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Cyclodextrin-calixarene nanosponges as potential platforms pH-dependent delivery of Tetracycline

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Abstract:	Four mixed cyclodextrin-calixarene nanosponges, were tested as possible Drug Delivery Systems, using Tetracycline antibiotic as a suitable model drug. The selected nanosponges featured a different composition ratio between the two host co-monomer components and by the possible presence of ionisable amine or carboxyl groups deriving from chemical post-modification. The pH-dependent absorption and release abilities of the materials were verified; in particular release kinetics showed the occurrence of a simple first-order profile. The antibacterial activity of nanosponge-tetracycline composites suitably prepared under sterile conditions was assayed towards both Gram-positive and Gram-negative typical bacterial strains, showing in some cases an interesting improvement of the biocidal activity.
Author Comments:	To the Editor of ChemistrySelect Palermo, June 28th 2019 Sir, I'm pleased to submit you our manuscript entitled "Cyclodextrin-calixarene nanosponges as potential platforms pH-dependent delivery of Tetracycline", by R.M. Fontana, N. Milano, L. Barbara, A. Di Vincenzo, G. Gallo and P. Lo Meo, for publication on ChemistrySelect. By means of an integrated approach joining chemical and biological methodologies, this communication is aimed at showing how the pH-responsivity of the nanosponge sorbent can be profitably exploited to build up smart

	systems for drug delivery. Therefore, in our opinion, our results might be of interest for researchers in the fields of Supramolecular Chemistry, Materials Chemistry and Medicinal Technologies. Sincerely yours. Prof. Paolo LO MEO
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FULL PAPER

Cyclodextrin-calixarene nanosponges as potential platforms pH-dependent delivery of Tetracycline

Rosa Maria Fontana, [a] Nicola Milano, [a] Lorenzo Barbara, [a] Antonella Di Vincenzo, [a] Giuseppe Gallo*[a] and Paolo Lo Meo*[a]

Abstract: Four mixed cyclodextrin-calixarene nanosponges, were tested as possible Drug Delivery Systems, using Tetracycline antibiotic as a suitable model drug. The selected nanosponges featured a different composition ratio between the two host co-monomer components and by the possible presence of ionisable amine or carboxyl groups deriving from chemical post-modification. The pH-dependent absorption and release abilities of the materials were verified; in particular release kinetics showed the occurrence of a simple first-order profile. The antibacterial activity of nanosponge-tetracycline composites suitably prepared under sterile conditions was assayed towards both Gram-positive and Gram-negative typical bacterial strains, showing in some cases an interesting improvement of the biocidal activity.

Introduction

The need of improving drug efficacy, and minimizing the dosage as well, has stimulated the development of novel Drug Delivery Systems (DDSs).[1] A DDS is a formulation or device designed for achieving effective pharmacokinetics, biodistribution and pharmacodynamics of a biologically or pharmacologically active compounds.[2] In recent times, nanostructured systems such as metal nanoparticles,[3] halloysite,[4] nano-clays[5] and mesoporous silica[6] based materials, graphene derivatives[7] and carbon nanoforms,[8] nanogels[9] and so on, have been largely employed to build DDSs with tunable and stimuli-sensitive properties.[10] These systems take advantage from their intrinsic small size, allowing them to reach easily any tissue or cell districts, as well as on their chemical versatility and ability to modify their physico-chemical properties in response to a specific condition, such as pH changes or light irradiation. In particular, Nanosponges (NS) have been increasingly employed to obtain effective DDS.[11] NSs, which constitute an emerging area of both supramolecular and materials chemistry, are hyper-cross-linked polymers obtained by reticulation of typical supramolecular host species (in particular cyclodextrins or calixarenes) with suitable linker reagents.[12] Therefore, NSs may exploit the intrinsic binding abilities of the host monomer in order to accomplish absorption or controlled release of diverse organic guest species. Some of us have recently reported on the preparation, characterization and pH-dependent sequestration and release abilities of mixed Cyclodextrin-Calixarene NSs (CyCaNSs, Scheme 1).[13] These materials can be easily prepared by means of the well-known *CuAAC* reaction approach, i.e. by reacting a poly-azido β-cyclodextrin (**ACD**) derivative and a poly-propargyloxy calix[4]arene derivative (**PCA**) in the presence of Cu(I) as the catalyst. Noticeably, the two co-monomers can be reacted in a non-stoichiometric ratio to afford materials which can be subjected in turn to chemical post-modification, exploiting the presence of unreacted azide or alkyne groups. The physico-chemical features of the materials, and their absorption and release abilities as well, can be largely varied depending on the particular structure and the molar ratio of the co-monomers.Some CyCaNSs (namely, materials **NS1** and **NS3** of Scheme 1) have been tested with promising results as potential DDS for Quercetin and Silibinin delivery towards some selected cancer cell lines.[14] So, in the present study we investigated the possible exploitation of CyCaNSs as DDSs for the antibiotic Tetracycline (**Tet**, Figure 1), used as tester compound. **Tet** is a broad-spectrum antibiotic used to treat various bacterial infections, including those associated to acne,[15] cholera,[16] brucellosis,[17] plague,[18] malaria,[19] and syphilis.[20] It is able to bind the 30S subunit of bacterial ribosome,[21] preventing aminoacyl-tRNA interaction with ribosome site A and the introduction of new amino acids to the nascent polypeptide chain, which ultimately results in inhibiting bacterial protein synthesis. We considered for this work two "pristine" materials, **NS1** and **NS3**, having a **ACD**/**PCA** co-monomer composition ratio as large as 0.93:1 and 0.29:1 mole/mole respectively, and two post-modified materials **NS2** and **NS4**, obtained respectively from **NS1** by Staudinger reduction of the excess azide groups, and from **NS3** by insertion of carboxyl functionalities exploiting the excess alkyne groups (Scheme 1). We verified the absorption and release abilities of materials **NS1**-**NS4** towards **Tet** at different pH values, and we assessed the antimicrobial activity of nanosponge-**Tet** composites towards four model bacterial strains that are routinely used in drug discovery and efficacy testing, namely *Escherichia coli* and *Pseudomonas aeruginosa* as representative Gram negative tester strains, *Staphylococcus aureus* and *Bacillus subtilis* as Gram positive tester strains.

