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Design, synthesis, and drug solubilising properties of the first folate–calix[4]arene conjugate†

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The first example of a folate–calix[4]arene conjugate was designed and synthesized *via* microwave-assisted click chemistry. In PBS medium at physiological pH, the conjugate formed soluble aggregates and showed the capability to improve the water-solubility of a hydrophobic drug model, such as indomethacin.

The development of molecular carriers capable of selectively delivering therapeutic agents into pathological cells is desirable to preserve healthy cells from toxic effects and to improve diagnosis and therapy of diseases such as cancer and chronic inflammation. In this context, the folate-targeting strategy holds great promise.[1] Folate receptor (FR) is a highly selective cancer cell and activated macrophage marker,[2] and folic acid vitamin (FA), which binds FR with high affinity ($K_d = 0.1$ nM), behaves as a "Trojan Horse"[3] that can promote the specific delivery of imaging and therapeutic agents into FR-positive cells.[1]

The finding that conjugation of foreign molecules to FA does not normally interfere with the high FA–FR affinity and cellular uptake,[4] has stimulated the development of a variety of FA-conjugates and some of them have provided encouraging results in preclinical and clinical studies.[5] FA-conjugates include monovalent structures in which FA is linked to proteins[4,6] chemotherapeutic[7] and imaging[8] agents, antisense oligonucleotides,[9] haptens,[10] and immunotherapeutics;[11] but also multivalent constructs in which multiple folate units are clustered by a core scaffold including polymers,[12] dendrimers,[13] organic and inorganic nanoparticles,[14] liposomes,[15] and virus.[16] Multivalent structures that can enable multiple FA–FR interactions are proving more efficient targeting systems than the monovalent analogs. As an example, it was demonstrated that FR binding avidity and biological targeting of folate–dendrimers was enhanced by increasing the degree of functionalization with FA moieties.[17]

It is known that FA receptors exist mainly as clusters of three or more molecules on the cell surface,[18] and not only the number of FA moieties but also backbone topology, linkers, conformational flexibility, shape, and size can affect the targeting properties of a multivalent folate-conjugate.[19] Therefore, research into the optimal clustering of FA ligands continues to be a challenge in the discovery of more efficient folate-based targeting systems.

The bowl-shaped calix[4]arene skeleton could deserve to be taken into account in the research of new molecular platforms for the clustering of FA ligands. It can allow an organization of multiple folate moieties in a well-defined tridimensional architecture with controlled cluster group number and spacing. Such a structure could carry covalently or non-covalently linked molecular entities (*i.e.* drugs or imaging agents).

Calix[*n*]arene macrocycles[20] possess peculiar features such as synthetic versatility, host properties, ability to cross biomembranes,[21] low cytotoxicity and immunogenicity, which are making them promising platforms for applications in medical and pharmaceutical fields.[22–25] Calixarene derivatives active as carriers for drug[26] and gene delivery[27] were also described.

The aim of this work is the design, synthesis, and structural characterization of the first example of a folate–calix[4]arene conjugate (**7**), and a preliminary investigation of its capability to improve the solubility of indomethacin, selected as a hydrophobic drug model.

In order to introduce four FA units onto a calix[4]arene skeleton, we planned to use the copper-catalyzed azide-alkyne cycloaddition (CuAAC), which belongs to the class of reactions, commonly termed "click chemistry" as coined by Sharpless *et al.*[28] The CuAAC has proven preferable to conventional methods for conjugation of biological ligands to macromolecules under mild reaction conditions. It results in highly specific and efficient formation of a 1,4-disubstituted 1,2,3-triazole linker, which is essentially stable to metabolic transformations such as oxidation, reduction, and both basic and acid hydrolysis.[29] Thus, folate–calix[4]arene conjugate **7** was synthesized by coupling azido-calix[4]arene **4** and γ-propargyl folate **6**.

