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Evaluation of a new dendrimeric structure as prospective drugs carrier for intravenous administration of antichagasic active compounds

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In the present work, we investigated a first generation of a new dendrimer as candidate for intravenous (iv) administration of a therapeutic compound (2′-(benzo[1,2-c] 1,2,5-oxadiazol-5(6)-yl (N-1-oxide) methylidene]-1-methoxy methane hydrazide). This compound presents antichagasic activity but low water solubility. Guest–host specific interactions results in good drug solubilization. These interactions can be controlled by varying the solution pH, allowing drug deliverance. Interaction between dendrimer and human serum albumin (HSA), and the hemolytic potential of dendrimer were evaluated. The dendrimer does not interact with blood proteins, is not hemolytic, and does not produce damage in the cellular membrane. The results demonstrate that the new dendritic structure could be an appropriate carrier for the novel antichagasic drug. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: dendrimers; antichagasic activity; host–guest interaction; hemolysis

INTRODUCTION

The hydrophobicity of therapeutic agents limits their application and formulation development, and the poor solubility in water of drugs often affects their ability to gain access to the action site in an appropriate dose.[1] It is possible to facilitate their transport by encapsulation of the active compound in a specialty designed molecular or supramolecular structure. The encapsulation of drugs consists on housing a guest in the microcavities present in the system that will act as host.[2]

Dendrimers are highly branched macromolecules studied as drug carriers. They have further precisely controlled structures, with a generally globular construct, a single molecular weight and a large number of controllable peripheral terminal groups.[3] This type of compounds have attracted the attention of the drug-delivery research community due in part to the belief that the control over the structure allows to advance in the development of studies about the absorption, distribution, metabolism, excretion, and toxicity of this compounds.[4] Dendrimers can act as drug carriers by conjugation of the guest with functional groups presented its surface,[5] or by encapsulation of hydrophobic active compounds in dendrimeric nanocavities, allowing the drug transport at concentrations exceeding their intrinsic water solubility.[6]

In recent years, much effort has been devoted to the preparation of dendrimers that are designed to be biodegradable, water soluble, and biocompatible.[7] Several dendrimers based on polylysine have been developed as promising vaccine, antiviral, and antibacterial candidates.[8] Frèchet *et al.*[9] have recently explored polyester dendrimers as candidates for development of anticancer drug delivery systems. Several other families synthesized by Schluter *et al.*[10] might also prove to be useful for biological applications.

Polyphosphazene-functionalized diaminobutane poly(propyleneimine) dendrimers have been prepared and investigated for their properties as prospective hydrophobic drug delivery system. These novel dendrimeric compounds exhibit useful protective and targeting properties.[11] Dendrimers based on melanine have been modified on their periphery with PEG (polyethylenglycol) units. The PEGylated dendrimers when evaluated *in vivo* showed no toxicity. Also they improve the solubility and reduce the hepatotoxicity of anticancer drugs methothexate and mercaptopurine.[12]

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Our previous studies using functionalized polyamide amine dendrimers were focalized in the analysis of the interactions between dendrimers and several biologically important guests.[13] Association constant obtained from changes in the ^{1}H-NMR chemical shifts of the amide protons in the host indicated two different interaction sites: inside and on the periphery of the dendrimer. Furthermore, we analyzed the solubilization and release of different hydrophobic compounds by interaction with polyamidoamine (PAMAM) and polypropylenimine (PPI) dendrimers.[14] We observed their ability to increase the solubility of oxadiazol derivatives, with antichagasic activity.[14]

However, to use of dendritic macromolecules in drug delivery, it is necessary to know their biological properties, such as toxicity and biocompatibility.[15] It is known that the circulation system is the most convenient way of drug administration, because an active compound is able to reach distant tissues within a relatively short time. Nevertheless, blood constituents can be the first and unwanted targets of drug action. The active drug binding to plasma proteins and blood cell walls may produce serious problems of toxicity, or a significant lost of the amount of drug available for therapy.[16] Human serum albumin (HSA) is the most abundant soluble protein constituent of circulatory system. It has many physiological functions and high affinity for negatively charged hydrophobic molecules. One of its primary functions is to participate in absorption, distribution, metabolism, and excretion of drugs.[17] In the human body dendrimers can interact with components of the blood and cells. Consequently, it is necessary to study the effects of dendrimers presence in the blood, their hematoxicity, and their interaction with HAS.

