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Molecular interactions between dimethoxycurcumin and Pamam dendrimer carriers

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Abstract

Dimethoxycurcumin, a lipophilic analog of curcumin found as a major pigment in the Indian species turmeric (*Curcuma longa* Linn.), is known to possess significant activity against various cancer cell lines, but its use as an anticancer drug is hindered by its poor water solubility. The conjugation of dimethoxycurcumin to water-soluble PAMAM dendrimers (generations 3.5 and 4) is demonstrated. The maximum drug–dendrimer incorporation efficiency is 4.3 and 5.0 molar for G3.5 and G4, respectively. The FTIR-ATR investigation of the neat compounds and the drug–dendrimer systems indicate that dimethoxycurcumin is in the enolic form, while its interaction with the integer generation dendrimer involves the major conformational change of the terminal ethylene amine groups.

Keywords: Dimethoxycurcumin; FTIR-ATR; PAMAM dendrimer

1. Introduction

Curcumin (diferuloylmethane) derived from the dried rhizoma of turmeric (*Curcuma longa* Linn.), has been used in Ayurvedic and Chinese medicine for centuries (Ishihara and Sakagami, 2005; Surh et al., 1999; Bhaumik et al., 1999; Khar et al., 1999). Curcuminoids such as curcumin, demethoxycurcumin, dimethoxycurcumin and bisdemethoxycurcumin are the bioactive substances of turmeric (Ohtsu et al., 2002). Much attention has been given lately to curcumin and its analogues that have been reported to reveal antioxidant, antiiflammatory and cancer prevention activities (Nogaki et al., 1998; Duvoix et al., 2005; Dorai and Aggarwal, 2004). Dimethoxycurcumin (**1**) (Fig. 1) is a lipophilic compound that has been investigated lately against various cancer cells lines. The results seem very promising as in many cases curcumin was inactive, while compound **1** exerted high activity (Ohtsu et al., 2002). Whereas compound **1** revealed significant *in vitro* pharmacological activity, further *in vivo* use is hindered by its water-insolubility. Systematic attempts to formulate compound **1** have not been published to the best of our knowledge, with the exception of a liposomal formulation of curcumin (Li et al., 2005) and a publication by our group (Gardikis et al., 2006) on the interaction of **1** with DPPC lipid bilayers.

An alternative approach in the formulation of drugs is the technology of dendrimers. Dendrimers have many attractive properties due to their well-defined structures and low polydispersity (Aulenta et al., 2003). The drug–dendrimer interactions determine the enhancement of the drug bioavailability, and are thought to involve electrostatic or covalent complexation on the surface of the dendrimer, or entrapment within the dendrimer architecture (D'Emanuele and Attwood, 2005).

To be used for drug delivery, a dendrimer must be non-toxic, non-immunogenic and biodegradable (Aulenta et al., 2003). The first complete dendrimer family which has been synthesized, characterized and commercialised is the poly(amidoamine) (PAMAM) dendrimers. They are considered safe, non-immunogenic and exhibit minimum cytotoxicity up to generation 5 (Malik et al., 2000), although there are concerns about the role of the amino groups at the periphery of the dendrimer (Dhanikula, 2006). As such, PAMAM dendrimers are

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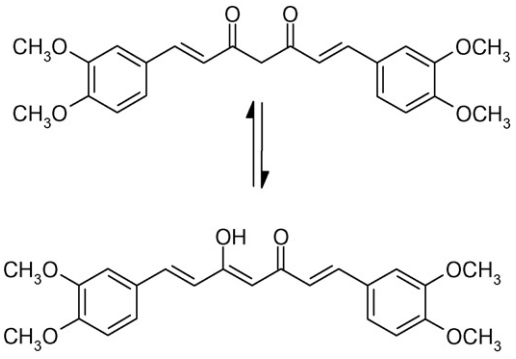

Fig. 1. Dimethoxycurcumin (**1**).

used in drug delivery, delivery of antisense nucleotides and gene therapy, both *in vitro* and *in vivo* (Eichman et al., 2001).

