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Solubilization and Release Properties of Dendrimers. Evaluation as Prospective Drug Delivery Systems

Luciana Fernandez[a]; Mercedes Gonzalez[b]; Hugo Cerecetto[b]; Marisa Santo[c]; Juana J. Silber[b]
[a] Departamento de Química, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina [b] Departamento de Química Orgánica, Facultad de Química—Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay [c] Departamento de Física, Universidad Nacional de Río Cuarto, Río Cuarto, Argentina

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Solubilization and Release Properties of Dendrimers. Evaluation as Prospective Drug Delivery Systems

LUCIANA FERNANDEZ[a], MERCEDES GONZALEZ[c], HUGO CERECETTO[c], MARISA SANTO[b,*] and JUANA J. SILBER[c]

[a]*Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, 5800 Río Cuarto, Argentina;* [b]*Departamento de Física, Universidad Nacional de Río Cuarto, Agencia Postal Nro 3, 5800 Río Cuarto, Argentina;* [c]*Departamento de Química Orgánica, Facultad de Química—Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay*

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The polarity and accessibility to the interior of several dendrimers using phenanthrene, anthracene and tetrabenzonaphtalene as probe molecules have been investigated. In addition the prospective application of the dendrimers as drug carriers was evaluated by incorporating 5(6)-methylbenzo[1,2-c]1,2,5-oxadiazole N_1-oxide] (1) and 2′-(benzo[1,2-c] 1,2,5-oxadiazol-5(6)-yl(N_1-oxide) methylidene]-1-methoxy methane hydrazide (2). These compounds have antichagasic therapeutic activity but very low water solubility, which limits their application. Polypropylene imine dendrimers with amine terminal groups (DAB-16AT and DAB-32AT) and polyamide amine (PAMAM) dendrimers with carboxylate terminal groups (PAMAM-32CT), with amine terminal groups, (PAMAM-8AT and PAMAM-32AT) and with hydroxyl terminal groups (PAMAM-32OHT) were chosen for this study. Approximately one molecule of phenanthrene or anthracene was encapsulated in PAMAM-32CT, PAMAM-32AT, PAMAM-32OHT and DAB-32AT dendrimers. However, slight encapsulation was observed working with PAMAM-8AT and DAB-16AT. The studies with tetrabenzonaphtalene show that the guest molecule might only be partially caged within the dendrimer host. However, for relatively insoluble solutes the efficiency to encapsulate can be dictated by the saturation in the aqueous phase besides the dendrimer capacity to dissolve it. These dendrimers are also able to encapsulate and consequently solubilize 1 and 2 oxadiazol. However, PAMAM dendrimers are better for encapsulation and retention due to guest-host specific interactions. These interactions can be diminished by lowering the pH to allow a controlled deliverance of the drug.

Keywords: Dendrimers; Polymers; Encapsulation; Antichagasic activity; Solubility; Host-guest interactions

INTRODUCTION

The efficacy of therapeutic agents is affected by their ability to gain access to the active site in an appropriate dose [1]. This ability habitually depends on the lipophilicity of molecules, a complex physicochemical property that is usually correlated with the capacity of therapeutic agents to penetrate the different hydrophobic barriers and with the effectiveness in its activity [2–4]. A large number of highly active pharmaceuticals are lipophilic, and the poor solubility in water affects their efficacy [5]. It is possible to adjust the lipophilicity of the drug by modifications in its structure, however; unfortunately even small structural changes can reduce the usefulness of the active compound. Another possibility is to encapsulate the drug into macromolecular carriers, in order to facilitate its transport [6]. The encapsulation of drugs consists on housing a guest in the lipophilic microenvironment present in the system that will act as a host. Dendrimers are highly branched macromolecules that were intensely studied with this purpose [7,8]. They have a single molecular weight, a large number of controllable peripheral functionalities and tendency to adopt a globular shape once a certain size is reached [9]. Dendrimers also offer unique opportunities in these applications since the structure of these macromolecules can be specifically tuned to the requirements of the delivery system [10–12]. The spherical geometry of these polymers defines a usually hydrophobic interior that provides a lipophilic microenvironment where a guest molecule is possible to be housed particularly drugs that are not soluble enough in water [13]. In contrast, the exterior of some dendrimers is hydrophilic, this property facilitates their circulation in the biological environments. With these characteristics, the

*Corresponding author. E-mail: msanto@exa.unrc.edu.ar

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dendrimers behave as unimolecular micelles capable of solubilizing hydrophobic compounds and carry out the controlled distribution of substances in the body [14]. It is interesting that a single dendrimer can act not only as a micelle but also be designed as a reverse micelle, being also attractive from the point of view of mimicking biological systems and having applications in entrapping and stabilizing protein molecules in organic solvents [15]. Another strategy for the encapsulation of guest molecules in dendrimers can be based in multi non covalent chemical interactions such as hydrogen bonding [7].

Dendrimers not only show an extraordinary structural control at nanoscale size but also have outstanding features such as: mimicry of globular proteins [16], lack of immunogenicity [17] and low toxicity, especially when their surface contains anionic or nonionic groups, such as carboxylic or hydroxylic functionalities [18]. These features have made their application in pharmaceutical and medicinal chemistry particularly attractive [19,20]. Dendrimers are actually studied mainly for these potentials applications in drug delivery [21,22]. The solubilization properties have been investigated for several probes. Thus, Richter-Egger *et al*. [23] primarily addressed the near-surface polarity and surface accessibility for diverse generations of amino terminal poliamidoamine (PAMAM) dendrimers using Reichardt's dye ($E_T(30)$). They found that the microenvironment polarity in the interior of these dendrimers is similar to 1-decanol. These authors also developed studies using the fluorescent, solvatochromic probe phenol blue in aqueous solutions [24], showing that the dye is associated inside the dendrimer and does not interact with the surface groups. Sideratou *et al*. [25] investigated the solubilization and release properties of functionalized PEGylated diaminobutane poly (propylene imine) (DAB) dendrimers using pyrene as probe. They reported enhanced solubility and increased protection of pyrene from aqueous solutions. They also evaluated the incorporations in these dendrimers of betamethasone corticosteroids as active ingredients. In another study besides PEG chains, guanidinium units were incorporated into the periphery to DAB dendrimers to make them useful for targeted drug delivery [26]. They found that these new dendrimers have higher loading capacity for guest encapsulation. Beezer *et al*. [1] recently published the synthesis of water-soluble dendrimers and they evaluated their ability to bind small acidic hydrophobic molecules. Spectroscopic data and pH behavior suggest that the guest was forming stable ion pairs with the basic tertiary nitrogen present in the interior of the dendrimer.