In the present work, we investigated the first generation of a new carboxylate ended dendrimer (Fig. 1a), with the purpose of using it as drug carrier for intravenous (iv) administration of novel antichagasic compounds. Previous studies about the polarity and accessibility of the dendrimeric structure showed that their lipophilic inner groups are able to specific association with hydrophobic guests.[18] Therefore, their potential application as drug carriers was evaluated by studying their interaction with 2′-(benzo[1,2-c] 1,2,5-oxadiazol-5(6)-yl(N_1-oxide) methylidene]-1-methoxy methane hydrazide (**OXMH**) (Fig. 1b). This compound presents antitrypanosomal activity. *Trypanosoma cruzi* is the etiologic agent of Chagas' disease, which is endemic in South America.[19] **OXMH** have trypanocidal activity but low water solubility, which limits their therapeutic application.[20] Also, the interaction between the dendrimer and plasma proteins was analyzed, and its hemolytic potential evaluated.

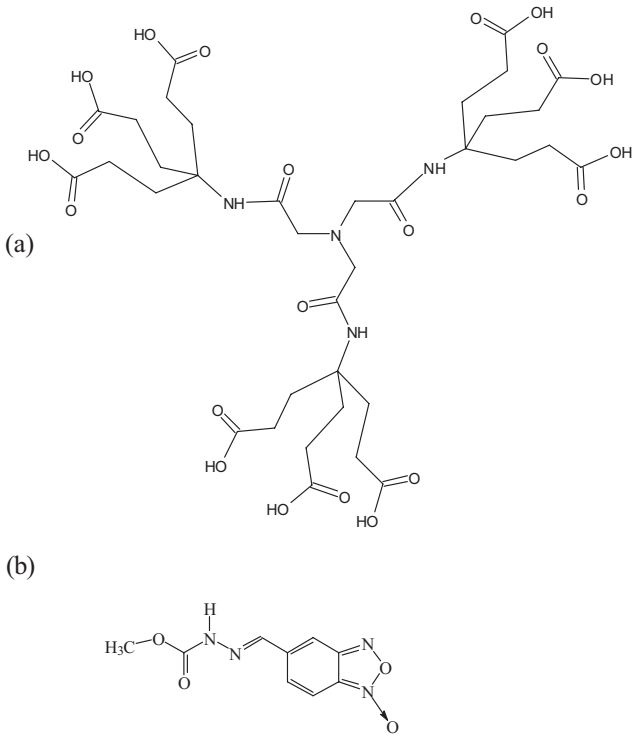


Figure 1. Chemical structure of (a) dendrimer and (b) 2′-(benzo[1,2-c] 1,2,5-oxadiazol-5(6)-yl(N1-oxide) methylidene]-1-methoxy methane hydrazide (**OXMH**)

EXPERIMENTAL

Materials

HSA (Sigma) was 99% fraction V, molecular mass about 66 kDa. Phosphate-buffered saline solution (PBS pH = 7.4) was prepared (150 mM NaCl; 1.9 mM NaH_2PO_4; 8.1 mM Na_2HPO_4) and methanol of HPLC grade was obtained from Aldrich. Standard buffers pH = 4 were obtained from Merck. All these compounds were used without further purification. Ultra-pure water was obtained from LABCONCO equipment model 90901-01. 2′-(benzo-[1,2-c]1,2,5-oxadiazol-5(6)-yl(N_1-oxide)methylidene]-1-methoxy-methane hydrazide (**OXMH**) was prepared as previously described[20] and stored at room temperature under vacuum.

Synthesis of first generation of carboxylic terminated dendrimer

Dendrimer was synthesized following the reactions shown in Scheme 1.

In the first step, a nonaester was synthesized by stirring a mixture of nitrilotriacetic acid (0.561 g, 2.4 mmol), Behera's amine (3.00 g, 7.2 mmol), dicyclohexyl carbodiimide (DCC, 1.488 g, 7.2 mmol), and 1-hydroxybenzotriazole (1-HBT, 0.973 g, 7.2 mmol) in DMF (50 ml) at room temperature for 48 h. Then, the reaction was filtered to remove dicyclohexylurea, the solvent evaporated in vacuum to give a residue, which was dissolved in CH_2Cl_2 (50 ml) and then sequentially washed with cold aqueous HCl (l0%), water, $NaHC0_3$ (l0%), and brine. The organic phase was dried ($MgSO_4$), concentrated *in vacuo*, and chromatographed, eluting with 10% acetone in CH_2Cl_2 to furnish.