The aim of the present work was to study the incorporation efficiency of dimethoxycurcumin (**1**) in PAMAM dendrimer formulations, as well as to characterize the molecular interactions between **1** and PAMAM. This is the first attempt on the incorporation of **1** in dendrimers, while little is known about the molecular interactions of bioactive molecules and dendrimers, which are crucial of the pharmaceutical formulations because of their ability to influence the *in vivo* pharmacokinetic parameters and the bioavailability of the drug.

2. Material and methods

2.1. Materials

Compound **1** was a gift from Prof. P. Pantazis (Foundation for Biomedical Research of the Academy of Athens). PAMAM G3.5 and G4 were purchased from Sigma–Aldrich Chemical Company (Germany).

2.2. Methods

2.2.1. Incorporation of 1 in PAMAM G3.5 and G4 dendrimers

Appropriate quantities of **1** and PAMAM G3.5 or G4 were dissolved in methanol, the solution was stirred for 24 h and then evaporated under vacuum in order to remove methanol completely. Buffer solution TES 10 mM (pH 7.4) was added and the mixture was stirred for 24 h in the dark. The separation of **1**:PAMAM complex from the non-incorporated **1** was performed by centrifugation at 4000 rpm, at 4 °C for 30 min. As compound **1** is not soluble in water, it is found in the precipitate, while the **1**:PAPMAM complex is dispersed in the aqueous medium. UV–vis Spectrophotometric calibration curves for compound **1**, PAMAM G3.5 and G4 in methanol were made at $\lambda_{max} = 415, 241$ and 244 nm, respectively. The amount of the non-incorporated **1** was determined**,** allowing for an estimate of the incorporation efficiency and the **1**:PAMAM molar ratio.

2.2.2. FTIR spectroscopy

Infrared spectra were recorded on a Fourier transform instrument (Equinox 55 s by Bruker Optics) equipped with a single reflection diamond Attenuated Total Reflectance (ATR) accessory (DuraSamplIR II by SENSIR). The spectra have been measured over the 525–5000 cm^{-1} range at a resolution of 2 cm^{-1}, and represent averages of 100 scans. One drop solutions (aqueous or methanolic) of the samples were introduced in contact with the diamond element and dried by N_2 purging to form a thin film. Powders were measured by using a special limited torque press accessory. Amorphous dimethoxycurcumin was measured by melting a small quantity of polycrystalline powder on a hot plate and quenching it on a metal block. The spectra are shown in the ATR formalism, *i.e.* after correction for the wavelength dependence of the penetration depth. Peak picking by second derivative analysis and spectral subtraction were performed by using routines of the OPUS software by Bruker Optics.

3. Results and discussion

3.1. Incorporation of 1 in PAMAM G3.5 and G4 dendrimers

The incorporation efficiency of **1** into PAMAM dendrimers has been studied using different **1**/PAMAM molar ratios in TES 10 mM buffer pH 7.5, (*i.e.* 2:1 to 10:1 molar ratios of **1**/PAMAM), as described in Section 2.2.1. The results indicate that initial molar ratios of 8 and 10 are necessary for reaching a maximum incorporation of 4.2 and of 5.0 for **1**/PAMAM G3.5 and **1**/PAMAM G4, respectively (Table 1).

3.2. FTIR spectroscopy

The identification of the vibrational signature of the drug–dendrimer interactions is only possible by comparison of the interacting systems to the spectra of the neat dendrimers and drug. Unfortunately, the vibrational investigation and structural understanding of PAMAM dendrimers is still very limited (Popescu et al., 2006, and references therein). Fig. 2 depicts the ATR spectra of PAMAM G3.5 and G4 films deposited by N_2 purging from aqueous solutions. Identical spectra have been obtained from methanolic solutions (not shown). The spectrum of PAMAM G4 compares favorably with the literature infrared transmission spectra of PAMAM G1 (Davis, 2002), G3 (Kolhe et al., 2003), G4 (Kolhe et al., 2003; Manna et al., 2001). As a result of strong H-bonding interactions, the spectra cannot

Table 1
Incorporation efficiency (%) of **1**/PAMAM at different **1**/PAMAM molar ratios

Dendrimer	Compound **1** incorporation			Compound **1** to dendrimer molar ratio	
	(μmoles)		%	(μmole/μmole)	
	Initial	Final		Initial	Final
PAMAM G 3.5	1.8[a]	1.6	90.0	2	1.8
PAMAM G 3.5	1.8	1.5	85.0	4	3.4
PAMAM G 3.5	2.0	1.1	53.0	8	4.2
PAMAM G 4	1.7	1.6	95.0	2	1.9
PAMAM G 4	2.4	1.2	50.0	10	5.0

[a] S.D. (±) never exceeded 5% of the mean value.

