be modulated at given pH values, and thus promoting the solubility of GlaB.

Keywords: resorc[4]arenes; supramolecular assembly; solubilizing agents; Glabrescione B; UV-vis spectroscopy

1. Introduction

A wide range of biologically active compounds suffers from poor aqueous solubility impairing their bioavailability and, as a result, their preclinical and clinical development. The self-assembly process of well-defined structures from various chemical building blocks have found exponential growth in the development of drug delivery and bio-nanotechnology systems [1]. In particular, these assemblies give the possibility to encapsulate pharmaceutically active compounds in their core (or at their surface) and to cargo them to the therapeutic targets [2]. Self-assembly can include different levels of complexity: it can be as simple as the dimerization of two small building blocks driven by hydrogen bonding or more complicated as a cell membrane [1], a remarkable supramolecular architecture created by a bilayer of phospholipids embedded with functional proteins. In addition to a vast series of natural amphiphilic structures, several “engineered” synthetic architectures have been designed as solubilizing agents, using a macrocyclic core such as cycloexetrins and crown-ethers [2–13]. Among the large pool of macrocycles available, calixarene-based macrocycles are one of the most ubiquitous host molecules in supramolecular chemistry. These macrocycles are cyclic oligomers characterized by a unique three-dimensional surface, featuring several phenolic units bound with methylene...
bridges, which can form large hydrophobic cavities. The high versatility of their chemical structure, which can be variously functionalized at both the upper and lower rims, allows the combination of hydrophilic and hydrophobic groups favoring the amphiphilic behavior of macrocycles and the formation of self-associates with a varying morphology [14]. Supramolecular complexation techniques using calixarene-based macrocycles as hosts may improve not only the solubility but also the stability of the guest molecules (including drugs), several examples of calixarene-guest aggregates have been reported [15–17]. In order to overcome the low aqueous solubility of these macrocycles, the upper or lower rim can be easily functionalized with water soluble groups containing positive or negative charges [18–24]. Among them, sulfonated calixarenes have dominated drug solubility studies [16]. Within the calixarene family, resorcinol-derived cyclic oligomers, namely resorc[4]arenes, endowed with cavities of molecular dimension, behave as an efficient artificial receptor [14,25,26]. The synthetic modularity and different complexation properties of the resorcarene-based macrocycles make them a promising building block for the design and synthesis of more sophisticated supramolecular architectures [27–31]. Efficient solubilizing agents were designed and developed by using several resorc[4]arenes [15,32,33]. Recently, Morozonova et al. reported that aggregates of amphiphilic calix-resorcinarenes, featuring amidoamino and dimethylamino peripheral groups on the upper rim and different aliphatic groups (penty1, octyl, and undecyl) on the lower rim, behave as effective solubilizing agents of hydrophobic drugs containing a carboxyl group (e.g., naproxen, ibuprofen, and ursodeoxycholic acid) [34]. The driving force of the association process is the ionization of organic acids and the peripheral nitrogen atoms of the macrocycles with the subsequent inclusion of hydrophobic acids into the macrocycle self-associates. Intriguingly, the solubilization of carboxylic acids in more than an equimolar ratio leads to the co-assembly of the macrocycle polydispers e associates into supramolecular monodisperse nanoparticles with the diameter of about 100 nm [34]. However, to date, a limited use of resorcarene derivatives as an amphiphilic host to complex poorly water-soluble drugs has been made with the purpose of enhancing their water-solubility and bioavailability. In a previous study, we demonstrated that physical descriptors, namely the aggregation polarity index (API), the cavitation Gibbs free-energy change (ΔΔGcav), and the decrease of the molecular surface (Å) after the exposure of solute to the solvent upon aggregation (%ΔA), were able to monitor the propensity of a double-spanned resorc[4]arene derivative (BSK), featuring a basket-like structure, to self-assembly in tetrahydrofuran (THF)/water solutions [29]. Based on these findings, with the aim to investigate the self-aggregation propensity of resorc[4]arene macrocycles, several architectures featuring a different pattern of substitution on the upper rim, including functional groups able to undergo acid dissociation, were synthesized (Figure 1). The self-assembly capability in polar solutions of amphiphilic resorc[4]arenes R1, R2, and R3, containing on the upper rim ester, carboxyl, and hydroxyl groups, respectively, were explored by UV-visible spectroscopy. Based on the ionization properties of R2 and R3, we demonstrated that, by varying the pH values, the supramolecular assembly of resorc[4]arene-based systems can be driven in polar solutions paving the way for the design and construction of new drug formulations.

Figure 1. Chemical structure of amphiphilic resorc[4]arenes R1, R2, and R3.
2. Results and Discussion

The construction of supramolecular assemblies by using amphiphilic molecular species is one of the most promising employable approaches to deliver hydrophobic pharmaceutically active compounds in physiological fluids. In principle, a suitable modulation of the self-assembly process can be effectively achieved in polar solvents by performing a proper chemical modification of the peripheral portion of the selected host, in terms of number and/or type of the hydrophilic groups. As such, the exploitation of supramolecular assembly of resorc[4]arenes represents a key approach to encapsulate hydrophobic bioactive compounds into their wide lipophilic and relatively flexible cavity-shaped architecture. In a previous study, we synthesized a cavity-shaped resorc[4]arene resembling a basket (BSK, Figure 2) via a ring closing metathesis reaction, and we investigated its self-aggregation propensity by UV-visible spectroscopy (Figure 2) [29]. To this aim, we developed a set of physical descriptors that, together, allowed us to calculate the hydrophilic–hydrophobic nature of the molecule and, thus, its amphiphilicity [29].