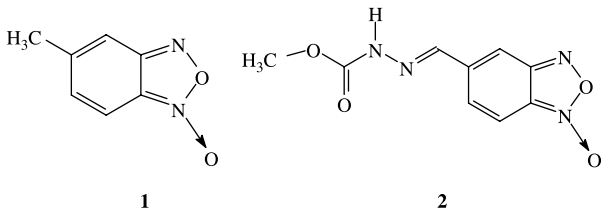
Our previous studies using functionalized polyamide amine dendrimers were focalized in the analysis of the interactions between dendrimers and several biologically important guests. Association constant obtained from changes in the ^{1}HNMR chemical shifts of the amide protons in the host indicated two different interaction sites inside and on the periphery of the dendrimer. Binding at the inner site is inhibited in the ester-terminated dendrimers [27].

An improved understanding of the solubilization properties of the interior regions of dendrimers is vital to their successful use in future applications as drug delivery carriers. With this aim, in the initial phase of this work we have examined the polarity, dimension and accessibility of dendrimers interior microenvironments using polycyclic aromatic hydrocarbons as probe molecules. According to the literature [28], polycyclic aromatic hydrocarbons should associate with dendrimers in aqueous solutions. Experimental evidences show that, for example pyrene and perylene derivatives are absorbed in the interior of carboxylate-terminated PAMAM dendrimers.

In this work, solubilization experiments using polycyclic aromatic compounds: phenanthrene, anthracene and tetrabenzonaphtalene, as probes guest, were performed to estimate the cavity accessibility of several type of dendrimers. These hydrocarbons are lipophilic compounds with low solubility in water and phenanthrene and anthracene were selected, in particular, for this study because they have similar size to that of the therapeutic compounds studied.

In fact, to evaluate the potential use of these macromolecules as drug delivery vehicles, the solubilization of oxadiazol derivatives (Fig. 1) in its lipophilic interior was investigated. These compounds exhibited potential antichagasic activity [29] but have poor solubility in water. Therefore, to encapsulate these compounds in a water-soluble carrier seems necessary in order to facilitate their application as drugs.

Two-dendrimer-family, polyamide amine (PAMAM) dendrimers and polypropylene imine (DAB) dendrimers were chosen for this study (Fig. 2). PAMAM dendrimers with carboxylate terminal groups (PAMAM-32CT), with amine terminal groups, (PAMAM-8AT and PAMAM-32AT), with hydroxyl terminal groups (PAMAM-32OHT) and (DAB) dendrimers with amine terminal groups (DAB-16AT and



1 **2**

FIGURE 1 Structure formula of the therapeutics compounds (guest molecules) **1**: 5(6)-methylbenzo[1,2-c]1,2,5-oxadiazole N_1-oxide and **2**: 2′-(Benzo[1,2-c]1,2,5-oxadiazol-5(6)-yl(N_1-oxide)-methylidene]-1-methoxymethane hydrazide.

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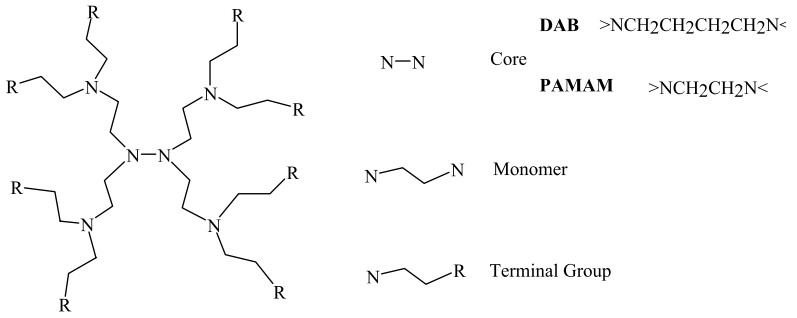

Dendrimer	Monomer	Terminal Group
PAMAM-8AT	>$NCH_2CH_2C(O)NHCH_2CH_2N$<	>$NCH_2CH_2C(O)NHCH_2CH_2NH_2$
PAMAM-32AT	3(>$NCH_2CH_2C(O)NHCH_2CH_2N$<)	>$NCH_2CH_2C(O)NHCH_2CH_2NH_2$
PAMAM-32OHT	3(>$NCH_2CH_2C(O)NHCH_2CH_2N$<)	>$NCH_2CH_2C(O)NHCH_2CH_2OH$
PAMAM-32CT	3(>$NCH_2CH_2C(O)NHCH_2CH_2N$<)	>$NCH_2CH_2COO^-$
DAB-16AT	2(>$NCH_2CH_2CH_2N$<)	>$NCH_2CH_2CH_2NH_2$
DAB-32AT	3(>$NCH_2CH_2CH_2N$<)	>$NCH_2CH_2CH_2NH_2$

FIGURE 2 Chemical structure of the dendrimer hosts I) PAMAM-8AT, II) PAMAM-32CT, III) PAMAM-32AT, IV) PAMAM-32OHT, V) DAB-16AT, VI) DAB-32AT.

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DAB-32AT) were compared. Since PAMAM and DAB have different structure in their interior a comparative study allows to analyze the effect of the chemical characteristics of the monomers unities in their guest association capacity. In addition, since the PAMAM dendrimers have different terminal groups the effect of their polarity and charge in the encapsulation and release properties was analyzed.

RESULTS AND DISCUSSION

In Table I several physicochemical properties [30,31] of importance to detect different type of interactions that could involve the guest molecules analyzed in this study are collected. The octanol–water partition coefficient (Log $P_{o/w}$) has been used as lipophilicity parameter [31,32] the molecular volume, V, and the dipole moment, μ, have been used as a measure of the size and the polarity respectively.

Solubilization of the Polycyclic Aromatic Hydrocarbons

The increase in solubility of the probe guest in the dendrimers solution with respect to water indicates how much hydrocarbon will associate with the dendrimer. The changes in solubility of the probes were followed either by absorption or fluorescence measurements depending on the sensitivity to detect such changes.