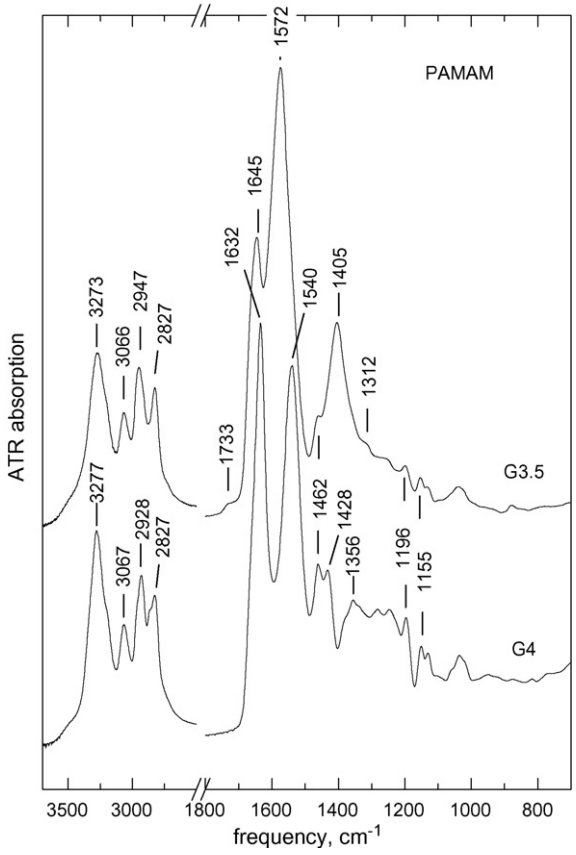

Fig. 2. ATR spectra of PAMAM G3.5 and G4.

be described as simple superpositions between a common secondary polyamide core and the surface carboxylic acid salt or primary amine terminal groups. By the same token, the main absorption bands observed represent mixed vibrations and cannot be uniquely assigned. The presence of the terminal -COO^- groups in PAMAM G3.5 is clearly inferred from the symmetric stretching at ca. 1400 cm^{-1}, which is absent from the spectrum of G4. The secondary "core" amide I band of G3.5 is observed at ca. 1645 cm^{-1}, but the corresponding amide II component overlaps with the asymmetric -COO^- mode and gives rise to the strong band at 1570 cm^{-1}. A weak shoulder at ca. 1730 cm^{-1} is assigned to a trace of –COOH terminal groups in G3.5, while the band at 1462 cm^{-1} is attributed to the scissoring mode of the methylene groups. The spectrum of G4 appears considerably simpler and is dominated by two sharp bands at 1632 and 1540 cm^{-1}. The former should be attributed to a convolution of the expected primary aliphatic amine deformation mode and the amide I mode, but its narrow bandwidth does not allow for the separation of these two modes and indicates a particularly ordered structural arrangement. The 1540 cm^{-1} band in the spectrum of PAMAM G4 is assigned to the amide II mode, and its low frequency is indicative of strong H-bonding interactions. The presence of the primary amine group in G4 is not reflected in any significant differentiation of the main N–H stretch observed at ca. 3275 cm^{-1} in both G3.5 and G4.

The spectral differences between PAMAM G3.5 and G4 extend to the $–CH_2–$ vibrations. The asymmetric stretching mode is active at ca. 2950 cm^{-1} in G3.5, but shifts below 2930 cm^{-1} in G4. In the frequency range for the $–CH_2–$ scissoring mode, G4 exhibits a clear doublet at ca. 1460 and 1428 cm^{-1} followed by a band at ca. 1360 cm^{-1} that has been assigned to a $–CH_2–$ twisting mode (Sabatini and Califano, 1960). These differences should be attributed to the presence and conformation of the outer ethyleneamine group in G4, which is not present in G3.5. Remarkably, it appears that this latter group has a profound influence on the amide I and II bands in G4.