![Figure 2. Sigmoidal aggregation profile of BSK as obtained in THF/water solvent system. The API parameter estimated for BSK from linear fitting of sigmoidal plot of ΔABS350–400 vs. δH is reported.](image)

The BSK resorcarene demonstrated a clear propensity to undergo self-aggregation in THF/water solvent systems. The aggregation phenomenon begins when the solvent composition shows a δH value of 16.0, i.e., THF/water = 52:48 (ν/ν), and stops when δH is about 17.1, i.e., THF/water = 44:56 (ν/ν) [29]. Specifically, the API index of BSK self-aggregation corresponded to a δH = 16.55 (kcal × dm³)⁻¹/₂ in the THF/water composition of 48/52 (ν/ν) [29]. The moderate API value found for BSK suggests that the hydrophilic nature of the macrocycle largely overcomes the hydrophobic one. In general, the linear relation between δH and the water percentage of the THF/water mixtures can be expressed by the following regression line achieved for the plot δH vs. ΨH2O% (δH values have been obtained by the linear combination shown here: ΨH2O% × δH-of-H2O + THF% × δH-of-THF, with the Hildebrand polarities δH-of-H2O and δH-of-THF amounting to 23.4 and 9.1, respectively) [35]:

\[
\delta_{H} = 0.1435 \times \Psi_{H2O} + 9.0929
\]  

(1)

Based on this evidence, by increasing the hydrophilic character of the resorc[4]arene macrocycles through their upper rim chemical modification, the API parameter, as well as
the range when the self-aggregation process occurs, should undergo a progressive shift towards greater values of $\delta_H$, corresponding to solutions largely rich in water which are able to give rise to a more effective solvation. Accordingly, we decided to synthesize three resor[4]arene derivatives ($R1$, $R2$, and $R3$, Figure 3), featuring the same four alkyl chains in the lower rim, but different upper rim functionalization, and to investigate their self-assembly tendency by UV-visible spectroscopy. With respect to $R1$ which contains four methyl ester groups, the $R2$ and $R3$ macrocycles own ionizable functions in their hydrophilic portion (i.e., carboxyl or phenolic groups). As predicted by theoretical pK$_a$ values calculated through the Marvin program [36] (Figure 3), the degree of deprotonation of such groups can be finely modulated by the employment of the THF/water mixtures at a fixed pH value $X$ of the aqueous component (pH$_X$). Accordingly, to perform the self-assembly investigation of the ionizable resor[4]arenes, the pH$_X$ was set by using a suitable buffer solution, i.e., THF/(buffer-pH$_X$) mixtures.

![Figure 3. Structure of amphiphilic resor[4]arenes $R1$, $R2$, and $R3$, and pK$_a$ of acid groups calculated by Marvin program.](image)

2.1. Synthesis of Amphiphilic Resor[4]arene Macrocycles

With the aim of constructing systems for pH-induced self-assembly of amphiphilic resor[4]arenes, we introduced in the resor[4]arene macrocycle scaffold four long non-polar hydrocarbon chains in the lower rim and polar groups in the upper rim. Resorcaren $R3$ was prepared according to the literature [37,38]. Tetramethoxyresorcarenes (3) and $R1$ were obtained by slight modifications of the synthetic procedures reported by Li et al [39]. The synthetic route to resor[4]arenes $R1$ and $R2$ is reported in Scheme 1. Compound 3 was obtained by a tetramerization reaction of 3-methoxyresorcinol (1) with dodecanal (2). Successively, the phenol groups of resorcaren 3 were functionalized with methyl bromoacetate in the presence of potassium carbonate as a base, to obtain resorcaren $R1$, which bears methyl ester moieties in the upper rim. Finally, the ester functionalities of $R1$ were hydrolyzed with 2 M of potassium hydroxide and then the solution was acidified with hydrochloric acid to obtain the resor[4]arene tetraacid $R2$. All these $^1$H NMR and $^{13}$C NMR spectroscopical data were identical to the literature for compounds 3 and $R1$ [39]. Compound $R2$, which was unknown, has been fully characterized by NMR and HRMS.
resorcarene 3 were functionalized with methyl bromoacetate in the presence of potassium hydroxide and then the solution was acidified with hydrochloric acid to moieties in the upper rim. Finally, the ester functionalities of moieties in the upper rim. Finally, the ester functionalities of

Scheme 1. Synthesis of amphiphilic resorc[4]arenes R1 and R2.

For all the resorc[4]arenes, the $^1$H and $^{13}$C NMR spectral data are featured by the presence of single signals for equivalent internal and external aromatic protons and carbons, suggesting a cone conformation with C4v symmetry in solution (Figure 4). Accordingly, in addition to having a greater hydrophilic character, resorc[4]arenes R1–R3 are less pre-organized and are more flexible systems with respect to BSK, in which a flattened cone conformation occurs for the presence of the two cyclic alkenes (Figure 4).