In the case of anthracene emission was used, its spectra in aqueous solutions are shown in Fig. 3. The changes observed in water solution reveal the

TABLE I Physicochemical properties of the guest molecules

Compounds	$\mu^{\dagger}$ (Debyes)	Volume† (Å)^3	Log $P_{o/w}$ Calc†	Log $P_{o/w}$ Obs	Water solubility (M)
Phenathrene	0.018	652	4.53	4.46[‡]	1×10^{-5}[§]
Anthracene	0.008	613	4.61	4.45[‡]	2×10^{-7}[§]
Tetrabenzonaphtalene	0	906	7.06	–	1×10^{-8}[‖]
1	4.776	451	3.42	2.18[¶]	1.5×10^{-3}[‖]
2	5.101	660	3.08	2.02[¶]	2×10^{-4}[‖]

[†] Calculated using AM1 Semiempirical calculations and Chem plus; [‡] from Ref [30]; [¶] from Ref [31], [§] from Ref [33], [‖] calculated from spectroscopic measurements.

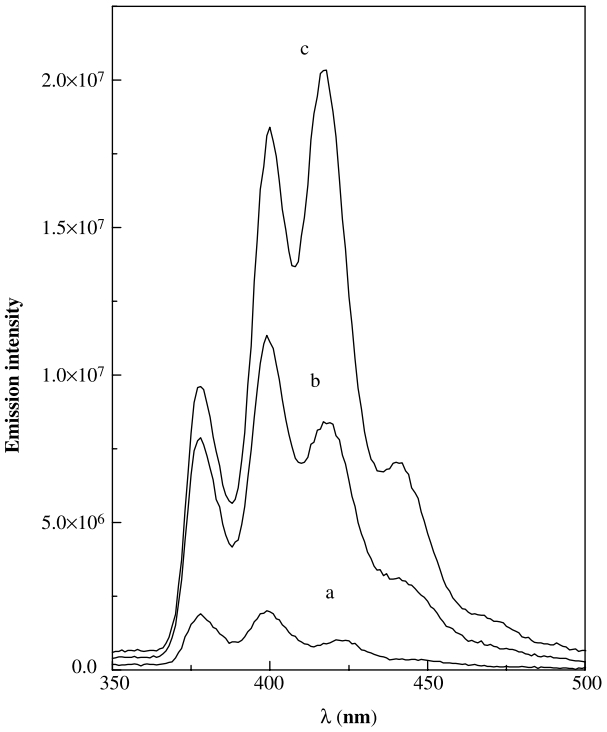

FIGURE 3 Fluorescence emission spectra of anthracene in water at a) 2×10^{-7} M, b) 6×10^{-6} M and c) 4×10^{-5} M.

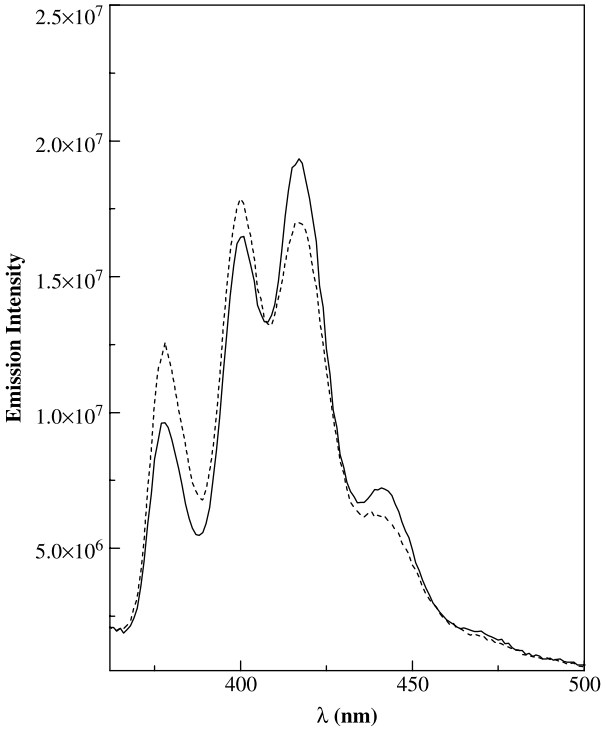

FIGURE 4 Fluorescence emission spectra of anthracene (—) in water and (----) in aqueous PAMAM-32AT solutions. [Anthracene] $= 3 \times 10^{-5}$ M, [PAMAM-32AT] $= 8 \times 10^{-5}$ M.

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formation of higher order species [33]. As it can be observed the fluorescence spectra of anthracene at low concentrations resemble the monomer emission [33]. As the concentration is increased the relative intensity of the band at $\lambda = 418$ nm respect to the one at $\lambda = 382$ nm increases notoriously. Dabestani *et al*. [34] reported that the increase of the emission band around 420 nm in the fluorescence of anthracene on dry silica as a function of the surface coverage, is due to a ground state stable pair formed between two anthracene molecules. The spectral changes observed in Fig. 3 are interpreted as a consequence of this type of aggregation of anthracene in water.

Figure 4 shows the effect of the addition of PAMAM-32 AT to these solutions. A decrease in the contribution of the emission of the stable pair accompanied by an intensity increase in the monomeric bands of anthracene is observed. This shows that the dendrimer breaks aggregated and increases its solubility.

The increase of solubility of anthracene in presence of PAMAM-32AT observed, afforded a concentration of 10 μM, this is a 50-fold improvement of the poliaromatic solubility in water without dendrimers (0.2 μM). The study of anthracene in PAMAM-32CT, PAMAM-32OHT and DAB-32AT dendrimers provides similar results showing increase of fluorescence emission of the monomer compared with pure water.

Aqueous solutions of phenanthrene also show increase in emission intensity when adding dendrimers. In addition, the absorbance data for this guest provide strong evidence that phenanthrene associates with dendrimers. For example, its absorbance increases three times in PAMAM-32AT aqueous solutions when compared with pure water (Fig. 5).

It should be noted that only a slight increase of solubility was observed for anthracene and phenanthrene working with small dendrimers such as PAMAM-8AT and DAB-16AT. Probably these dendrimers are incapable of encapsulating the guest. This observation is consistent with the globular structure required for encapsulation by a dendrimer [14].

Tetrabenzonaphtalene shows the increase of fluorescence emission intensity in the presence of PAMAM-32AT, PAMAM-32CT, PAMAM-32OHT and DAB-2AT dendrimers compared with pure water. For example the increase of fluorescence intensity of tetrabenzonaphtalene in PAMAM-32AT aqueous solutions is showed in Fig. 6.

The increase of intensity in either the fluorescence or absorbance observed in the spectra of these compounds was attributed to additional solubilization of the aromatic molecules in the lipophilic interior of dendrimers. The guest isolates itself from the outer interface of the host to afford minimum contact with polar and aqueous domains.