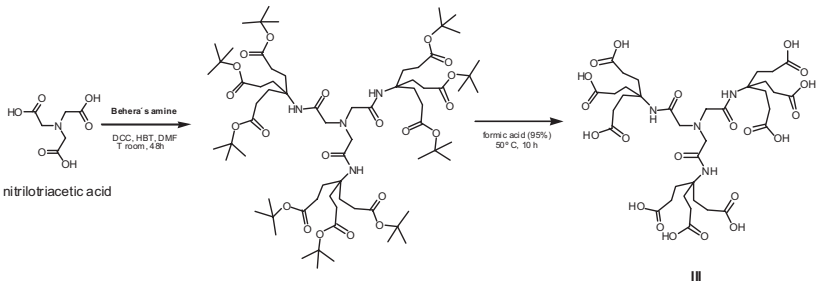
Amidation of nitrilotriacetic acid was supported (^{13}C NMR) by the shift of the signal assigned to the quaternary carbon moiety (CONHC) of Behera's amine from 52.8 to 57.1 ppm and the MALDI-TOF molecular peak at *m/z* 1406.4 ([M + Na^+]).

FT-IR (cm^{-1}): Show two new signals at 1667 and 1535 assigned to Band I (C═O stretching vibration) and Band II (N—H bending vibrations), corresponding to amide group. The carbonyl absorption band of ester appeared at 1739.

^{13}C-NMR ($CDCl_3$): (δ ppm) = 172.5 (C═O ester); 169.8 (C═O amide); 80.3 (CH_3CO); 58.1 (NCH_2CO); 57.5 (CONHC); 29.6 (CH_2CH_2CO); 29.4 (CH_2CH_2CO); 27.8 ($OCCH_3$).

^{1}H-NMR ($CDCl_3$): (δ ppm) = 3.26 (s, 6H, NCH_2CO); 2.15 (m, 18H, CCH_2CH_2CO); 1.92 (m, 18H, CCH_2CH_2CO); 1.37 (s, 81H, $OC(CH_3)_3$).

In a second step, a solution of the product nonaester (1.061 g, 0.77 mmol) in 95% formic acid (50 ml) was stirred at 50 °C for 10 h. After concentration, toluene was added and the solution was again evaporated in vacuum to azeotropically remove residual



Scheme 1. Synthesis of the dendrimer

formic acid. The crude solid was dissolved in water (20 ml) and then sequentially washed with CH_2Cl_2. The formation of dendrimer was supported by the signal at 179.9 ppm (COOH) in the ^{13}C NMR spectrum and the molecular peak at *m/z* 878.3 ([M–H$^-$]) in neg-FAB.

FT-IR (cm^{-1}): The carbonyl absorption band of acid occurred at 1720.

^{13}C-NMR (D_2O): (δ ppm) = 179.9 (C=O acid); the signals at 80.9 ($OCCH_3$) and 27.9 ($OCCH_3$) were absent. The rest of the signals did not change with respect to nonaester.^1H-NMR (D_2O): (δ ppm) = the signal at 1.36 (s, 54H, OC $(CH_3)_3$) was absent.

Methods

UV-Visible spectroscopic measurements were performed using Shimadzu U.V.2401PC spectrofotometer at 20.0 ± 0.2 °C. Spex Fluoromax apparatus was employed for the fluorescence measurements at the same temperature. Dendrimer/HAS fluorescence spectra were taken using excitation wavelength at $\lambda = 285$ nm, where the albumin tryptophan residue absorbs, and the emission registered in the 290–500 nm range. NMR data were recorded using Bruker 400 MHz Advance II. Energy-minimized structures of **OXMH** and protonated dendrimer were obtained using Program SPARTAN '04. The structures were initially minimized with PM3 followed by the calculation of the equilibrium structure with Hartree–Fock 3.21G(*).