Figure 4. 3D chemical structure of BSK and of amphiphilic resorc[4]arenes R1, R2, and R3.

2.2. The Self-Association of Resorc[4]arene R1 in THF/Water Solution

The resorc[4]arene R1, which features similar structure to R2 but endowed with a non-ionizable upper rim (-COOCH$_3$ in place of -COOH), was used as a reference system to compare its self-assembly behavior with that of the ionizable resorc[4]arenes. Accordingly, we investigated the aggregation propensity of resorc[4]arene R1 in THF/water mixtures by varying progressively the non-polar and polar solvent components in the δ$_V$ range from 9.1 (100% of THF) to 21.3 (THF/water = 15:85, $v/v$). For this purpose, the concentration of R1 was kept at a constant value of 3.2 × 10$^{-3}$ M. The self-association process was monitored by registering the changes in the UV-absorbance at the wavelengths of 350 and 400 nm ($\Delta$ABS$_{350-400}$) as a function of the δ$_H$ value of the corresponding solvent system. The scatter plot of $\Delta$ABS$_{350-400}$ vs δ$_H$ was further fitted by using the following equation [29,40]:

$$\Delta\text{ABS}_{350-400} = \frac{b}{(1 + 10^{(\delta_H - \text{API})/a})}$$

with b and a parameters representing the maximum value assumed by $\Delta$ABS$_{350-400}$ and the slope of the sigmoidal curve, respectively. As depicted in Figure 5, the self-aggregation of R1 is featured by an API value of 17.3 which corresponds to a water amount of 57.3% (with a = 0.42). This means that the resorc[4]arene assembly starts approximately when the THF/water composition yields δ$_H$ =16.9 (54.6% of water) and stops when δ$_H$ =17.7 (60.1% of water composition). At the end of the R1 self-association process, a suspension with visible turbidity is formed (Figure 5). Although R1 does not possess ionizable groups, to further characterize the structure in terms of lipophilic/hydrophilic balance of the macrocycle and to allow the comparison of its lipophilicity with that of resorc[4]arenes R2 and R3, the distribution coefficient in the logarithmic form, Log(D), was calculated through the Marvin program [36]. In general, Log(D) is a widely used descriptor measuring
the lipophilicity of ionizable biologically active compounds, where the partition in two immiscible solvents (octan-1-ol/water) is a function of the pH. Lower values of Log(D) correspond to structures endowed with higher aqueous solubility. Specifically, the Log(D) value of the resorc[4]arene R1 was established to be 21.8.

2.3. The Self-Association of Ionizable Resorc[4]arenes R2 and R3 in THF/(Buffer-pHx) Solution

Due to the presence of four carboxyl groups in the upper rim, the R2 degree of lipophilicity can be modulated in the Log(D) range from 21.6 to −5.2 by inducing the formation of the ionized forms at different pH values (in the range of 2.0–11.4). Accordingly, to investigate the self-assembly behavior of resorc[4]arene R2, the UV-visible spectroscopic analysis was performed by using the THF/(buffer-pHx) mixtures. To establish the final pH value (i.e., apparent pH) in the resulting THF/(buffer pHx) solution, the THF effect was experimentally measured up to its total amount of 50% in the mixture (see Figure S1). Specifically, as highlighted in Figure S1, the pH variation in the aqueous solution was overall rather modest reaching the maximum deviation at pH 8.4 (ΔpH = 0.54 units). To explore the influence of the R2 deprotonation degree on the δH ranges at which the self-assembly process begins and finishes, three different pH values (i.e., 1.9, 6.2, and 8.7) of the aqueous component employed in the THF/(buffer-pHx) mixtures were chosen. The ratio between the non-polar and the polar components of the solvent system was progressively varied in the δH range from 9.1 (100% THF) to 21.3 (15% THF/85% water), when using buffer-pH1.9 and buffer-pH6.2 solutions, and in the δH range from 9.1 (100% THF) to 22.7 (5% THF/95% water), when using a buffer-pH8.7 solution. In all cases, the R2 concentration was maintained at a fixed value of 3.0 × 10⁻⁵ M. The self-aggregation plots of R2, registered as a function of the THF/(buffer pHx) mixtures at the three above-mentioned pH values, are reported in Figure 6. By using a buffer-pH1.9 solution, the ionization of the resorc[4]arene R2 is substantially suppressed and the Log(D) value accounts for 21.6. The total charge on the upper rim of R2 was estimated to be −0.2, corresponding to the following distribution of each unionized and ionized species in water: 85% of the uncharged form; 14% of the mono-anionic form; and 1% of the di-anionic form. In the THF/(buffer-pH1.9) mixture, the aggregation process of R2 starts at δH = 17.3 (57.3% of water) and stops at δH = 20.9 (82.5% of water). By comparing the self-assembly of R2 with that of R1, the more hydrophilic resorc[4]arene R2 (Log(D) = 21.6 vs Log(D) = 21.8) begins the aggregation at a little bit greater δH value (δH = 17.3 vs. δH = 16.9, corresponding to a difference of +2% in water), and completes the process to a higher δH value (δH = 20.9 vs. δH = 17.7, corresponding to a difference of +23% in water).