To quantify the effectiveness of a particular dendrimer in solubilize the studied aromatic compounds, the enhancement solubilization factor (ESF) defined as the number of moles of compound solubilized per number of moles of dendrimers was

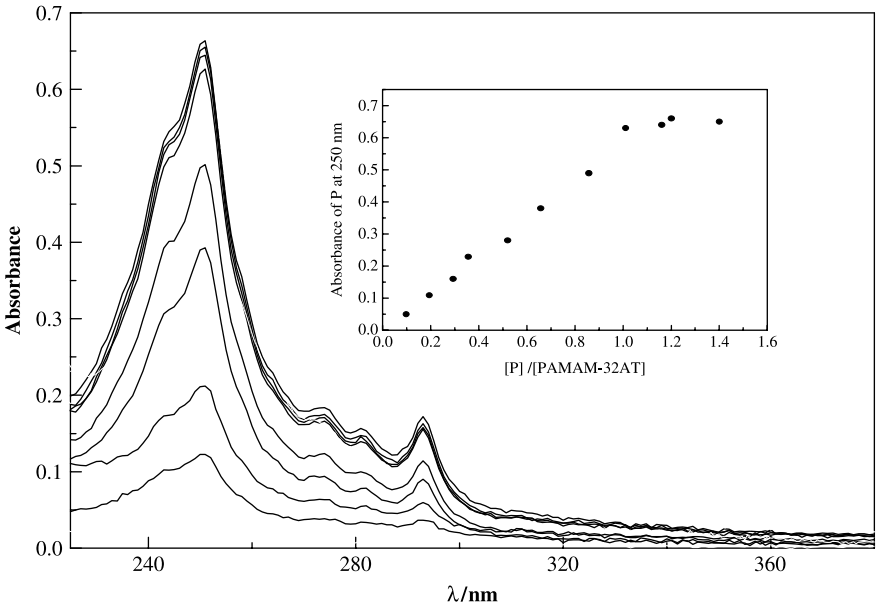

FIGURE 5 Absorption spectra of phenanthrene in water and in aqueous solutions of PAMAM-32AT. [Phenanthrene]: a) 3×10^{-5}, b) 4.5×10^{-5}, c) 7.6×10^{-5}, d) 9.5×10^{-5}, e) 11×10^{-5}, f) 12.5×10^{-5}, g) 15×10^{-5} M. [PAMAM-32AT] $= 1 \times 10^{-4}$ M. Insert: Comparison of absorbance of phenanthrene, at 250 nm, for various ratios of Phenanthrene/PAMAM-32AT.

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calculated, using Eq. (1).

$$\mathrm{ESF} = \frac{[\mathrm{H}]_d}{[\mathrm{D}]_w} = \frac{[\mathrm{H}]_o - S_w}{[\mathrm{D}]_w} \quad (1)$$

Where $[\mathrm{H}]_d$ is the guest concentration in aqueous solutions of dendrimers, $[\mathrm{D}]_w$ is the concentration of dendrimers in aqueous solutions, $[\mathrm{H}]_o$ is the analytical concentration of the guest and S_w is the water solubility of the guest. A similar factor, namely molar solubilization ratio was utilized by An et. al to quantify the solubilization of polycyclic aromatic hydrocarbons in micelles [35].

The results are summarized in Table II. As it can be observed the lipophilic interior of dendrimers allows a variable degree of encapsulation of these hydro-

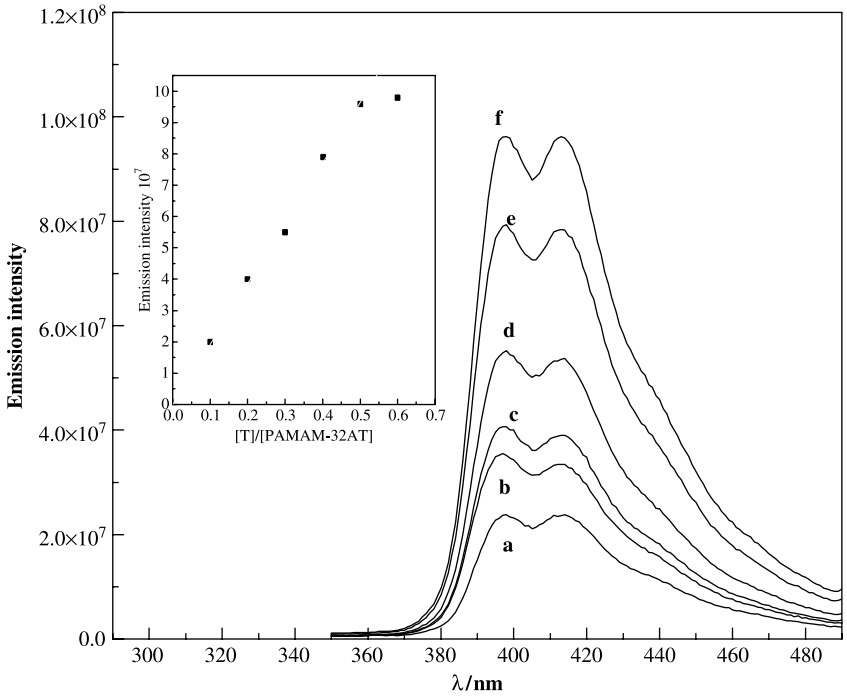

FIGURE 6 Emission spectra of tetrabenzonaphtalene in PAMAM-32AT/water solutions. [Tetrabenzonaphtalene]: a) 1.7×10^{-5}, b) 2.1×10^{-5}, c) 2.7×10^{-5}, d) 3.9×10^{-5}, e) 5.2×10^{-5}, f) 6.5×10^{-5} M. [PAMAM-32AT] $= 1.3 \times 10^{-4}$ M. Insert: Comparison of emission intensity of T, at 395 nm, for various ratios of Tetrabenzonaphtalene/PAMAM-32AT.