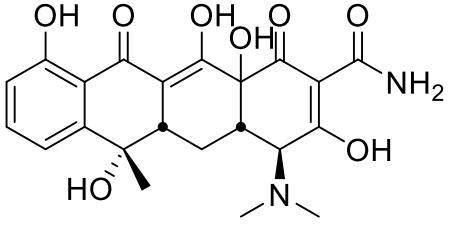

Figure 1. Structure of Tetracycline.

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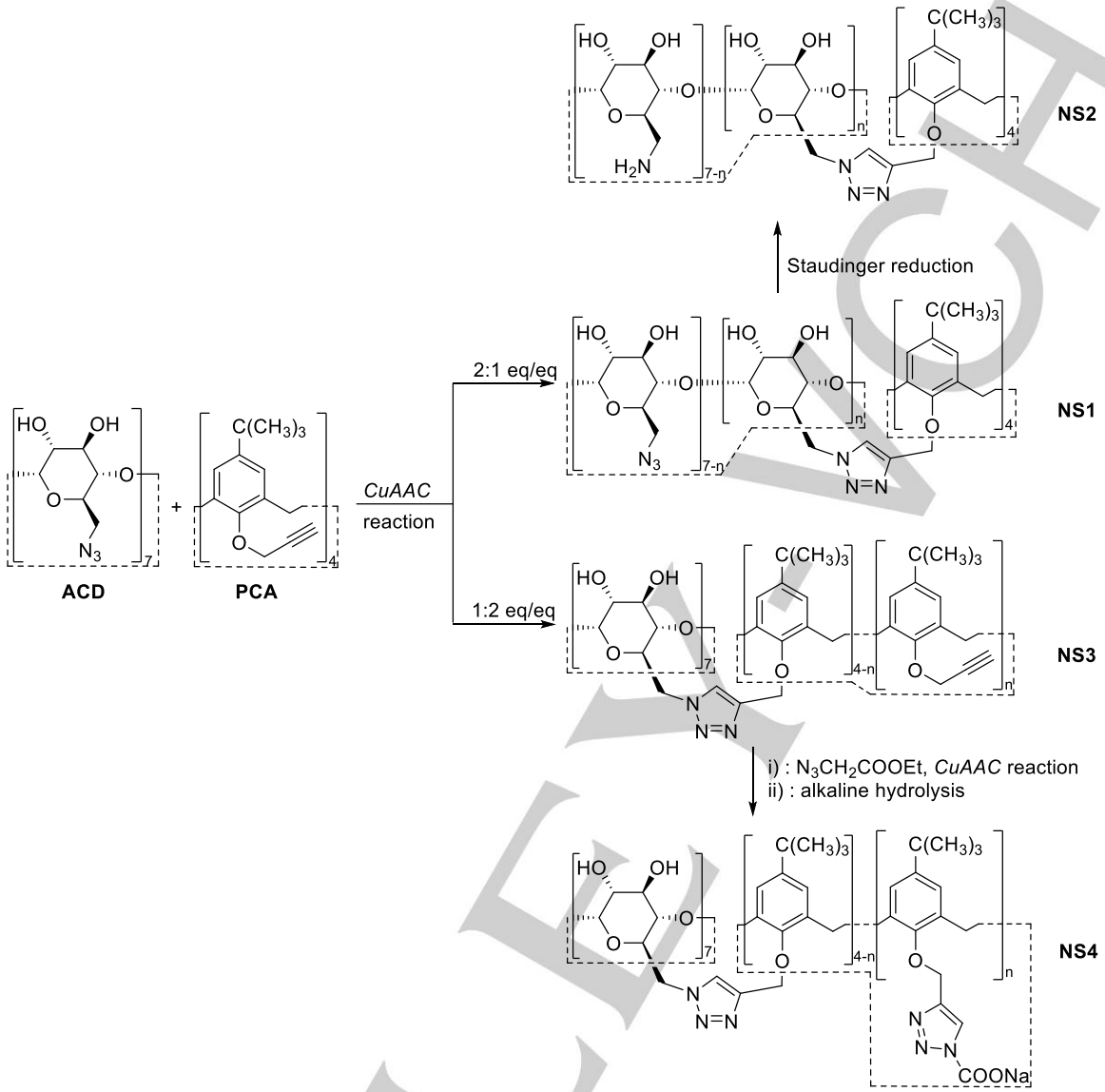