Figure 5. On the left, the sigmoidal aggregation profile of resorc[4]arene R1 as obtained in THF/water solvent system. Regression analysis to fit the experimental data was performed according to Equation (2). On the right, the UV—visible spectra of resorc[4]arene R1 (3.2 × 10⁻⁵ M) in different THF/water solvent systems.
Figure 6. The sigmoidal aggregation profile and the corresponding UV-visible spectra of resorc[4]arene R2 as obtained in THF/water solvent systems at fixed pH values of the aqueous component. Regression analysis to fit the experimental data was performed according to Equation (2). Subfigures (A–E) represent the deprotonation states of R2 at the analyzed pH values of 1.9, 6.2 and 8.7 (the relevant percentages are shown next to the respective aggregation plots).

When the aggregation process of R2 was performed at the higher pH values (i.e., 6.2 and 8.7), marked changes in the δH values, as well as in the API and a parameters, were found. In particular, by employing a buffer pH 6.2 solution, the self-assembly of R2, featuring an estimated Log(D) of 10.0, is comprised in the δH range from 18.9 to 20.0, with API and a parameters of 19.5 and 0.56, respectively. At the end of this aggregation process, the solution appears slightly turbid, with the self-assembled molecules of R2 showing a surfactant action evidenced by the formation of a small foam layer (Figure 7). When the self-aggregation process of R2 was carried out by using the THF/(buffer-pH 8.7) solvent mixture, more drastic changes on the API and a parameters, as well as on the δH value at which the assembly starts, were observed. In a buffer-pH 8.7 solution, the resorc[4]arene R2 is characterized by a Log(D) of 0.1, and it is completely deprotonated. As such, the process is triggered when δH reaches the value of 20.5 (i.e., 86% of water), with the API and a parameters assessed equal to 22.5 (i.e., 94% of water composition) and 2.17, respectively. In these conditions, unlike in buffer-pH 1.9 and buffer-pH 6.2 systems, at the end of the self-assembly process a clear solution appears, featured by a very low ∆ABS350–400 value of 0.01 (about ten times lesser than that registered for the aggregated form of R2 at pH = 6.2). The self-assembled molecules of R2 show a strong surfactant action, as evidenced by the formation of a thick layer of foam (Figure 7). These results suggest that, in such an experimental condition, the resorc[4]arene R2 might act as an effective molecular shuttle of
hydrophobic structures. Interestingly, the Log(D) value of 0.1 assessed for R2 at pH = 8.7 corresponds to the one owned by the stearic acid at pH = 11.5 and by the palmitic acid at pH = 10.5 (values calculated by Marvin [36]). The sodium salts of these fatty acids, which are the common components of natural soaps, are typically characterized in water by pH values close to 11. Thus, resorc[4]arene R2 at a pH of around 9 is featured by a similar lipophilic/hydrophilic balance to that of components of natural soaps.

Figure 7. Turbid solution of R2 at pH = 1.9; clear solution of R2 at pH = 6.2 with some foam at liquid surface; clear transparent solution of R2 at pH = 8.7 with consistent formation of a foam at the liquid surface.

Further investigation was focused on the self-assembly behavior of resorc[4]arene R3, featuring eight ionizable phenolic groups on the upper rim. The aggregation process was monitored in the pH range from 2.4 to 11.8 by using specific THF/(buffer-pH\_X) mixtures (i.e., \(X = 2.4, 6.2, 8.5, 10.0, 11.8\)), in order to allow a selective modulation of R3 hydrophilicity in response to an appropriate pH value. Accordingly, the Log(D) values of resorc[4]arene R3 were assessed by the Marvin program [36] as a function of the selected pH (Figure 8) and the theoretical pK\_a values of the phenolic groups (Figure 3), thus reflecting the different percentages in which R3 is neutral or in the charged forms. The \(\Delta A B S\_{350-400}\) values plotted as a function of \(\delta H\) for each THF/(buffer-pH\_X) mixture are collected in Figure 8. From the sigmoidal plots, an initial induction of R3 self-aggregation is followed by a progressive disaggregation step, except for the THF/(buffer-pH\_2.4) mixture which preserves R3 in its uncharged form. To experimentally explain this trend, Dynamic Light Scattering (DLS) measurements were carried out by analyzing the diameters \(\varnothing\) of the R3 aggregates in solutions prepared from a THF/(buffer-pH\_10.0) mixture in the \(\delta H\) range from 18.8 to 22.7 (i.e., from 68% to 95% of water composition). As outlined in Figure 9, the variation of \(\varnothing\) (blue line) is perfectly related to the \(\Delta A B S\_{350-400}\) changes at the same \(\delta H\) range (gray line). After one hour, the DLS measurements were performed on the same solutions, showing how the R3 aggregates significantly increase in dimensions by a factor of 1.8 at the water composition of 75%, while to a lesser extent at 68% of water (Figure 8, orange line). Interestingly, the measured diameters of the R3 aggregates are linearly related to the \(\Delta A B S\_{350-400}\) values at the same \(\delta H\) index (\(R^2 = 0.9869\)), according to the following equation:

\[
\varnothing = 18,532 \times \Delta A B S\_{350-400} + 207.49 \quad (3)
\]