TABLE II The enhancement solubilization factor (ESF) for the polycyclic aromatic compounds calculated using Eq. (1)

Guest	PAMAM- 8AT	PAMAM- 32CT	PAMAM- 32AT	PAMAM- 32OHT	DAB-16AT	DAB-32AT
Phenanthrene	0	2	1	1	0	0.5
Anthracene	0	0.5	0.5	0.1	0	0.5
Tetrabenzonaphtalene	0	1	0.5	0.4	0	0.1

phobic guests. Considering the comparable lipophilicity, as measured by log $P_{o/w}$ and the molecular volume of anthracene and phenanthrene (Table I), a similar amount of guest encapsulation is expected, however no regular tendency in the maximum amount of entrapped molecules is observed. Probably anthracene pairing competes with the hydrophobic interactions that permit the guest encapsulation. The autoassociation of anthracene changes the size of this guest molecule and restrains its incorporation in the dendrimer. On the other hand, tetrabenzonaphtalene, which is more lipophilic than the other aromatic studied but has a larger size compared with the dimensions of the dendrimers interior, might only be partially caged within the dendrimer host. It is possible as well, that the dendrimers associated around tetrabenzonaphtalene [28].

The storage space of the dendrimers is controlled by geometrical parameters of the branch cell (branching angles, rotation angles and repeat-unit segment length) by the shape and size of available internal dendrimer microenvironment that influence the host-guest interactions [36]. The maximum amount of entrapped guest molecules is proportional to the shape and size of the guest molecules. As seen in Table II the encapsulation of phenanthrene is more favorable than that of anthracene or tetrabenzonaphtalene in all the dendrimers studied. Therefore, the molecular volume of the guest molecules, (Table I), affects the accessibility into the dendrimer. Consequently, it can be speculated that the interior space size suitable for guest encapsulation is between the molecular volume of phenanthrene and tetrabenzonaphtalene. Since phenanthrene has a similar size to **1** and **2** we expect that the dimension of the therapeutic guests will be adequate for the encapsulation in the lipophilic interior of dendrimers.

Nevertheless, the fact that the solubilization power of the dendrimers as measured in this work, may not only be due to the intrinsic affinity solute-dendrimer but also to the saturation solubility of the solute in the aqueous phase should be considered. Melo *et al*. have shown [37] how the latter effect influences the solubilization power of detergents defined as the moles of solute dissolved at saturation per mole of micellized surfactants. As the comparison between dendrimer and micelles holds, it can be expected that for relatively insoluble solutes the ESF can be dictated by the saturation in the aqueous phase besides the capacity of the dendrimer to encapsulate it. Although we have not explored this matter further it can be seen that the polyaromatic least water soluble, tetrabenzonaphthalene, is also the less solubilized in the studied dendrimers (Table I). In any case, when the solutes aside from lipophilicity interact with the dendrimers by specific interactions such as hydrogen bonds (compound **2**, Table III) the ESF does not correlate with water solubility, as it is shown below for the therapeutic compounds.

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Encapsulation of Therapeutic Compound

In order to evaluate the application of the studied dendrimers as prospective drug delivery systems, the solubilization of 5(6)-methylbenzo[1,2-c]1,2,5-oxadiazole N_1-oxide, guest **1,** and 2′-(Benzo[1,2-c]1,2,5-oxadiazol-5(6)-yl(N_1-oxide)methylidene]-1-methoxymethane hydrazide, guest **2**, was investigated (Fig. 1).

Changes in the solubility of **1** and **2**, in the presence of dendrimers were studied by UV spectroscopy as shown in Figs. 7 and 8.

The ESF factor calculated using Eq. (1), is summarized in Table III. As can be observed, even the smallest generations of dendrimers increase the solubility of these compounds. Since the small dendrimers lack globular structure to allow the encapsulation of guest, the increase of solubility of guest **1** and **2** suggests the existence of guest-dendrimer specific interactions. The values of ESF for compound **1** are always smaller than for compound **2** in all the dendrimers studied and, in most cases, the increase in the size of dendrimers increases the amount of guest encapsulated, nevertheless, the tendency is not as regular as could be expected.

TABLE III The enhancement solubilization factor (ESF) for therapeutic compounds **1** and **2**, calculated using Eq. (1)

Guest	PAMAM- 8AT	PAMAM- 32CT	PAMAM- 32AT	PAMAM- 32OHT	DAB-16AT	DAB- 32AT
1	1	9	2	3	3	1
2	2	10.5	4	4	3.5	1.5

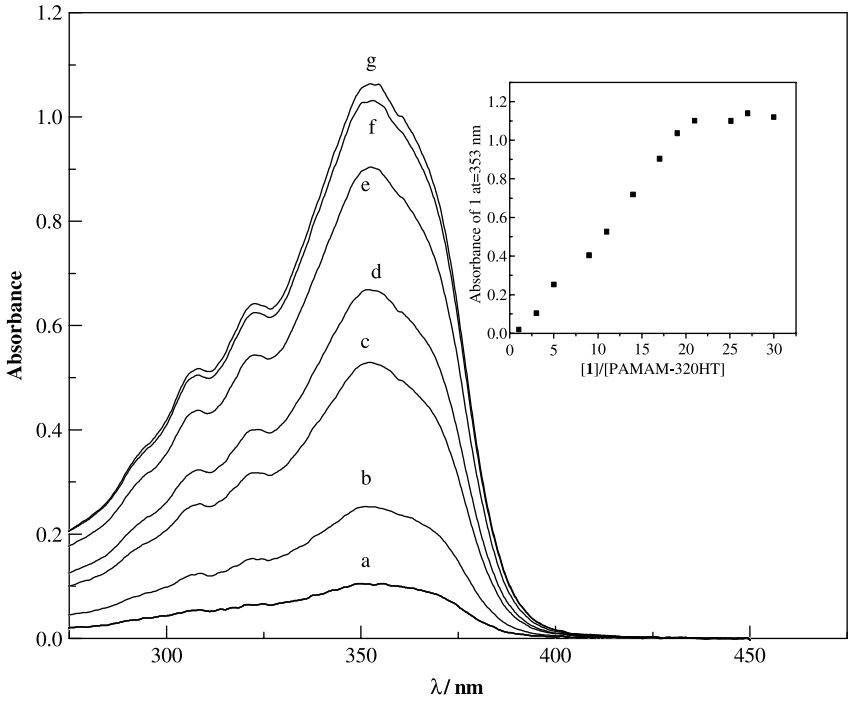

FIGURE 7 UV-vis. Spectra for guest **1** in PAMAM-32OHT/water solutions. [1]: a) 2.16×10^{-4}, b) 4×10^{-4}, c) 8.8×10^{-4}, d) 1.1×10^{-3}, e) 1.36×10^{-3}, f) 1.5×10^{-3}, g) 1.7×10^{-3} M. [PAMAM-32OHT] $= 8 \times 10^{-5}$ M. Insert: Comparison of absorbance, at 353 nm, for various ratios of **1**/PAMAM-32OHT, used to calculate the maximum number of guest molecules **1** encapsulated within the dendrimer.