Association OXMH/dendrimer

Stock solutions of **OXMH** were prepared dissolving the guest in methanol at 4×10^{-3} M, and stored in darkness. Appropriate amounts of **OXMH** stock solution were transferred into 5 ml volumetric flasks, and the solvent evaporated off under nitrogen atmosphere. The samples were diluted to the appropriate volume with stock dendrimers/buffers solution (dendrimer concentration 1×10^{-4} M), sonicated for 20 min and allowed to equilibrate in darkness overnight. In this way, the samples were prepared at constant dendrimer concentration and **OXMH** concentration varied from 6×10^{-5} to 7×10^{-4} M, keeping pH and ionic strength constant. It is worthy to note that sample preparation conditions clearly affect the nature of **OXMH**/dendrimer association. Only the guest/dendrimers complexes are reproducibly form when **OXMH** is added slowly to the system which has the dendrimer and the system is allowed to equilibrate. Stirring samples, with and without heat, results in further association of **OXMH**/dendrimer complexes into a suspension of higher order species. Sonication prevents the formation of such aggregates. These results have been observed previously with other guest dendrimers systems.[14]

Interactions between the dendrimeric structure and HSA

Before examining the fluorescence properties of the protein, the dendrimer fluorescence emission spectrum was controlled. However, no background signal due to the dendrimer was noticeable in the wavelength region of interest. HSA was dissolved in phosphate buffer at a concentration of 6 μmol/L and stored at room temperature in the darkness. Appropriate aliquots of the dendrimer stock solutions in methanol were transferred into 5 ml volumetric flasks, and methanol evaporated under nitrogen. Then, samples were diluted to volume with HSA buffer phosphate solutions, stirred for 10 min, and stored at room temperature in darkness. Dendrimer concentrations ranged from 3×10^{-6} to 6×10^{-4} M.

Interaction dendrimer–blood red cell

From human blood collected at the UNRC Health Center. A 2% w/v red blood cell (RBC) solution was prepared and centrifuged at 1500 rpm for 10 min at 4 °C. The plasma supernatant was removed and the erythrocytes were suspended in ice cold PBS. The cells were again centrifuged at 1500 rpm for 10 min at 4 °C. This procedure was repeated two more times to ensure the removal of any released hemoglobin. Once the supernatant was removed after the last wash, the cells were suspended in PBS to get a 2% w/v RBC solution. The dendrimers were also prepared in PBS via serial solutions. Dendrimers solution (400 μl) in PBS (1×10^{-5}–2×10^{-3} M) or the reference (solution of PBS) were added to 400 μl of the 2% w/v RBC solution in 10 microcentrifuge tubes for each concentration and incubated for 1 or 24 h at 37 °C. Complete hemolysis was attained using neat water yielding the 100% control value (positive control). After incubation, the tubes were centrifuged and the supernatants were transferred to new tubes. The release of hemoglobin was determined by spectrophotometric analysis of the supernatant at 414 nm. Results were expressed as the amount of hemoglobin release induced by the conjugates as a percentage of the total. On the other hand, the cell samples were viewed under Axiocam HRC Zeiss optical microscope using a magnification of 1000×.

RESULTS AND DISCUSSION

Association OXMH–dendrimer

In order to evaluate the potential use of the new dendrimeric structure as drug delivery vehicle, the interaction with a novel oxadiazol derivative (**OXMH**), with therapeutic activity, was investigated. Since, as stated before, this compound exhibits potential antichagasic activity. The therapeutic activity can be

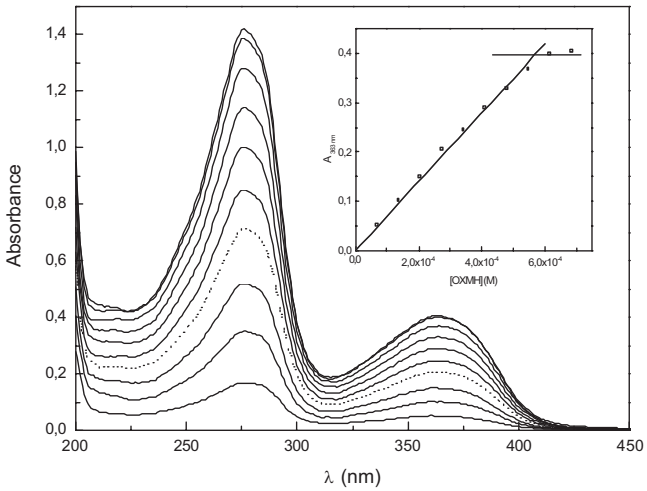


Figure 2. Absorption spectra of **OXMH** in aqueous solution of dendrimer at pH = 7.4 Dendrimer concentration 1×10^{-4} M. [**OXMH**] increases from 6.8×10^{-5} to 6.8×10^{-4} M. (-------) Solubility of **OXMH** in water. Inset: Comparison of absorbance of **OXMH**, at 363 nm, for various ratios of [**OXMH**]/[dendrimer] at pH = 7.4

observed at concentrations close to their water solubility.[20] We study the association of **OXMH** with a new water soluble dendrimer that will increase its solubility and improve its pharmaceutical action.