Scheme 1. Synthesis of **CyCaNS**s **NS1**-**NS4**.

Results and Discussion

Sequestration and release tests. As a first step, we verified the ability of our CyCaNSs to interact with **Tet**, by performing a series of sequestration tests, *i.e.* by equilibrating a given amount of each nanosponge with a fixed volume of a standard buffered aqueous solution of **Tet**. The tests were performed at two different pH values, namely 4.4 and 6.7; the relevant results are collected in Table 1. The two pH values were chosen in order to be consistent with our previous works,[13c] and because they represent conditions occurring in different districts of the intestinal tract. In general, it has been shown that the sequestration properties of

Table 1. Sequestration tests.[a]

	NS1	NS2	NS3	NS4
pH 4.4	36	15	65	38
pH 6.7	71	86	73	80

[a] All data given within a ± 3% indetermination.

CyCaNSs may be significantly affected by pH variations, and largely depend on the electrostatic interactions between the guest and the co-polymer matrix.[13] Accordingly, we can observe that the affinity of **Tet** towards the materials largely decreases on

passing from pH 6.7 to 4.4. This can be easily explained considering the occurrence of an unfavorable Coulomb interaction between the positively charged guest and the average positive charge of the matrix due to protonation of the ionizable functional groups (1,2,3-triazole, amine, carboxylate) present in the polymer. For the sake of clarity, it is worth recalling here that, on the grounds of its pK_a values,[22] **Tet** is in its zwitterionic form at both the operational pH values.

We subsequently prepared four composites by equilibrating each CyCaNS with a 20% w/w amount of **Tet** (see Supporting Information for experimental details). The actual resulting drug loading was determined evaluating the amount of **Tet** eluted away from a given amount of composite by repeated washing with methanol. In detail, loadings resulted as large as 19.2 ± 0.2 % w/w for **NS1**, 10.2 ± 0.2 % w/w for **NS2**, 13.4 ± 0.2 % w/w for **NS3**, 9.9 ± 0.2 % w/w for **NS4**, respectively. The composites obtained were then subjected to controlled release tests at 37 °C, under the same pH conditions (4.4 and 6.7) used for sequestration tests, in order to study the relevant kinetics. Typical release curves are shown in Figure 2, whereas relevant kinetic data are collected in Table 2. All the release curves can be rationalized according to simple first-order-like kinetics; relevant non-linear data regression analysis enabled to obtain the apparent kinetic constant (k_{app}) for the release process and the concentration of drug at equilibrium (C_0).