The azido-calix[4]arene (**4**) was prepared by following the synthetic route depicted in Scheme 1. To arrange four folate units on the same side with respect to the macrocycle medium plane (all-*syn* orientation), the *p*-*tert*-butylcalix[4]arene skeleton was blocked in a *cone* conformation by introducing four triethylene glycol moieties at the lower rim (compound **1**).[30] The triethylene glycol chains were also selected to increase the water-solubility of the planned folate–calix[4]arene conjugate (**7**). After that, the *tert*-butyl groups at the upper rim of **1** were converted to nitro groups (compound **2**) and then amino groups (compound **3**) by an ipsonitration reaction followed by

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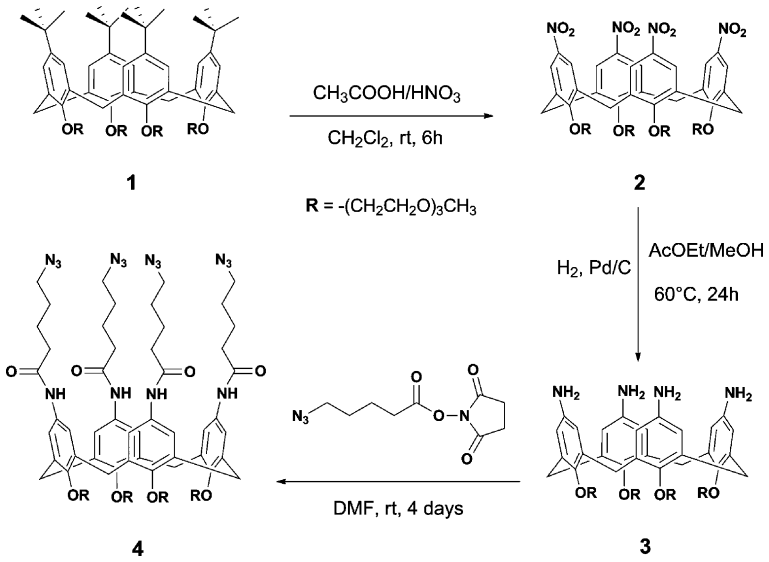
† Electronic supplementary information (ESI) available: Synthetic procedures and NMR spectra for compounds **4**, **6**, **7**, ESI-MS spectra for compounds **6** and **7**, DLS measurements for compound **7**. See DOI: 10.1039/c1ob06032e



Scheme 1 Synthesis of the azido-derivatized calix[4]arene (**4**).

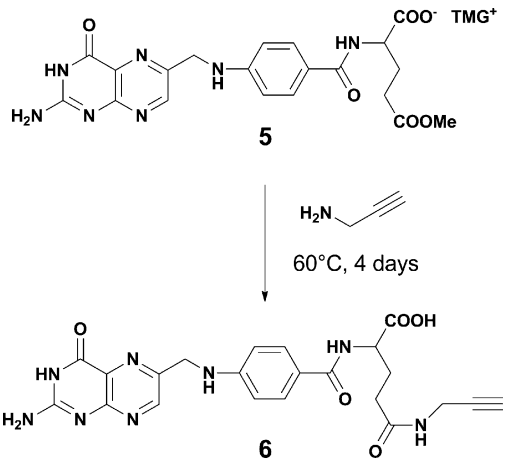
catalytic hydrogenation.[31] *p*-Amino-calix[4]arene **3** was reacted with purposely synthesized succinimidyl 5-azidopentanoate,[32] and pure azido-derivatized calix[4]arene **4** was obtained in 80% yield. The structural characterization of compound **4** was carried out by ^{1}H- and ^{13}C-NMR experiments.

The presence of a pattern of signals at 1.62, 1.69, 2.28, 3.28, relative to the CH_2 protons of the four equivalent 5-azidopentanoate chains in the proton spectrum of **4** confirmed the exhaustive functionalization of the calixarene macrocycle. The presence of the CH_2N_3 groups was also corroborated by the resonance at 52.1 ppm in the carbon spectrum of **4**.