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In fact if the PAMAM family is analyzed, it is observed that higher amounts of guest molecules are encapsulated in PAMAM-32AT and PAMAM-32OHT compared to PAMAM-8AT. This can be attributable to changes in the macromolecular shape, when there is a shift from one generation to the next one, that produces an increase of the storage space of dendrimers. However, DAB-16AT encapsulated more guest molecules than DAB-32AT. Although the size of the dendrimer interior is expected to increase

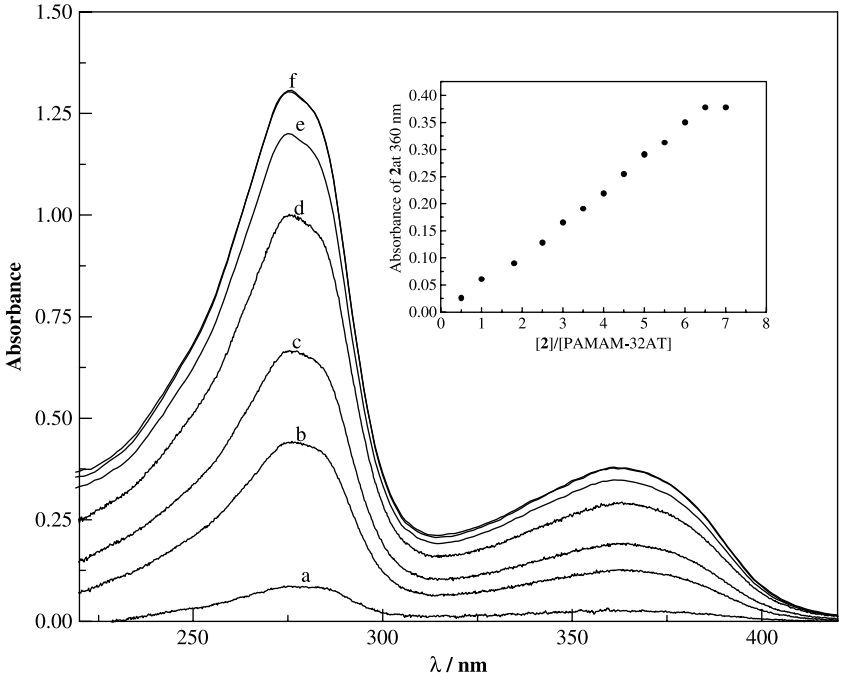

FIGURE 8 UV-vis. Spectra for guest **2** encapsulated within PAMAM-32AT dendrimers at various ratios. Concentration of **2** in dendrimeric solutions is a) 9.8×10^{-4}, b) 2.45×10^{-4}, c) 3.4×10^{-4}, d) 4.4×10^{-4}, e) 4.9×10^{-4}, f) 6.4×10^{-4} M. [PAMAM-32AT] $= 9.8 \times 10^{-5}$ M Insert: Comparison of absorbance, at 360 nm, for various ratios of **2**/PAMAM-32A, used to calculate the maximum number of guest molecules **2** encapsulated within the dendrimer.

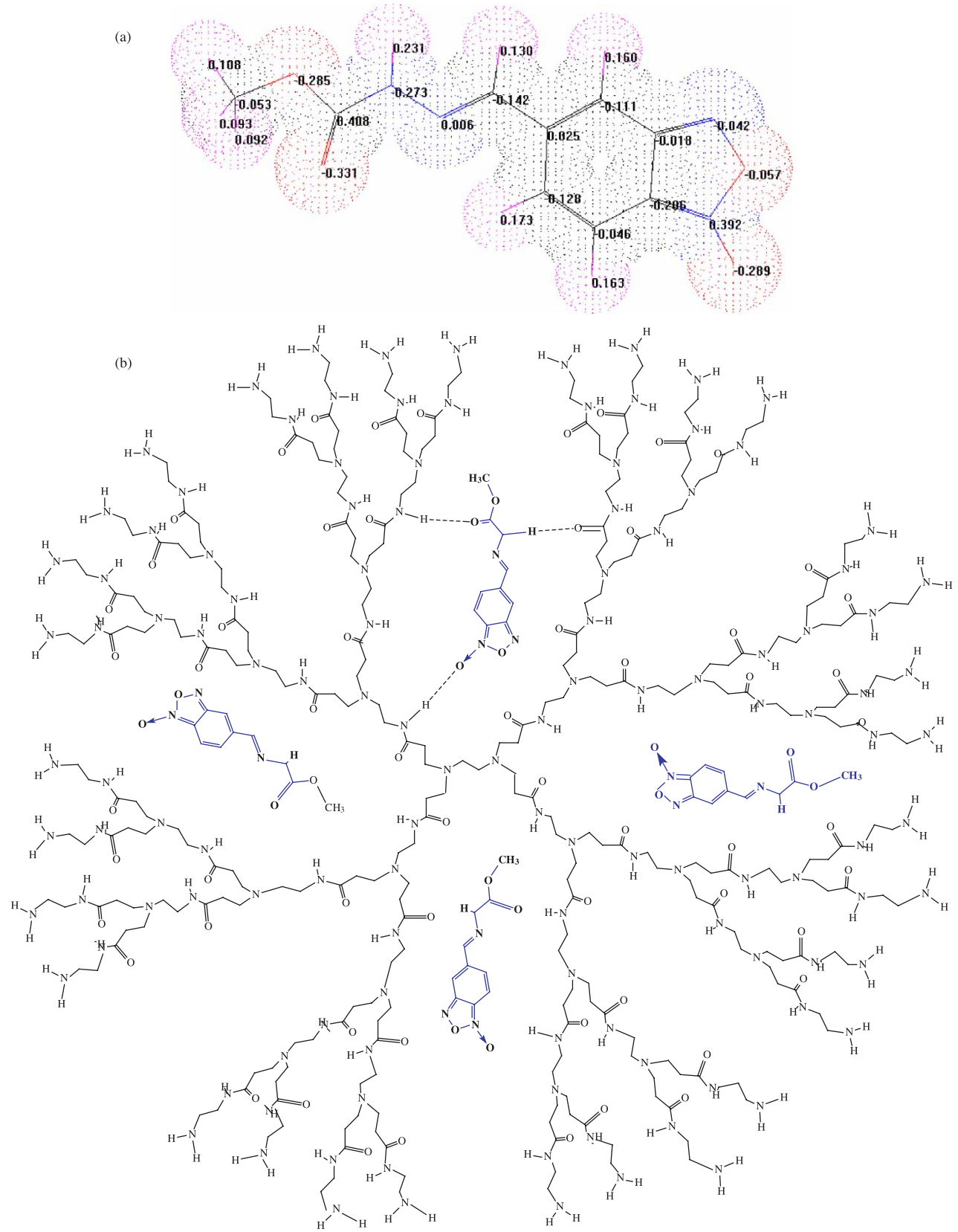

FIGURE 9 a) Charge distribution of compound **2** calculated using semiempirical AM1 method; b) schematic overview of the interaction of guest **2** with PAMAM-32AT.