An indication of the existence of the dendrimer-therapeutic compound association has been obtained by an increase in solubility of the guest in the dendrimer solution, with respect to water. Thus, the changes in the guest solubility were followed by UV–Visible absorption measurements (Fig. 2). The solubility of **OXMH** in PBS solution is *ca.* 2.6×10^{-4} M. As can observed in Fig. 2, the presence of 1.4×10^{-4} M dendrimer increases absorbance of **OXMH** in dendrimer aqueous solutions, when compared with PBS, and the concentration of **OXMH** reaches up to *ca.* 5.7×10^{-4} M. This is an evidence that the therapeutic compound associates with the dendrimeric structure. Dendrimer concentration is fixed at 10^{-4} M because it is the optimum value to perform the spectroscopic measurements in this study.

In order to quantify the dendrimer effectiveness to solubilize the studied compound, the enhancement solubilization factor (ESF) defined as the number of moles of compound solubilized per number of moles of dendrimer was calculated, using Eqn (1).[14]

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

where $[H]_d$ is the guest concentration in aqueous solutions of the dendrimer and it is obtained from the leveling of the plot of the absorbance as a function of the **OXMH** concentration, as shown in the inset in Fig. 2. $[D]_w$ is the concentration of the dendrimer in the aqueous solutions. $[H]_o$ is the analytical concentration of the guest and S_w is the aqueous solubility of the guest.

Hence by using Eqn (1), we obtain an ESF factor equal 2 (Fig. 2 inset) for **OXMH** in the dendrimer at pH = 7.4. Since the dendrimer studied lacks of globular structure to allow the encapsulation of the guest, the observed increase in solubility suggests the existence of **OXMH**–dendrimer specific interactions.[21] Hydrogen bonding between the *N*-oxide moiety in the oxadiazol and the hydrogen in the dendrimer amide group

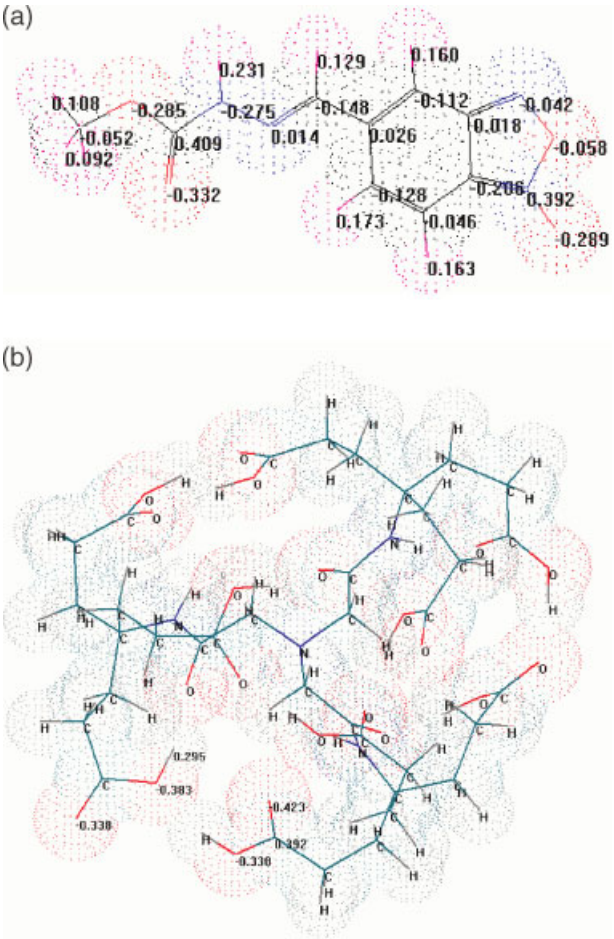


Figure 3. (a) Charge distribution of **OXMH** and (b) simulated configuration of dendrimer using semiempirical method

facilitates the association of this host–guest system. Moreover, also oxadiazol derivative is able to donate hydrogen to the amine and carbonyl groups of the dendrimer. We propose that hydrogen bond specific interaction could be the possible mechanism for the solubility enhancement. The hydrogen bonding donor and acceptor nature of the guest groups, that can be appreciated from the analysis of the charge density of the different groups involved in the interaction (Fig. 3a), supporting this conclusion.