The choice of a C5 chain as a spacer between the calixarene scaffold and the targeting folate moieties arose by the consideration that a longer and more flexible spacer can enhance the host hydrophobic cavity and by reducing ligand steric interference facilitate multiple simultaneous ligand–receptor bindings.[33]

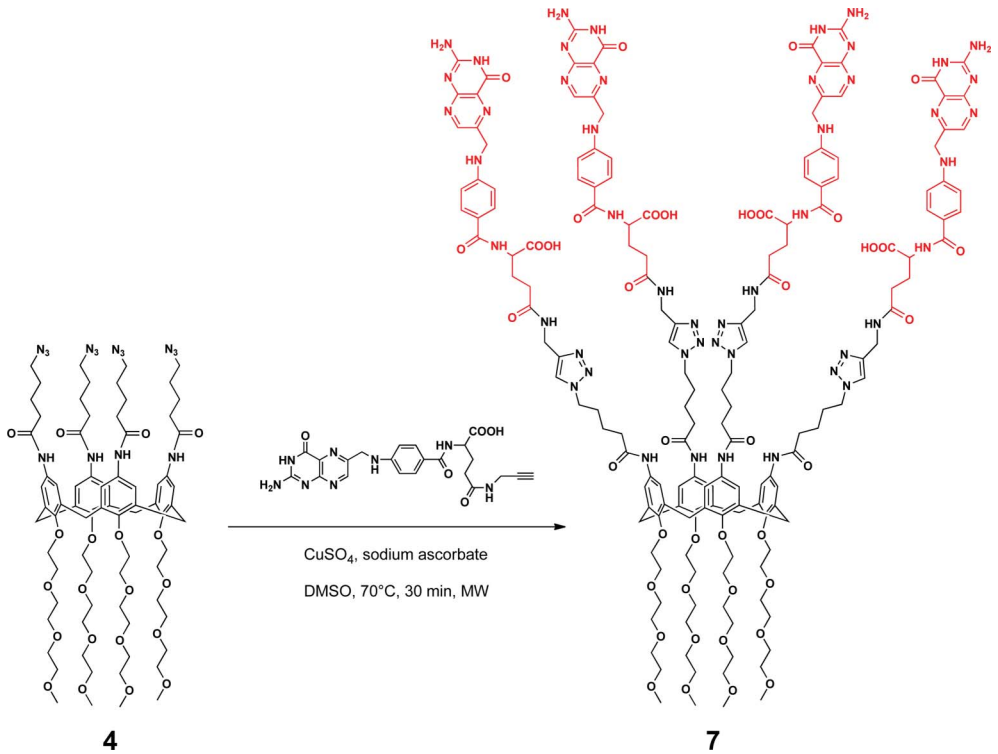
Since the α-carboxyl group of FA is crucial for retaining a high FA–FR binding affinity,[34] we planned to introduce a propargyl functionality exclusively at the FA γ-carboxyl group. This was also essential to prevent the formation of a complex mixture during the succeeding exhaustive functionalization of the azido-calixarene derivative (**4**). In principle, a mixture of α- and γ-propargyl folate might give rise to six possible calix[4]arene derivatives, generated by the combination of α- and γ-attached folate pendants (α_4, γ_4, $\alpha_3\gamma$, $\alpha\gamma_3$, and $\alpha_2\gamma_2$ in vicinal and distal positions). The regiospecific γ-functionalization starting from unprotected folic acid is difficult to obtain and a mixture of both α- and γ-conjugate, often accompanied with the bis-functionalized derivative, is frequently formed.[34b,35] Complete γ-regioselectivity has been accomplished by multistep procedures, which entail the coupling of pteroic acid precursors with glutamic acid derivatives selectively functionalized at the γ[34b,36] or α-carboxyl group.[37]

By using a multistep procedure, we prepared tetramethylguanidinium L-methyl folate **5**[36] which treated with an excess of propargyl amine (Scheme 2) provided only γ-propargyl folate **6**[38] (66% yield). Compound **6** was fully characterized by 1D- and 2D-NMR experiments, ESI-MS spectroscopy, and HPLC analysis.[39]



Scheme 2 Synthesis of the propargyl folate (**6**).