Within the \(\Delta A B S\_{350-400}\) range of 0.001–0.03, this equation was employed to estimate the variation in the aggregate size of the resorc[4]arenes R1, R2, and R3 as a function of \(\delta H\). The \(\delta\) of the most significant aggregates are indicated in the sigmoidal profiles of Figures 4, 5 and 7. The propensity of the macrocycle to self-assembly with the formation of
colloidal aggregates is clearly demonstrated from the aggregation plots of R3. At higher pH and δH values (water percentages greater than 85%), their diameters are lesser than 300 nm, giving rise to lyophobic colloids and thus to clear solutions. Nevertheless, by using a THF/(buffer-pH11.8) mixture at δH > 20 (water composition greater than 90%), the diameter of the colloid system exceeds the above limit, reaching the value of 647 nm in 100% of buffer and leading to a perfectly clear solution. Similarly to resorc[4]arene R2 in the THF/(buffer-pH8.7) solvent system at δH greater than 20.5, the R3 solutions at specific pH and δH values might favor the solubilization of hydrophobic compounds.

Figure 7. Turbid solution of R2 at pH = 1.9; clear solution of R2 at pH = 6.2 with some foam at liquid surface; clear transparent solution of R2 at pH = 8.7 with consistent formation of a foam at the liquid surface.

Figure 8. The sigmoidal aggregation profiles and the corresponding UV-visible spectra of resorc[4]arene R3 as obtained in THF/water solvent systems at fixed pH values of the aqueous component. Regression analysis to fit the experimental data was performed according to Equation (2). Subfigures (A–I) represent the deprotonation states of R3 at the analyzed pH values of 2.4, 6.2, 8.5, 10.0 and 11.8 (the relevant percentages are shown next to the respective aggregation plots).
2.4. Lyophilic Colloids Based on Self-Aggregated Resorc[4]arenes \( R_2 \) and \( R_3 \)

The ability of lyophilic colloids based on resorc[4]arenes \( R_2 \) and \( R_3 \) to capture hydrophobic compounds in wide polar media was investigated towards Glabrescione B (GlaB, \( \log(D) = 5.14 \)) (Figure 9), a naturally-occurring isoflavone which proved to be a good preclinical candidate for the treatment of Hedgehog (Hh) dependent tumors [41–44]. Based on the above-mentioned results, the self-aggregation process of \( R_2 \) was induced in its completely deprotonated form by using a THF/(buffer-pH 8.7) mixture at \( \delta_H \) values of 21.97 and 22.69 (corresponding to 90% and 95% of water composition, respectively). As showed in Figure 10, while GlaB alone gives rise to cloudy suspensions in both selected \( \delta_H \) conditions, by using the \( R_2 \) lyophilic colloids, the turbidity, although present, appears strongly reduced.

![Figure 9](image_url)

**Figure 9.** DLS profiles of \( R_3 \) aggregates in solutions prepared from a THF/(buffer-pH 10.0) mixture: variation of \( \bar{\Theta} \) (blue) related to the \( \Delta \text{ABS}_{350-400} \) changes at the same \( \delta_H \) range (gray); variation of \( \bar{\Theta} \) (orange) after one hour.

![Figure 10](image_url)

**Figure 10.** The ability of lyophilic colloids based on resorc[4]arene \( R_2 \) to encapsulate GlaB by using a THF/(buffer-pH 8.7) mixture at different \( \delta_H \) values.
The observed behavior of resorc[4]arene R3 was rather different. The aggregation test of GlaB alone and in the presence of R3 was performed by employing THF/buffer mixtures featuring different δH values: (a) 19.83, 21.97, and 22.69, with the use of buffer-pH10.0 solution; (b) 21.97 and 22.69, with the use of buffer-pH11.8 solution. As outlined in Figure 11, by using an equimolar concentration of GlaB and R3 (3.0 × 10⁻⁵ M), clear colloidal solutions at both the analyzed pH values were obtained. Coherently, the aggregate dimensions established by the DLS measurements indicate that the lyophilic colloids (R3 + GlaB) at δH = 22.69 reach diameters very close to the ones measured for the self-aggregated R3, at both the pH values of 10.0 and 11.8. In addition, the dimensional stability of lyophilic colloids (R3 + GlaB) in the THF/(buffer-pH11.8) solvent system at δH = 22.69 was analyzed over time by DLS measurements (Figure 11). The R3-GlaB aggregates were stable within the first hour (h), characterized by a diameter of 276 nm. Later (4 h), a reduction occurred, reaching a δ of 136 nm, and a further increment towards the value of 400 nm was observed within 24 h.

![Figure 11](image_url). The ability of lyophilic colloids based on resorc[4]arene R3 to act as a molecular shuttle of GlaB by using THF/(buffer-pH10.0) and THF/(buffer-pH11.8) mixtures at different δH values.