On the other hand, as the interior of the studied dendrimer consists of amido groups and tertiary amino core (Fig. 1a), the protonation of these groups will certainly modify the specific interactions with **OXMH**. Thus, the specific binding sites in the interior of dendrimeric structure and in their terminal groups should be affected by changes in the pH medium, as is actually observed.

Therefore, it is possible that a portion of the final ramification of dendrimer is folded into the macromolecule, building an intramolecular hydrogen bonding between the carboxylic and internal amide groups. This intramolecular interaction modifies the dendrimer conformation, and diminishes its ability to associate with the studied oxadiazol molecule. Semiempirical calculations were carried out with the protonated form of the dendrimer (Fig. 3b). As can be observed the dendrimer shows the

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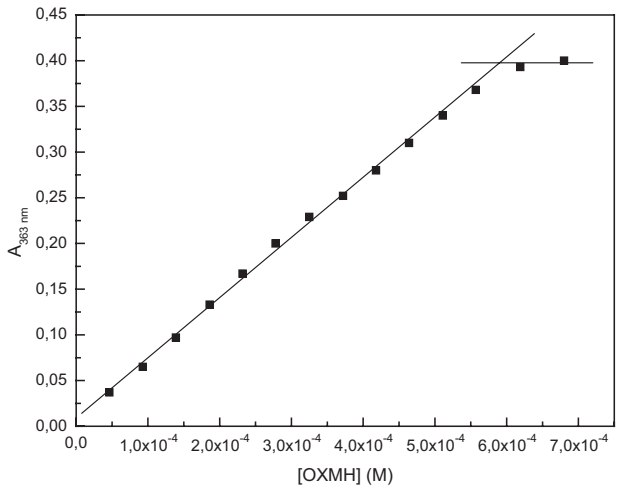

Figure 4. Comparison of absorbance of **OXMH**, at 363 nm, for various ratios of [**OXMH**]/[dendrimer] at pH = 4

terminal groups folding to the center of the structure as proposed.

The comparative study of **OXMH**–dendrimer association at pH = 7.4 and pH = 4 was analyzed as representative systems to examine more closely the pH influence in the interaction guest dendrimer. At pH = 4, the ESF factor obtained using Eqn (1) is 3 (Fig. 4). The observed difference in the number **OXMH** associated to the dendrimer at pH 4 and pH 7.4 is explained as due to the change of the interactions guest/host with the grade of protonation of the system. The pKa values of the carboxylic acids like the ones in the dendrimer terminal groups are close to 5 thus, at pH 4 most of them are protonated while at pH 7.4 practically all are deprotonated. Since the internal amide groups of the dendrimer can be involved in intramolecular hydrogen bond, and therefore are not available to interact with the host, only some surface acid groups can associated with the host. Moreover, the interaction of carboxylate groups is greater when the group is protonated and the density of the negative charge is not delocalized. Consequently, we can explain that the association **OXMH**–dendrimer increases when the pH decreases (3:1 for pH 4 to 2:1 for pH 7) because when the terminal groups are protonated increases the possibility of interaction with the host. Figure 5 shows the possible association between **OXMH** and dendrimer at pH 4 and pH 7.

However, others interaction effects, like solvation energy of the carboxylic and internal groups, as well as solvation of the **OXMH** molecule can play a important role in the solubilization effect here observed.

At pH = 2 (obtained adding hydrochloric acid to solution) **OXMH** solubility decreases to similar values to that observed in the same media without dendrimer. Protonation of the guest molecule and of the dendrimer is expected which precludes the host–guest specific association.

^{1}H-NMR spectroscopy studies on the systems allowed to verify the proposed region for hydrogen bonding interaction. The ^{1}H-NMR assignments for the H bound to carbon in the dendrimer in water are the following ^{1}H-NMR (D_2O): (δ ppm) $H_A = 3.30$ (s, 6H, NCH_2CO); $H_B = 2.15$ (m, 18H, CCH_2CH_2CO); $H_C = 1.94$ (m, 18H, CCH_2CH_2CO). Assigning these protons is critical because the shift of the signal can be used to define the interaction zone. When the guest associated with the dendrimer, the methylene protons B