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in DAB-32AT respect to DAB-16AT dendrimer probably the surface of these macromolecules has become sufficiently crowded as to hinder the guest migration to the inner cavities of hosts [38].

On the other hand, by comparing PAMAM dendrimers of the higher generation the amount of guest encapsulated in PAMAM-32CT is observed to be greater than the others. This could be indicating that the association guest-host takes place not only with the groups in the inner cavities of the dendrimer but also with the terminal groups.

It is interesting to note that the guest encapsulations remain stable at least for 20 days in all dendrimers studied. However, **1** and **2** caged in the interior of DAB dendrimers can be released after extensive shaking, while those in PAMAM dendrimers were unable to escape after the same treatment.

Free and complex guest molecules can be distinguished only by small finite energy barriers related to the ease of entry and departure to dendrimer, for that reason perhaps the vigorous agitation releases the guest when the interaction is a weak hydrophobic one. Consequently in the DAB systems it is evident that the host-guest interactions are weaker.

Being PAMAN a polyamide polymer it has hydrogen bond donors and acceptor sites which could favor the host-guest interaction and particularly for guest **2.** Consequently, hydrogen-bonding interactions between the O of the *N*-oxide group in the drug and H in the dendron amide groups are likely to be key in determining the interaction in this guest-host system. Hydrogen bonding acceptor nature of O of the *N*-oxide group can be predicted from the analysis of the charge density calculated by semiempirical methods, (Fig. 9a) whereas the schematic overview of the possible interaction of guest **2** with PAMAM-32AT is showed in Fig. 9b.

In the case of DAB, although tertiary amine groups are hydrogen bond acceptors, the crowded dendrimer structure in water solution seems to make the specific interactions weaker and mostly lipophilic.

On the other hand, **1** and **2** encapsulated within PAMAM dendrimers can be released by lowering the pH of the solution. For example, more than 60% of the guest **2** encapsulated were released from PAMAM-32CT dendrimers upon protonation as shown in Fig. 10.

It should be noticed that previous studies of the solubility of **1** and **2** in water showed that it is independent of the pH of the solution.

In the case of DAB solutions, very small changes in the solubility were observed by lowering the pH in the same manner. Thus, the protonation of the system is significant in the guest-PAMAM interaction but not in the guest-DAB family.

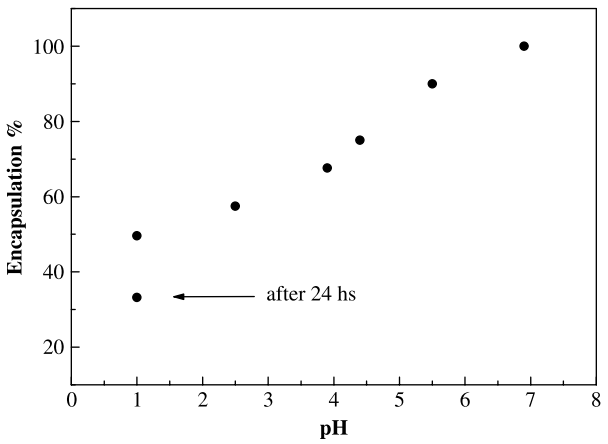

FIGURE 10 Variation of % encapsulation of **2** in PAMAM-32CT by lowering the pH of the solution with hydrochloric acid. Initial concentration of **2** in PAMAM-32CT/water 1.2×10^{-3} M, [PAMAM-32CT] $= 1 \times 10^{-4}$ M.

In order to obtain more insight about the effect of pH in the encapsulation of **2**, a systematic study was performed.

pH Effect on Guest-dendrimers Interactions

The interior of the studied dendrimers consists of amidoamine groups and tertiary amino groups, (Fig. 2), therefore protonation will certainly modify their environmental properties such as the specific interaction between the guest and specific binding-sites in the interior of hosts and terminal groups [39]. Cakara *et al*. [40] have made a detailed study about the behavior of dendrimers. These studies show that dendrimers protonate in different steps. Around pH = 10 protonation is observed at the outermost primary amine groups and at the odd shells of tertiary amine groups, while the central tertiary amines in the inner cavities are protonated at lower pH, i.e. pH = 5. Thus these dendrimers behave as polyprotic bases, which contain sets of equivalently basic groups. Ottaviani *et al*. [41] calculated the pK_b values for equivalent NH_2, NR_3 and COO^- groups. The obtained pK_b values show that the protonation occurs in the sequence: $NH_2 > NR_3 > COO^-$.

The encapsulation of compound **2** in PAMAM-32CT at pH = 7 and pH = 4 was studied as representative systems for the analysis of the pH influence in the capacity of dendrimers to encapsulate and release guests. The different range of protonation involved determined the choice of these two pH values. At pH = 7 the protonation of the first layer of NR_3 groups occurs, whereas at pH = 4 more internal NR_3 layers and most of the terminal groups are also protonated. The results are illustrated in Fig. 11.