In general, release occurs faster and leaves at equilibrium a larger amount of guest at the more acidic pH value, in perfect agreement with the decrease in absorption abilities of the materials observed hereinabove. The release rates from the material **NS4** seem anomalously large as compared to the other cases. This could be reasonably explained considering that **NS4** is subjected to severe stress during its preparation,[13b] resulting in large mechanical fragmentation; consequently, faster release might simply result from the increased particle surface exposed to the extraction medium. On the other hand, the effect of basic ionizable groups in the material matrix is apparent from the comparison between **NS1** and **NS2**. In fact, the latter one releases **Tet** nearly three times faster than the former at both pH values. Noticeably, on the grounds of data reported in Table 2 one can easily calculate that **NS1** releases at equilibrium a significantly smaller amount of **Tet** (29% at pH 4.4, 9 % at pH 6.7 of the loaded guest) in comparison with the other materials; on the contrary, **NS2** shows the largest percent releases (93% at pH 4.4, 72% at pH 6.7). It is worth noting here that the behavior of **Tet** is different from the one found in a previous work for the release of a neutral drug guest such as Quercetin.[14] In the latter case, indeed, release was much slower, and provided a short first-order step followed by a long-lasting apparent zeroth-order step, which was justified assuming a relatively fast release of the guest adsorbed on the surface shell of the material grains followed by a much slower release from the inner core, controlled by mass transfer phenomena. In the present case the occurrence of a simpler trend is consistent with the much larger hydrophilic character of **Tet** and the occurrence of relatively unfavorable Coulomb interactions with the nanosponge matrix, in comparison with the hydrophobic interactions prevailing for the inclusion of a neutral guest such as Quercetin. Finally, from the k_{app} values reported in Table 2 it can be calculated that at pH 6.7 (*i.e.* under the pH conditions closer to the ones used for biological assays), the release half-times for the materials **NS1**, **NS2**, **NS3** and **NS4** are as large as 64 min, 29 min, 23 min and 6 min respectively.

The whole of these results helped us to choose the most suitable composites to proceed with antimicrobial assays. In particular, we first selected the composite **Tet**-**NS1** because it presents the slower and less abundant release of the biocidal agent. On the opposite, **Tet**-**NS2** showed the largest release, with a relatively quick kinetics. We considered **Tet**-**NS4** unsuitable for our purposes because its release kinetics appeared too fast to show significant differences in behavior with the use of the free antibiotic agent; on the other hand, **Tet**-**NS3** shows similar features as **Tet**-**NS2**. Hence, keeping into consideration also the

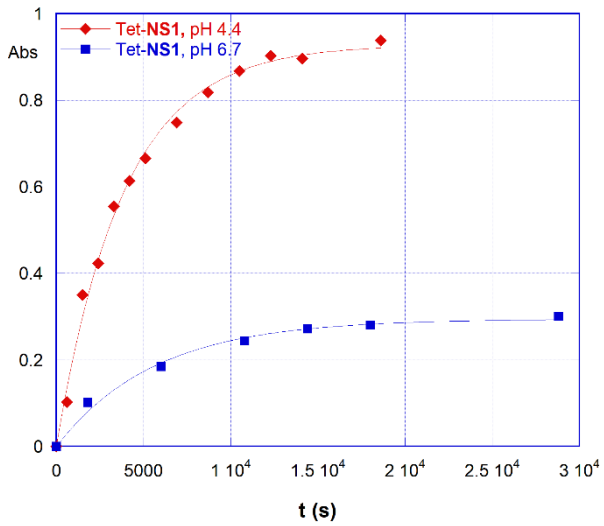

Figure 2. UV-vis release kinetic profiles for the **Tet**-**NS1** composite.

Table 2. Kinetic parameters for Tet release from the composites with CyCaNSs.

	Tet-NS1		**Tet-NS2**	
included Tet (% w/w)	19.2 ± 0.2		10.2 ± 0.2	
pH	4.4	6.7	4.4	6.7
C_0 (μM)	58 ± 1	18.4 ± 0.5	98 ± 2	76 ± 1
k_{app} (10^{-4} s^{-1})	2.6 ± 0.1	1.8 ± 0.2	7.4 ± 0.3	5.0 ± 0.2
	Tet-NS3		**Tet-NS4**	
included Tet (% w/w)	13.4 ± 0.2		9.9 ± 0.1	
pH	4.4	6.7	4.4	6.7
C_0 (μM)	90 ± 9	73 ± 7	68 ± 2	60 ± 2
k_{app} (10^{-4} s^{-1})	3.5 ± 0.4	4.0 ± 0.4	28.3 ± 0.6	18.0 ± 0.4

similarity in the co-monomer composition of the materials, we selected composites **Tet**-**NS1** and **Tet**-**NS2** for biological tests.