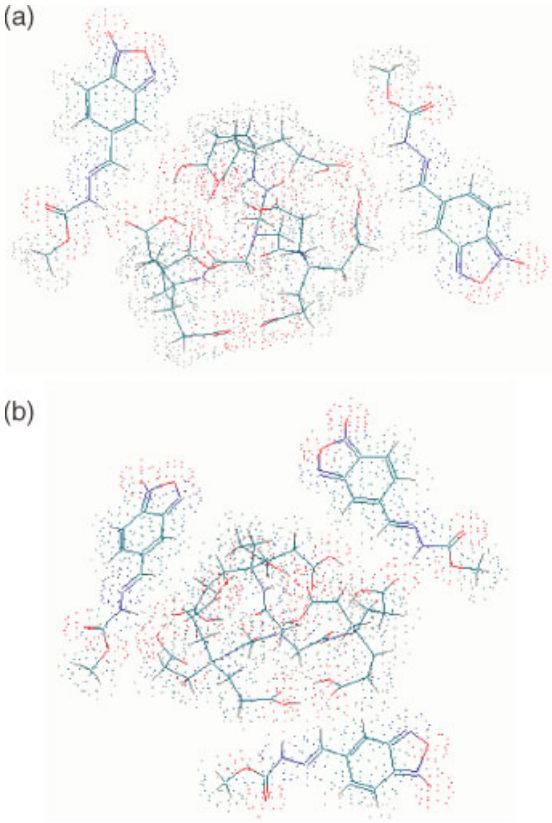

Figure 5. Schematic overview of the hydrogen bonding intra and intermolecular interactions in **OXMH**–dendrimer system at (a) pH = 7.4 and at (b) pH = 4

(CCH_2CH_2CO) adjacent to the carboxylic terminal groups shifted upfield from 2.27 to 2.14 ppm. This is probably due to ring current effects produced by the aromatic ring of **OXMH** when this compound associates with the terminal groups of the dendrimer.

Interaction dendrimer–human serum albumin

HSA is a monomeric protein comprising 585 amino acids, in which the principal regions of ligand binding sites are located in the subdomains IIA and IIIA hydrophobic cavities. In this structure tryptophan residue (Trp-214) is in subdomain IIA. Compounds bound to HSA could alter the intermolecular forces involved in the substantiation of the secondary structure, which results in conformational changes of the protein.[22] Thus, tryptophan fluorescence measurements usually give information about the binding of substance to the protein.[17] For HSA, there are only three intrinsic fluorescent residues: tryptophan, tyrosine, and phenylalanine. In fact, the observed fluorescence of HSA is mostly originated by the contribution of tryptophan, because phenylalanine have very low quantum yield, and the fluorescence of tyrosine is almost totally quenched if it is ionized or near to an amino group or to tryptophan.[23]

The effect of the presence of the dendrimer first generation in HSA was evaluated following the tryptophan residues fluorescence intensity, before and after addition of the dendrimeric systems. The protein showed a strong fluorescence emission band at 342 nm by fixing the excitation wavelength at 285 nm,

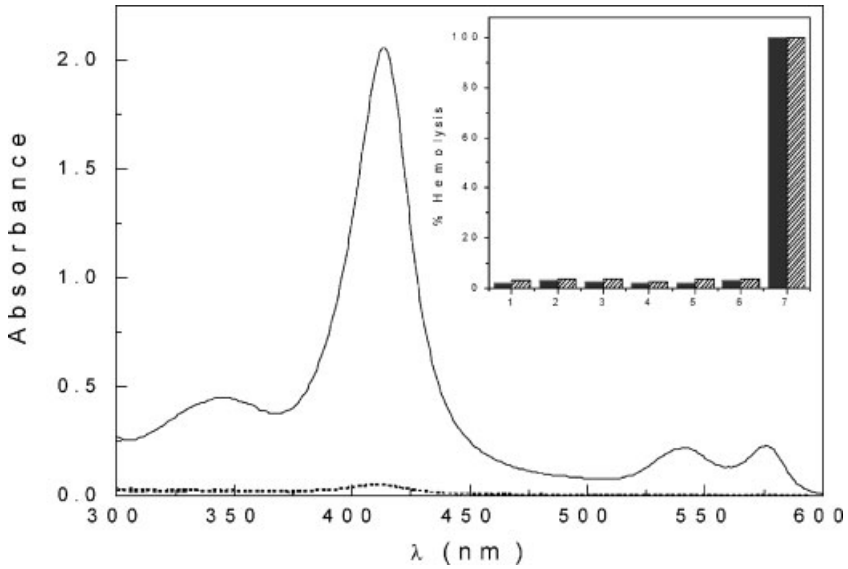

Figure 6. Absorption spectra of hemoglobin in (—) red blood cells treated with PBS and (----) red blood cells treated with double-distilled water. Inset: Hemotoxicity of dendrimer at 1 h and 24 h. **1**-Red blood cells 2% w/v treated with PBS and **7**-red blood cells 2% w/v treated with double-distilled water. Doses of dendrimer increase from **2** to **6** in 1×10^{-5}–2×10^{-3} M