In this respect, we note that the study of PAMAM G0 and G1 by vibrational sum frequency techniques has suggested that the adjacent $–CH_2–$ units are not in trans conformation to each other, and appear to be distributed perpendicular to the surface plane of the dendrimer (Davis et al., 2003). Furthermore, a recent ab initio study (Tarazona-Vasquez and Balbuena, 2004) concluded that in the lowest energy PAMAM G0 conformers, the secondary amide group has a trans configuration.

Based on the analysis of our spectra, and in agreement with the above cited literature, we conclude that the terminal primary amine of neat PAMAM G4 is not exposed on the surface of the dendrimer but, instead, is folded inwards to interact strongly with the amide groups and expose an outer sphere of cis $–CH_2CH_2–$ moieties. This folding is thought to result in the formation of a

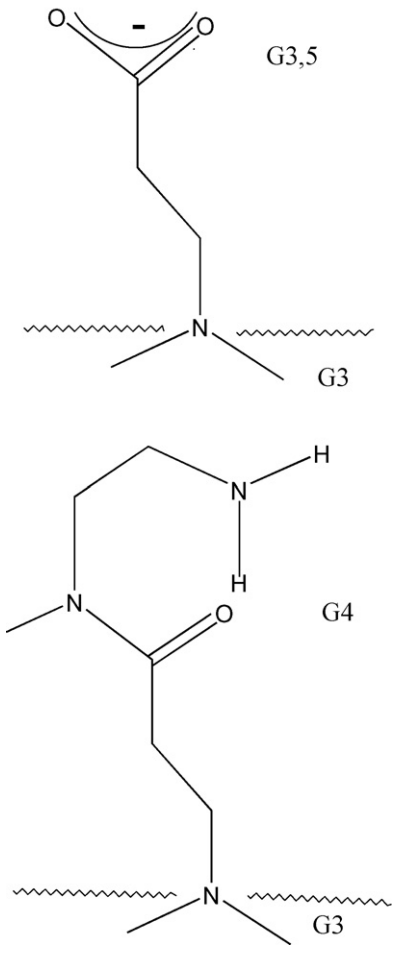

Fig. 3. Suggested conformation of the terminal half or integer generation of PAMAM dendriners.

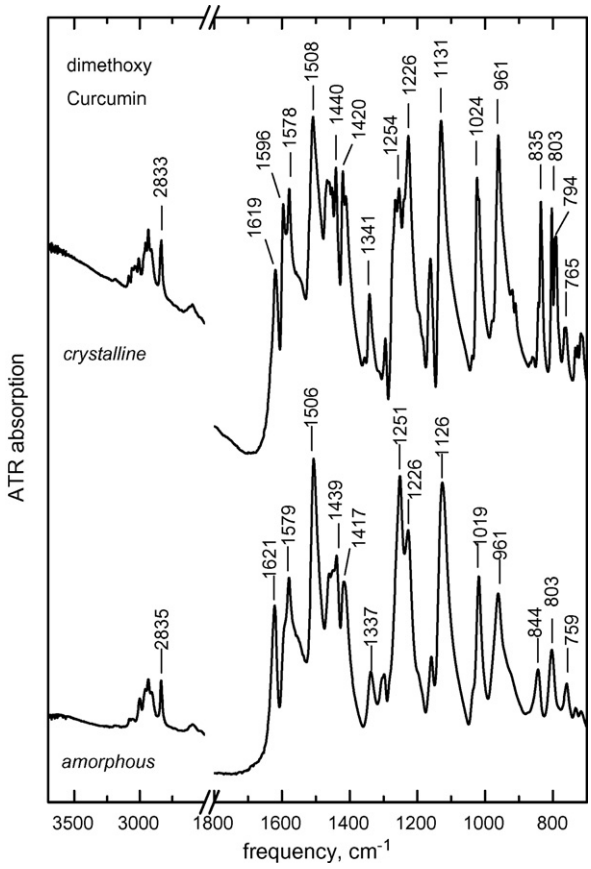

Fig. 4. ATR spectra of polycrystalline and melt-quenched **1**.