Antibacterial activity assays. The antibacterial activities of **Tet**-loaded CyCaNS composites were quantified by evaluating the minimum concentration of substance able to inhibit 90 % of bacterial growth (MIC_{90}) against the selected bacterial tester strains. From a methodological point of view, it is worth stressing here that the need to perform antimicrobial assays under sterile conditions forced us to suitably modify the procedure for the preparation of the composites (see experimental details in the Supporting Information), which resulted in significantly different loadings as compared to the previously described preparation (namely, 2.5 % w/w for **Tet**-**NS1** and 7.5 % for **Tet**-**NS2**). Moreover, because the composites were subjected to pasteurization, pasteurized **Tet** solution suspended in PBS buffer (indicated hereinafter as *PT*), was necessarily used as a positive control for the assays.

Table 3. Diameters (mm) of *E. coli* growth inhibition halos.

	1 µg	5 µg	10 µg
ET	9 ± 1	12 ± 1	17 ± 1
UT	7 ± 1	13 ± 1	15 ± 1
PT	7 ± 1	12 ± 1	15 ± 1

Finally, we tested the efficacy of our composites towards the selected tester bacterial strains by evaluating the relevant MIC_{90} values (Table 4); for this purpose, both *PT* and *ET* were used as controls. Bacterial growth inhibition assays revealed that on average the **Tet**-**NS1** composite was able to exert a comparable antimicrobial activity with respect to *PT*; on the other hand, the **Tet**-**NS2** composite interestingly showed an improved activity in comparison with both *PT* and *ET* with all the tester strains. The latter finding might be tentatively explained as resulting from the combined effect of the relatively fast release discussed previouslyand the possible occurrence of a favorable coulomb interaction between the negatively charged peptidoglycan component of the bacterial cell wall and the averagely positive charge on the surface of the nanosponge grains due to the presence of partly ionized amine groups.

Table 4. MIC_{90} values (µg/mL) against the tester bacterial strains.

	NS1-**Tet**	**NS2**-**Tet**	*PT*	*ET*
E. coli	3.5	0.5	2.5	1
P. aeruginosa	5.5	4	10	5
B. subtilis	3.5	2	2.5	5
S. aureus	10	2	10	5

Conclusions

In the present work we showed how pH-responsive cyclodextrin-calixarene nanosponges (CyCaNSs) may constitute an appealing platform for obtaining smart DDS systems with improved release abilities. In particular, these materials were tested as carriers for a typical model antibiotic such as tetracycline (**Tet**). Both absorption and release abilities of the materials were proven to be pH-dependent and consistently with previous literature[12b,13] could be easily rationalized in terms of prevailing coulomb interactions between the positively charged guest molecule and the average charge density on the polymeric framework. Moreover, the materials proved to be perfectly treatable under sterile conditions and showed no biocidal activity. On the other hand, antibacterial assays towards typical Gram-positive and Gram-nagative tester bacterial strains showed that **Tet**-CyCaNS composites may show comparable or ever improved activity in comparison to **Tet** alone. Once again, the latter result might be tentatively justified on the grounds of particularly favourable coulomb interactions occurring between the NS carrier and the bacterial cell wall.

In perspective, in our opinion this work opens potential opportunities for the development of new DDS with improved performances. Further biological studies to clarify the possible mechanisms implied in the CyCaNS-bioactive molecule-bacterial cell interaction are currently in progress.

Supporting Information Summary

Supporting Information reports the complete Experimental Section: Materials, Bacterial strains, Instrumentation, Sequestration tests, Preparation of the non-sterile composites, Release kinetics, Tetracycline pasteurization and preparation of the sterile composites, Agar diffusion assays, Determination of Minimal Inibitory Concentration (MIC_{90}).

Keywords: Calixarene • Cyclodextrin • Drug delivery • Nanosponges • Tetracycline

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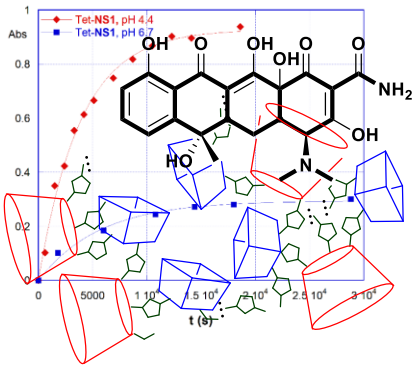

The pH-tunable adsorption and release abilities of cyclodextrin-calixarene mixed nanosponges, make them ideal candidates as new versatile Drug Delivery Systems. We tested this possibility using a well-known model antibiotic such as Tetracycline. The antibacterial activity of nanosponge-tetracycline composites was successfully assayed towards typical bacterial tester strains.