A maximum of ten molecules of compound **2** for dendrimer are encapsulated as it is observed working at pH = 7. Lowering the pH of the final solution

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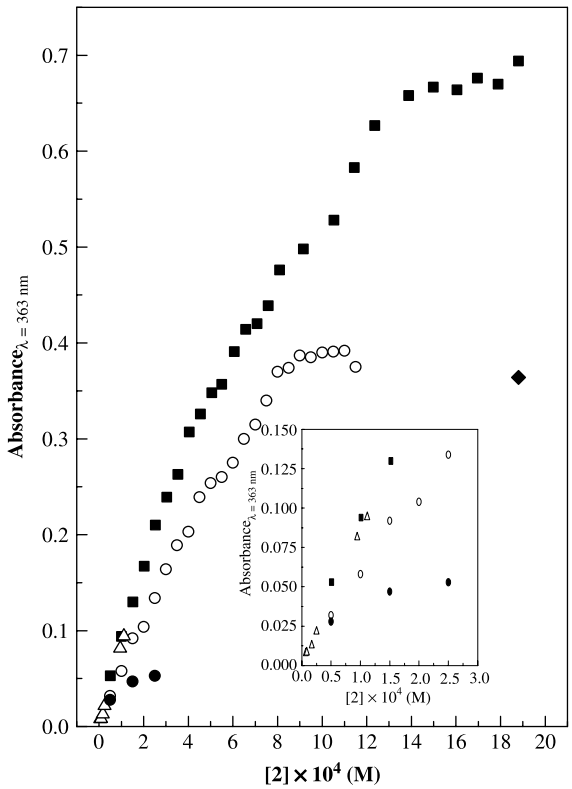

FIGURE 11 Comparison of absorbance of compound **2** in PAMAM-32CT aqueous solution for various ratios of **2**/PAMAM-32CT, (○) in citrate buffer at pH = 4; (●) in potassium acid phthalate buffer at pH = 4. (■) in phosphate buffer at pH = 7, (◆) lowering the pH of this system at pH = 2 with hydrochloric acid. Compound **2** in pure water (Δ).

to pH = 2 with hydrochloric acid the release of the guest is observed and only five molecules stay encapsulated. After 24 h only four molecules remain incorporated.

On the other hand, when the solubilization is studied at pH = 4 the encapsulation depends on the buffer solution used. Considerable reduction in the solubility of **2** with respect to pure water was observed when the encapsulation was studied in acid phthalate buffer at pH = 4. However, with citrate buffer the encapsulation is lower than at pH = 7 but still five molecules remain associated as occurs in the experiment of lowering the pH with hydrochloric acid. It seems that not only the pH but also the chosen buffer could determine important changes in the drug release. In this case the aromatic phthalate could be competing in the association of the compound with the dendrimer.

The results reveal that although the hydrophobic properties of the interior cavities of the dendrimers are important, the host-guest specific interactions control the association. Hydrophobic interaction helps the incorporation of guests in the lipophilic interior of dendrimers but hydrogen bonding between the O of the *N*-oxide group in the drug and H in the dendron amide groups facilitates the association of this host-guest system. Moreover, compound **2** is also able to donate H to the amine and carbonyl groups of the host. The hydrogen bonding donor and acceptor nature of the guest groups can be appreciated from the analysis of the charge density of the different groups involved in the interaction (Fig. 9a) supporting this conclusion.

It is evident that compound **2** and also **1** have an additional interaction with the COO- terminal groups in PAMAM-32CT which could be responsible of the high increase of solubility observed in this system respect to PAMAM-32AT.

In summary, both types of dendrimers are able to encapsulate the analyzed therapeutic compounds. However, particularly PAMAM dendrimers favor not only drug encapsulation but also retention due to the existence of strong specific host-guest interactions. This interaction can be controlled with the pH of the solution to allow the deliverance of the drug, which gives an additional biological importance.

EXPERIMENTAL SECTION

Materials

The polycyclic aromatic hydrocarbons, phenanthrene, anthracene and tetrabenzonaphtalene, were obtained from Sigma-Aldrich. Stock solutions of the aromatic compounds were made by dissolving them in methanol at 1×10^{-3} M for phenanthrene and anthracene and 1×10^{-4} M for tetrabenzonaphtalene. They were then stored in darkness.

PAMAM 8AT, PAMAM 32AT, PAMAM 32CT and PAMAM 32OHT dendrimers in methanol solution were obtained from Sigma-Aldrich. DAB 16AT and DAB 32AT dendrimers were obtained as neat liquids from Sigma-Aldrich. They were stored at 4°C under nitrogen. Methanol HPLC grade from Merck was utilized for stock solutions. Ultra pure water was obtained from Labonco equipment Model 90901-01. Standard buffers pH = 4 and pH = 7 were obtained from Merck. All chemicals were used as received. 5(6)-Methylbenzo[1,2-c]1,2,5-oxadiazole N_1-oxide] (**1**) and 2′-(benzo[1,2-c]1,2,5-oxadiazol-5(6)-yl(N_1-oxide)methylidene]-1-methoxymethane hydrazide (**2**) prepared as previously described [42], were stored at room temperature under vacuum.

Solubilization Experiments

To prepare solute/water stock solutions, aliquots of the solute stock solutions in methanol were transferred into 5 mL volumetric flasks and methanol evaporated off under nitrogen. Samples were then diluted to volume with HPLC grade water, sonicated for 2 h, and allowed to equilibrate in darkness overnight.

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The dendrimers/water blank solutions were prepared transferring the appropriate volumes of dendrimer commercial methanol solutions into 5 mL volumetric flasks, and the solvent was evaporated off under nitrogen. They were then diluted to volume with HPLC grade water, sonicated for 2 h, and allowed to equilibrate in darkness overnight.

To prepare the solute/dendrimer/water samples, appropriate volumes of the solute stock solutions and dendrimer commercial solutions were transferred into 5 mL volumetric flasks, and methanol solvent was evaporated under nitrogen. They were then diluted to volume with HPLC grade water, sonicated for 2 h, and allowed to equilibrate in darkness overnight. These samples were prepared at constant dendrimer concentration of approximately 1×10^{-4} M. In the studies with phenanthrene, **1** and **2** the solute concentration was varied from 10^{-6} M to 10^{-4} M. In anthracene/dendrimer/water and tetrabenzonaphtalene/dendrimer/water systems the solute concentration was increased from 2×10^{-7} M to 0.5×10^{-4} M and from 10^{-8} M to 0.5×10^{-4} M respectively.

Instrumental Methods

The absorption spectra were measured by using Shimadzu 2401 at 25.0 ± 0.1°C. Although no background signal was apparent in the wavelength region of interest, due to the dendrimers; spectra were blank corrected for the possible absorption of the solvent and the aqueous dendrimers [43]. A Spex Fluoromax apparatus was employed for the fluorescence measurements. Corrected fluorescence spectra were obtained using the correction file provided by the manufacturer. Spectra were also solvent-blank-corrected. Solutions were excited at $\lambda = 294$ nm for phenanthrene, $\lambda = 340$ nm for anthracene and $\lambda = 350$ nm for tetrabenzonaphtalene. Slit widths for excitation and emission monochromators were set to 1 nm. The emission scan interval was 1 nm.

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