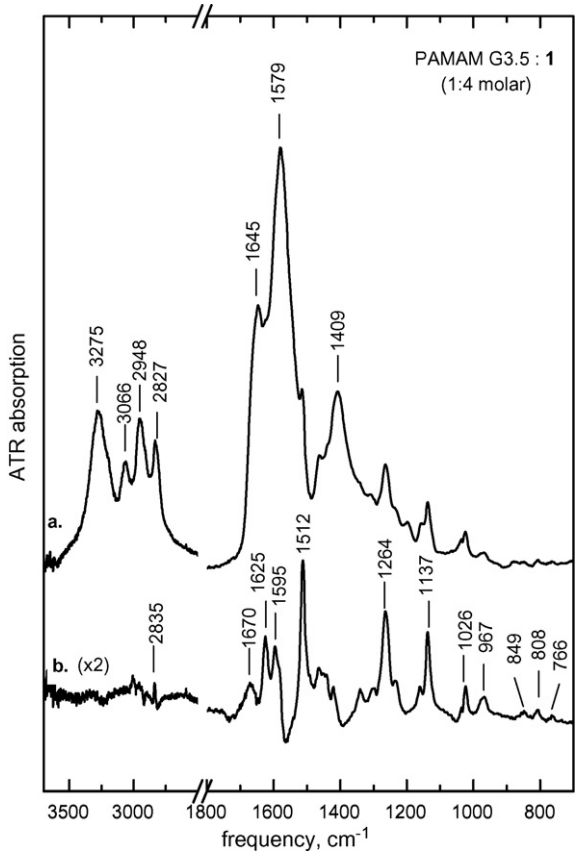

Fig. 5. (a) ATR spectrum of the 4:1 molar **1**:PAMAM G3.5 complex, and (b) difference spectrum obtained by scaling and subtracting the spectrum of neat PAMAM G3.5 from (a).

pseudo six-membered ring as in Fig. 3, which can only exist in integer generations of PAMAM.

The ATR spectra of polycrystalline and amorphous dimethoxycurcumin are compared in Fig. 4. The spectrum of the polycrystalline sample exhibits the splitting of several vibrational modes due to periodicity, as well as spectral distortions attributed to the Christiansen effect (Duyckaerts, 1959). Assignments for most of the observed bands can be suggested on the basis of a recent vibrational analysis of curcumin (Kolev et al., 2005). What is important in the context of the present study is the observation that neat dimethoxycurcumin is clearly found in its enolic form in both polycrystalline and amorphous preparations. Evidence for this is provided by the low frequency of the carbonyl stretching mode (ca. 1620 cm^{-1} instead of 1715–1745 cm^{-1} expected for the diketo form).

Figs. 5a and 6a depict the ATR spectra of the PAMAM G3.5 and G4 complexes with dimethoxycurcumin (1:4 and 1:5 molar, respectively). Both spectra are obtained from films deposited from aqueous solutions and do not differ from those of the corresponding methanolic solutions. In both cases, the presence of dimethoxycurcumin is manifested by strong bands at ca. 1510, 1260, 1135 cm^{-1}. These bands are observed at frequencies slightly higher than in the neat drug, suggesting the presence of interactions between the drug and the PAMAM molecules. A better picture is obtained by subtracting the spectrum of PAMAM from that of the corresponding complex (Figs. 5b and 6b). In the case of the G3.5 system, this treatment produces a spectrum that clearly corresponds to dimethoxycurcumin despite the aforementioned shifts of peak maxima and some broadening. A weak new peak is observed at 1670 cm^{-1} (Fig. 5b) that was not present neither in the spectrum of PAMAM G3.5 nor in the spectrum of dimethoxycurcumin. The origin of this band cannot be fully understood, but could be related to a change of H-bonding of the enol functional group and/or the amide groups and therefore be suggestive of the kind of interactions between the drug and the dendrimer. The situation is much more diagnostic in the case of PAMAM G4 (Fig. 6b). While the signature of the dimethoxycurcumin in the subtracted spectrum does not differ significantly from that of its G3.5 counterpart, the strong bands due to the dendrimer do not fully subtract. Indeed, negative bands at 1630, 1535, 1430 and 1355 cm^{-1} dominate the spectral difference, indicating that the conformation of the dendrimer exhibits pronounced changes upon incorporating the drug molecule. It was earlier argued that these four bands are resulting from the strong interaction of the terminal primary amine with the amide group via the formation of a six-member ring. If this assignment is correct, the difference spectrum in Fig. 6b is suggesting that the interaction between PAMAM G4 and dimethoxycurcumin involves a conformational change of the terminal $–CH_2CH_2NH_2$ segment.