3. Materials and Methods

3.1. Synthesis of Resorc[4]arenes

General remarks: melting points were recorded with a Büchi melting point B-545 and are not corrected. The ¹H and ¹³C NMR spectra have been acquired with a Bruker Avance 400 spectrometer operating at 400.13 and 100.6 MHz, respectively, at 300 K in CDCl₃ or DMSO-d₆, using 5 mm diameter glass tubes. Chemical shifts were expressed in ppm and coupling constants (J) in hertz (Hz), approximated to 0.1 Hz. The residual solvent peak was used as an internal reference for ¹H and ¹³C NMR spectra. Data for ¹H NMR are reported as follows: chemical shift, multiplicity (br = broad, ovrlp = overlapped, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet), coupling constant, and integral. Spectra were processed with the program MestReNova version 6.0.2-5475, FT and zero filling at 64 K. High-resolution (HR) mass spectra were obtained using a Thermo Fischer Exactive mass spectrometer equipped with an ESI source and an Orbitrap analyzer: capillary temperature 275 °C, spray voltage 3.5 kV, sheath gas (N₂) 10 arbitrary units, capillary voltage 65 V, and tube lens 125 V. Analytical TLC were performed using 0.25 mm Fluka F254 silica gel. The compounds on TLC were revealed by quenching fluorescence (at 254 and 365 nm) using a 4 W UV lamp. Otherwise, plates were stained [with...].
with an acidic solution of p-anisaldehyde or a 10% phosphomolybdic acid solution in EtOH and heated (T = 120 °C). The product mixture purifications were carried out with silica column chromatography using Fluka 60 Å silica gel (0063—0200 mm, 70—230 mesh). Flash chromatography was performed using 200—400 mesh silica gel. Commercially available reagents were supplied by Sigma-Aldrich and used without further purification. Dry solvents were purchased from Sigma-Aldrich or dried by distillation. Resorcarene R3 [37,38] and GlaB [45] were synthesized according to the literature. Yields of synthesized compounds are referred to chromatographically and spectroscopically pure compounds, unless otherwise stated.

3.2. Synthesis of Tetraundecanyl Tetra-O-methyl Resor[4]arene (3)

Boron trifluoride etherate (2.3 g, 2 mL, 16.2 mmol) was added to a solution of 3-methoxyphenol (1) (1 g, 0.88 mL, 8.0 mmol) and dodecanal (2) (1.47 g, 1.76 mL, 8.0 mmol) in anhydrous dichloromethane (40 mL), and the reaction was kept under stirring at room temperature for 2 h. The reaction mixture was then washed with water (2 × 40 mL) and brine (1 × 40 mL). The organic layer was dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure to give a dark red oil. The crude was recrystallized from hot ethanol to give a reddish solid. The product was recrystallized from hot methanol to obtain pure compound 3 (0.687 g, 80% yield) as a pale pinkish solid. 

\[ {^1}H\text{ NMR (400 MHz, CDCl}_3 \] \( \delta \) 7.51 (s, 4H), 7.22 (s, 4H), 6.35 (s, 4H), 4.27 (t, J = 7.4 Hz, 4H), 3.83 (s, 12H), 2.19 (d, J = 6.7 Hz, 8H), 1.27 (M, 72H), and 0.89 (t, J = 6.3 Hz, 12H).

\[ {^{13}}C\text{ NMR (101 MHz, CDCl}_3 \] \( \delta \) 153.7, 153.1, 124.9, 124.7, 123.8, 100.1, 77.5, 77.2, 76.8, 56.0, 34.1, 33.2, 32.1, 29.9, 29.9, 29.6, 28.2, 22.8, and 14.3.

3.3. Synthesis of Tetraundecanyl-tetra(methoxycarbonylmethoxyl)-tetra-O-methyl Resor[4]arene (R1)

Methyl bromoacetate (0.225 mL, 0.364 g, 2.38 mol) was added to a stirred solution of resor[4]arene 3 (0.554 g, 0.476 mmol) and K₂CO₃ (0.654 g, 4.76 mmol) in dry acetonitrile (65 mL), and the reaction mixture was heated at reflux for 24 h under inert atmosphere. Then, the reaction mixture was cooled down and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (40 mL), and the organic layer was washed with 1 M HCl (10 mL), with water and brine. The organic layer was dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure. The pure product R1 was obtained as a solid (0.398 g, 0.274 mmol) in 58% yield and used without further purification.

\[ {^1}H\text{ NMR (400 MHz, CDCl}_3 \] \( \delta \) 6.61 (s, 4H), 6.29 (s, 4H), 4.50 (t, J = 7.4 Hz, 4H), 4.21 (d, J = 15.9 Hz, 4H), 4.02 (d, J = 15.9 Hz, 4H), 3.77 (s, 12H), 3.61 (s, J = 5.5 Hz, 12H), 1.85–1.77 (m, 8H), 1.32–1.21 (ovrlp m, 18H), and 0.87 (t, J = 6.9 Hz, 12H).

\[ {^{13}}C\text{ NMR (101 MHz, CDCl}_3 \] \( \delta \) 170.3, 155.7, 155.0, 128.4, 127.6, 126.5, 99.7, 77.5, 77.2, 76.8, 56.0, 34.1, 33.2, 32.1, 29.9, 29.9, 29.6, 28.2, 22.8, and 14.3.