Click reaction between azido-calix[4]arene derivative **4** and γ-propargyl folate **6** was performed in DMSO and in the presence of catalytic amounts of $CuSO_4$ and sodium ascorbate (Scheme 3). The reaction mixture was stirred in a microwave synthesizer at 70 °C for 30 min, and pure folate–calix[4]arene conjugate **7** was obtained in 40% yield after gel permeation chromatography. The reaction time increased to 24 h when the same reaction was performed in the absence of microwaves. Compound **7** was characterized by 1D- and 2D-NMR experiments, and ESI-MS spectrometry.[39] A single pattern of signals compatible with a totally symmetric structure (C_4 symmetry) and a 4 : 1 integral ratio between folate and calix[4]arene resonances in the proton spectrum of **7**, corroborated the exhaustive functionalization of the azido-calix[4]arene scaffold (Fig. 1). The folate–calix[4]arene conjugation was confirmed by the disappearance of the signal relative to the alkyne groups in both ^{1}H and ^{13}C-NMR spectra, and by the appearance of a proton signal at 7.85 ppm and a carbon resonance at 122.6 ppm relative to the CH protons of the triazole rings. The 1,4-substitution of the triazole



Scheme 3 Synthesis of the folate–calix[4]arene conjugate (7) *via* click chemistry.

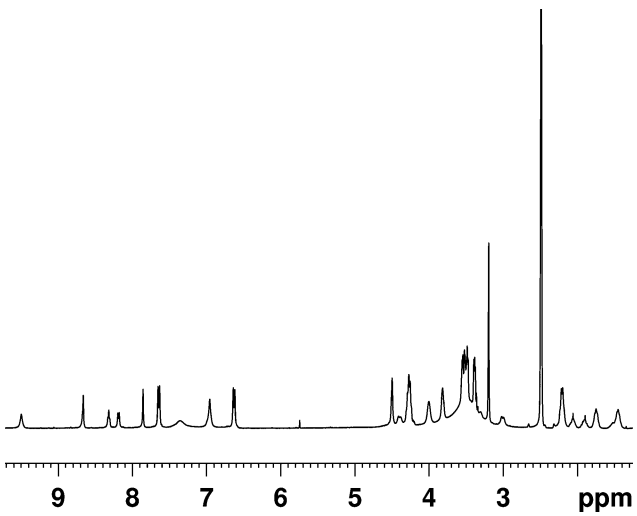
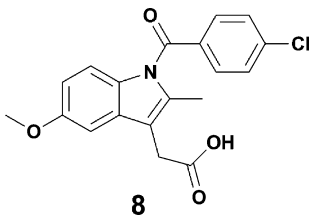


Fig. 1 ^{1}H NMR spectrum of **7**, DMSO-d_6, 297 K, 400.13 MHz.



ring was evidenced by a positive chemical shift difference ($\Delta\delta$ 22 ppm) between the C_4 and C_5 triazole carbons. The ESI-MS spectrum of compound **7** showed strong peaks at mass of 1740.2, 1159.85, and 869.2, which corresponded to $[M - 2H]^{2-}$, $[M - 3H]^{3-}$, and $[M - 4H]^{4-}$ ions, respectively. In addition, peaks at masses of 1652.6, 1101.6, and 825.7 were also observed, derived from each of the above cited ions after the fragmentation of one pteridinyl radical (176) from the folate residues.

As predicted, the ionization state of the carboxylic acid group is critical to the water solubility of folate–calix[4]arene **7**. Solutions of **7** were completely clear under alkaline conditions, but the solubility gradually decreased under acid conditions, forming a precipitate at about pH 5. The good water-solubility of folate–calix[4]arene **7** in PBS buffer (pH 7.4) was important for prospective biological applications.

In order to test the potential of the folate–calix[4]arene (**7**) as a molecular carrier, we decided to investigate its capability to interact with indomethacin, selected as hydrophobic drug model. Indomethacin (**8**) is a non-steroidal anti-inflammatory drug characterized by toxic side effects. For this reason the development of selective delivery systems for this drug is nowadays a challenge.[40]

The solubility method of Higuchi and Connors[41] showed that compound **7** improves indomethacin solubility in PBS buffer (pH 7.4). The drug solubility increased linearly with compound **7** concentration (0–2 mM range) and the phase–solubility profile (Fig. 2) showed the folate–calix[4]arene **7** formed a type A_L (1 : 1 molar ratio) complex with the drug. The slope value was less