Given the fact that PAMAM G4 has 64 terminal ethylene amine groups, while the **1**:PAMAM G4 molar ratio is only 5:1, we speculate that either further incorporation of the drug is lim-

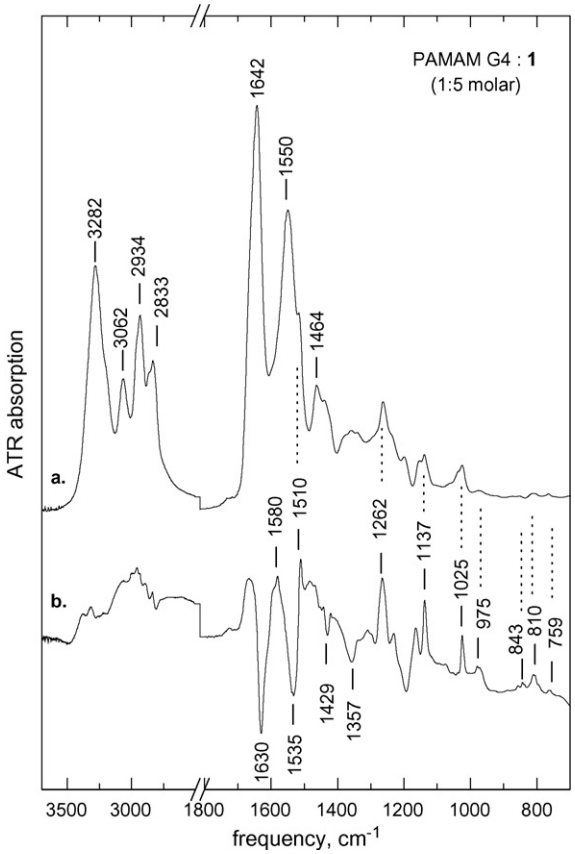

Fig. 6. (a) ATR spectrum of the 5:1 molar **1**:PAMAM G4 complex, and (b) difference spectrum obtained by scaling and subtracting the spectrum of neat PAMAM G4 from (a).

ited by, *e.g.* steric factors, or, that several ethylene amine groups are involved in the encapsulation of a single dimethoxycurcumin molecule. Either possibility is very different to the simple acid-base chemistry that determines the interaction between several acidic non-steroidal anti-inflammatory drugs and integer generations of PAMAM (Kolhe et al., 2003; Yiyun and Tongwen, 2005; Asthana et al., 2005).

4. Conclusions

The incorporation of dimethoxycurcumin into hydrophilic and biocompatible carriers is of prominent importance due to its recently discovered anti cancer activity. This work demonstrates that PAMAM dendrimer generations G3.5 and G4 are able to conjugate compound **1**, albeit with small incorporation efficiencies. FTIR spectroscopy by the ATR technique has been employed to study the structures of the neat dendrimers and drug, as well as to shed light on the nature of the drug–dendrimer interactions. These studies have demonstrated that, in all cases, **1** is found in the enolic form, and its interaction with PAMAM G4 involves the rearrangement of the terminal ethylene amino groups of the latter in a manner that affects markedly the whole amide spectrum of the dendrimer. It is anticipated that these interactions may be relevant to other non-acidic medicinal compounds and will allow for the design of optimized drug carriers.

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