3.4. Synthesis of Tetraundecanyl-tetra(hydroxycarbonylmethoxyl)-tetra-O-methyl Resor[4]arene (R2)

A 2 M aqueous solution of potassium hydroxide (15 mL) was added to a solution of resor[4]arene R1 (0.400 g, 0.276 mmol) in THF (40 mL), and the reaction mixture was stirred for 24 h at room temperature. Then the solution was acidified with 2 M HCl (40 mL) and the THF was removed under reduced pressure. The white precipitate was filtered, washed with water, and dried under a vacuum at 80 °C for 3 h. Then it was dissolved in THF and the solution was filtered. The THF was removed under reduced pressure to give R2 as a white powder (0.366 g, 0.262 mmol) in 95% yield.

\[ {^1}H\text{ NMR (400 MHz, DMSO-d}_6 \] \( \delta \) 6.65 (s, 4H), 6.38 (s, 4H), 4.51 (t, J = 7.0 Hz, 4H), 4.42 (d, J = 16.1 Hz, 4H), 4.25 (d, J = 16.1 Hz, 4H), 3.57 (s, 12H), 1.69 (br s, 8H), 1.16 (s, 72H), and 0.79 (t, J = 6.7 Hz, 12H).

\[ {^{13}}C\text{ NMR (101 MHz, DMSO-d}_6 \] \( \delta \) 170.3, 155.7, 155.0, 128.4, 127.6, 126.5, 99.7, 77.5, 77.2, 76.8, 56.4, 55.6, 52.0, 50.9, 35.6, 34.8, 32.1, 30.1, 30.0, 29.9, 29.87, 29.83, 29.5, 28.2, 22.8, and 14.2.

ESI-HRMS: \( m/z \) [M−H]⁻ \( C_{84}H_{127}O_{16} \) requires 1391.9130, found 1391.9100; [M−2H]⁻ \( C_{84}H_{126}O_{16} \).
requires 695.4528, found 695.4524; and [M−3H]− 3 C84H125O16 requires 463.2995, found 463.2993.

3.5. UV-Vis Spectroscopical Analyses

General remarks: all spectroscopic analyses were performed with the JASCO V-550 spectrometer with a Peltier thermostat at 25 °C using a quartz cuvette (cell length 1 mm). The HPLC grade THF (tetrahydrofuran) and H2O (water) were obtained from Sigma Aldrich, St. Louis, MO, USA.

3.6. Preparation of Solutions

Stock solution of R1 (C88H136O16, Mw 1449.76 g/mol; 6.1 × 10^-3 g, 4.2 × 10^-3 mmol) at a concentration of 2.1 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples used for the UV spectrophotometric analysis were obtained with a different THF/H2O ratio (from 0% to 85% of water) having a final concentration of R1 equal to 3.2 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/H2O ratio as the samples. Modifications of the pH at 11.78 were obtained by an aqueous solution of sodium tetraborate decahydrate (Na2B4O7·10 H2O, Mw 381.49 g/mol, 5.6 × 10^-3 g, 4.0 × 10^-3 mmol) at a concentration of 2.0 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples used for the UV spectrophotometric analysis were obtained with a different THF/H2O ratio (from 0% to 85% of water) having a final concentration of R1 equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/H2O ratio as the samples. Modifications of the pH at 11.85 were obtained by using an aqueous solution of phosphate buffer (NaH2PO4, Mw 119.98 g/mol, 8.2 × 10^-3 g in 50 mL of H2O, 10^-3 M and H3PO4, Mw 97.99 g/mol) at a different ratio. The baseline was obtained with the same THF/buffer phosphate ratio as the samples. Stock solution of R2 (C84H126O16, Mw 1392.92 g/mol; 5.6 × 10^-3 g, 4.0 × 10^-3 mmol) at a concentration of 2.0 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples used for the UV spectrophotometric analysis were obtained with a different THF/H2O ratio (from 0% to 85% of water) having a final concentration of R2 equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/H2O ratio as the samples. Modifications of the pH at 11.82 were obtained by an aqueous solution of sodium hydroxide (NaOH, Mw 39.99 g/mol, 1 N). The baseline was obtained with the same THF/buffer borate ratio as the samples. A stock solution of GlaB (C72H112O38, Mw 1105.65 g/mol; 10.5 × 10^-3 g, 9.5 × 10^-3 mmol) at a concentration of 1.9 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples used for the UV spectrophotometric analysis were obtained with a different THF/H2O ratio (from 0% to 85% of water) having a final concentration of R3 equal to 2.85 × 10^-5 M and final volume of 2 mL. The baseline was obtained with the same THF/H2O ratio as the samples. Modifications of the pH at 11.82 were obtained by an aqueous solution of sodium tetraborate decahydrate (Na2B4O7·10 H2O, Mw 381.49 g/mol, 5.63 × 10^-2 g in 100 mL of H2O, 1.5 × 10^-3 M) and sodium hydroxide (NaOH, Mw 39.99 g/mol, 1 N). The baseline was obtained with the same THF/buffer borate ratio as the samples. A stock solution of GlaB (C72H112O38, Mw 1105.65 g/mol; 10.5 × 10^-3 g, 9.5 × 10^-3 mmol) at a concentration of 1.9 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples were diluted in THF in a ratio of 1:2 (final concentration 2 × 10^-4 M) and used for the UV spectrophotometric analysis were obtained with a different THF/buffer phosphate (pH 11.82) ratio (from 0% to 85% of water) having a final concentration of GlaB equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/buffer phosphate ratio as the samples. Stock solution of GlaB (C72H112O38, Mw 1105.65 g/mol; 10.5 × 10^-3 g, 9.5 × 10^-3 mmol) at a concentration of 1.9 × 10^-4 M in 20 mL of THF was prepared. Starting from this solution, the samples were diluted in THF in a ratio of 1:2 (final concentration 2 × 10^-4 M) and used for the UV spectrophotometric analysis were obtained with a different THF/buffer phosphate (pH 11.85) ratio (from 0% to 85% of water) having a final concentration of GlaB equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/buffer phosphate ratio as the samples. Starting from this solution, the samples were diluted in THF in a ratio of 1:2 (final concentration 2 × 10^-4 M) and used for the UV spectrophotometric analysis were obtained with a different THF/buffer phosphate (pH 11.85) ratio (from 0% to 85% of water) having a final concentration of GlaB equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/buffer phosphate ratio as the samples. Starting from this solution, the samples were diluted in THF in a ratio of 1:2 (final concentration 2 × 10^-4 M) and used for the UV spectrophotometric analysis were obtained with a different THF/buffer phosphate (pH 11.85) ratio (from 0% to 85% of water) having a final concentration of GlaB and R3 equal to 3.0 × 10^-5 M and a final volume of 2 mL. The baseline was obtained with the same THF/buffer phosphate ratio as the samples. Starting from the stock solutions of R3 and GlaB at a concentration of 1.2 × 10^-3 M, 0.100 mL of sample was taken and solubilized in 0.100 mL of THF and 1.8 mL of buffer to obtain the concentration of water at 90%. A 0.100 mL of solubilized sample in 1.9 mL of buffer was used to obtain a 95% water concentration. In both cases the sample had a concentration of 6 × 10^-5 M. A 1 mL of solution of R3 was added to a 1 mL of solution of GlaB to give a final volume of 2 mL, with a final equimolar concentration of the compounds equal to 3 × 10^-5 M.
3.7. DLS Analysis