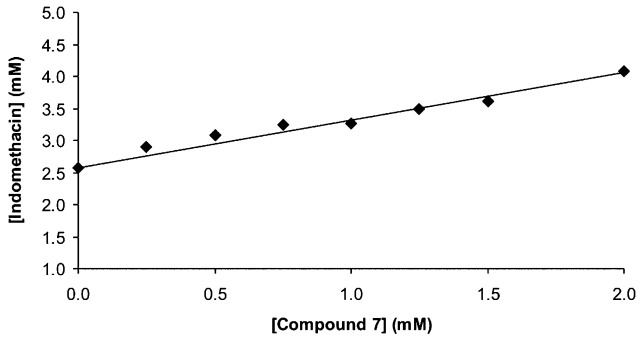


Fig. 2 Phase–solubility diagram of indomethacin in PBS, pH 7.4, at increasing concentration of folate–calix[4]arene (**7**).

than one suggesting, as for other calixarene–drug complexes,[42] the formation of 1 : 1 stoichiometry complex in solution.[43]

But, considering the amphiphilic nature of compound **7** and the presence of folate moieties, whose self-assembling properties are known,[44] aggregate formation in PBS medium can easily be imagined. This was confirmed by DLS measurements[45] showing that in the concentration range 2–0.04 mM, compound **7** formed large aggregates, in any case 99% of the mass of the sample had an average hydrodynamic radius of 5 nm.[39] Loftsson *et al.* reported that in the presence of aggregate species in solution, the stoichiometry of a complex cannot be derived from simple phase solubility diagram; an A_L type diagram does not necessarily indicate formation of 1 : 1 host/guest inclusion complex.[46] Therefore, as for other amphiphilic calixarene aggregates,[47] it is likely that drug solubilisation by compound **7** is based on drug–aggregate interaction.

^{1}H NMR experiments evidenced that compound **7**–drug interactions occur in PBS medium. The aromatic proton resonances of the drug solubilised in the presence of **7** appeared broader and upfield shifted ($\Delta\delta$ 0.05 ppm) compared to those of the free drug. A very broad ^{1}H NMR spectrum of compound **7** in PBS medium, consistent with its aggregate state, prevented the determination of the stoichiometry of the **7**–drug complex *via* NMR spectroscopy.

A more resolved proton spectrum was observed for the sodium salt of **7**, obtained by ionic exchange or dissolution in aqueous NaOH solution. The presence of monomeric species of **7** sodium salt (1 mM aqueous solution, pH 9) was detected by diffusion-ordered NMR spectroscopy (DOSY),[39] which provided a diffusion coefficient (D) of 1.69 ± 0.03 × 10^{-10} m^2 s^{-1} corresponding to a hydrodynamic radius (r_{exp}) of 1.27 ± 0.02 nm. This value was congruent with a medium radius (r_{calc}) of 1.29 nm, calculated by computer modeling[48] for the monomeric species of **7** sodium salt.

When an excess of indomethacin was added to a 1 mM basic aqueous solution of compound **7**, no change of the proton resonances and diffusion coefficients of both compound **7** and drug in ^{1}H NMR spectrum and 2D-DOSY experiment was observed.[49] That indicated that no **7**—drug interaction occurred.

Thus, as predictable, at high salt concentrations (PBS, pH 7.4) hydrophobic interactions are favoured and self-association of **7** and **7**–drug recognition occur, whereas at basic pH values hydrophobic interactions are disadvantaged probably as a result of an increase in charged groups, and only the free monomeric species of compound **7** and drug are detected. Further studies will elucidate the aggregate architecture and the drug solubilisation mechanism of **7** under physiological conditions.

Conclusions

In summary, a multivalent folate conjugate (**7**) in which four folate units are clustered by means of a calix[4]arene platform has been designed and synthesized. The presence of multiple folate homing moieties and a good water-solubility at physiological pH, in addition to the capability to increase indomethacin water-solubility are prerequisites that make **7** potentially appealing for targeted drug delivery. The efficiency of compound **7** in FR binding and cell uptake as well as targeted drug delivery has to be demonstrated and it will be matter of a future work.

The calix[*n*]arene family offers a variety of oligomers which might be engineered to meet the specific needs of novel targeted drug delivery systems with controlled characteristics. Therefore, compound **7**, as the first example of a calixarene-based folate conjugate, may open the way to a novel class of multivalent and multifunctional targeting systems in which drugs and/or imaging agents are covalently or non-covalently conjugated to multiple homing moieties by means of a calix[*n*]arene scaffold.

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