while the dendrimer had no intrinsic fluorescence. With the addition of 100-fold dendrimer concentration only slight reduction in the fluorescence intensity of tryptophan residue in HSA is observed. The results may be indicating that the interaction between the dendrimer and HAS is very small. As has been reported,[17] the fluorescence of tryptophan residues is very sensitive to the changes in their vicinity, thus is widely used to study variations of the molecular conformations of proteins, and allow to detect if some substance is added to HSA. The strength of the interaction between proteins and the dendrimeric structure depends of the dendrimer surface, as exemplified by the study of the PAMAM dendrimers on bovine serum albumin (BSA).[24] These studies showed that the interaction between BSA and dendrimers are of electrostatic nature and depends largely on the type of the dendrimers surface groups. Since at pH 7.4, the pH of blood, HSA present anionic domains,[25] the terminal negatively charged carboxylic groups of the dendrimers studied in this work, seem to have low impact on the protein, probably because they are folded into the molecule.

Interaction dendrimer–blood red cell

RBC lysis is a simple method widely used to study polymer–membrane interaction. It gives a quantitative measure of hemoglobin release.[26] The data obtained in such assays also give a qualitative indication of that potential damage to RBC's are due to the dendrimers administered. Regardless of internal repeat unit structure, cationic dendrimers were generally hemolytic (producing changes red cell morphology) and cytotoxic and this depends on molecular weight, generation, and the number of terminal groups of dendrimer. Furthermore, they produce changes in red cell morphology after 1 h, even at low concentration.[27] Conversely, anionic dendrimers, with carboxylated (—COONa) terminal groups were neither hemolytic nor cytotoxic over a broad concentration range. In the study performed in this work hemolysis was controlled spectrophotometrically at 1 and 24 h. The first generation dendrimer studied resulted non-hemolytic up to concentration of 2×10^{-3} M (Fig. 6), and does not show time-dependent hemolysis. The possible changes in the shape of RBC in response to interaction with the dendrimer were followed by optical microscopy. Control cells were discocytes (Fig. 7a). Figure 7b shows that there is no change of the shape of erythrocytes with increasing dendrimer concentration. The surface of normal erythrocytes is negatively charged due to the presence of glycolipids and some glycated integral and peripheral proteins. The first generation of carboxylic terminated dendrimer studied has as well a negative periphery, consequently the electrostatic repulsion prevents the adhesion to the walls of blood vessels.

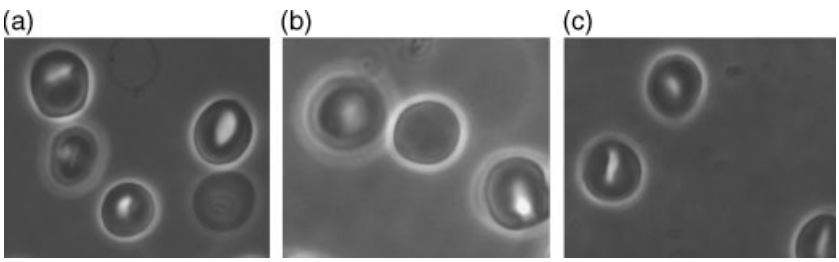

Figure 7. Control erythrocytes (A), erythrocytes in the presence of 2×10^{-3} M of dendrimer (B) at 1 h and (C) at 24 h. (Magnification, 1000×)

CONCLUSIONS

The results obtained show that the terminal groups of the new dendrimeric structure are able to associate with the antichagasic compound. Furthermore, no interaction between dendrimer and blood constituents was observed. The dendrimer is not hemolytic and does not produce damage in the cellular membrane. We conclude that the first generation of carboxylic terminated dendrimer studied could be an appropriate carrier with potential use in drug delivery and it is also that there is a good prospect for releasing it by changing the pH. Therefore, the results encourage us to conduct the synthesis and evaluation of higher dendrimer generation.

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