The size and z potential values of resorc[4]arene R3 were measured by using a 90Plus/BI-MAS ZetaPlus multiangle particle size analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA). For size measurements, the autocorrelation function of the scattered light was analyzed assuming a log Gaussian distribution of the vesicle size. The mean size and polydispersity index have been obtained. The z potential values were calculated from the electrophoretic mobility by means of the Helm-holtz-Smoluchowski relationship.

4. Conclusions

In conclusion, we carried out a detailed characterization of the self-assembly process of amphiphilic resorc[4]arene-based architectures featuring long aliphatic side chains and a different pattern of substitution on the upper rim, including functional groups able to undergo acid dissociation. Based on the hydrophilic features and the ionization properties of the upper rim of the macrocycles, these amphiphiles revealed a strong propensity to self-assembly in a specific THF/water composition. The combination of theoretical calculations with the experimental results highlighted that the supramolecular assembly of ionizable resorc[4]arenes is strictly dependent on the pH values, when using solutions largely rich in water (i.e., 10% THF/90% H₂O and 5% THF/95% H₂O), leading to the formation of lyophilic colloids with characteristic diameters. Based on these properties, we demonstrated that the resorc[4]arene-based systems can entrap the poorly water-soluble isoflavone GlaB, most probably due to inclusion complexation between the guest molecules and the hydrophobic alkyl chains of the macrocycles. The next steps of the study will be: (i) the NMR investigation to characterize the inclusion complexation more deeply; (ii) the design of novel amphiphilic architectures featuring ionizable functional groups with improved ability to supramolecular self-assemble in water at specific pH values. In addition, we will investigate the in vitro bioactivity of GlaB-resorcarene aggregates in the anticancer efficiency towards the Hh-dependent tumors.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222111785/s1, Figure S1: pH variations of THF/water solutions depending on the mixture composition and different prefixed pH values, Figure S2: 1H NMR spectrum of compound 3 (CDCl₃, 400 MHz), Figure S3: 13C NMR spectrum of compound 3 (CDCl₃, 101 MHz), Figure S4: 1H NMR spectrum of compound R1 (CDCl₃, 400 MHz), Figure S5: 13C NMR spectrum of compound R1 (CDCl₃, 101 MHz), Figure S6: 1H NMR spectrum of compound R2 (DMSO-d₆, 400 MHz), Figure S7: 13C NMR spectrum of compound R2 (DMSO-d₆, 101 MHz), Figure S8: ESI-HRMS spectrum of compound R2, Table S1: R3 pH 9.96 from 68% to 95% H₂O, Table S2: R3 + GlaB pH 11.8, 95% H₂O, Table S3: R1, Table S4: R2 pH 6.21, Table S5: R2 pH 1.91, Table S6: R2 pH 8.70, Table S7: R3 pH 6.21, Table S8: R3 pH 2.41, Table S9: R3 pH 8.52, Table S10: R3 pH 10.0, Table S11: R3 pH 11.8.

Author Contributions: F.B. performed the UV-vis studies. F.G., I.R. and A.C. contributed to analyze the data and fully reviewed the manuscript. M.P. and D.Q. design the experiments, analyze the data, and fully reviewed the manuscript. G.S. and S.P. performed DLS analysis. M.P. and B.B. conceived the project and provided overall guidance. The manuscript was written with contribution of all authors. All authors have read and agreed to the published version of the manuscript.

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