Formation and characterization of Langmuir and Langmuir-Blodgett films of Newkome-type dendrons in presence and absence of a therapeutic compound, for the development of surface mediated drug delivery systems

Nahir Dib [a], Ana Lucia Reviglio [a], Luciana Fernández [a], Gustavo Morales [a], Marisa Santo [a], Luis Otero [a,*], Fabrisio Alustiza [b], Ana Cecilia Liaudat [b], Pablo Bosch [b], Marcelo Calderón [c], Marisa Martinelli [d], Miriam Strumia [d]

[a] *Departamento de Física, Departamento de Química, Universidad Nacional de Río Cuarto, CONICET, Agencia Postal 3, X5804BYA Río Cuarto, Argentina*
[b] *Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Agencia Postal 3, X5804BYA Río Cuarto, Argentina*
[c] *Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany*
[d] *IPQA-CONICET, Departamento de Química Orgánica, Facultad Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Edificio de Cs. II, Ciudad Universitaria, Córdoba 5000, Argentina*

GRAPHICAL ABSTRACT
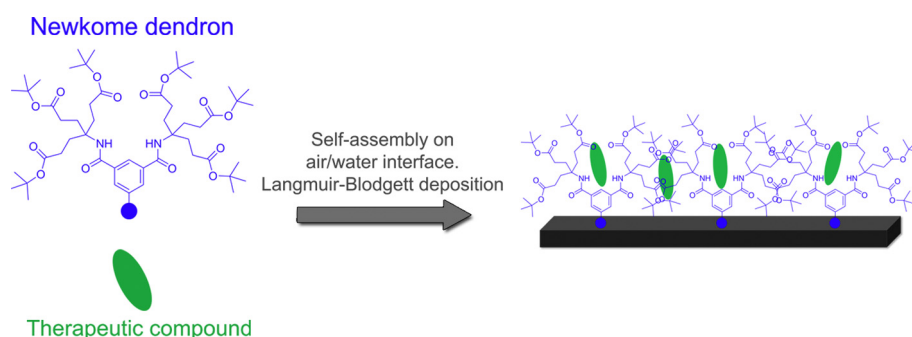
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ABSTRACT

Organic macromolecules with dendrimeric architectures are polymeric materials potentially useful as nanocarriers for therapeutic drugs. In this work, we evaluate a series of Newkome-type dendrons in Langmuir and Langmuir-Blodgett films as platforms capable of interacting with a potential antitumoral agent. The nanocomposite is proposed as model for the development of surface mediated drug delivery systems. We were successful in the formation and characterization of pure (dendrons) and composite (drug-dendron) stable and reproducible monolayers, and their transfer to solid substrates. A detailed study of topographic characteristics of the generated surfaces by atomic force microscopy was conducted. Furthermore, we probed dendron monolayer films as anchorage surfaces for mammalian cells. Normal cell attachment and proliferation on the surfaces were observed. No evident cytotoxic effects were detected, demonstrating the adequate biocompatibility of the surfaces.

* Corresponding author.
    E-mail address: lotero@exa.unrc.edu.ar (L. Otero).

1. Introduction

Dendrimeric structure is a general category of macromolecules that constitutes the fourth class of polymeric architectures [1]. Dendrimers belong to this family, which are characterized by a well-defined molecular weight and a precise three-dimensional structure [2,3]. Dendrimers are formed by dendrons, which possess a focal point, branches and numerous end functional groups [4]. The structural properties and the monodisperse nature of dendrons and dendrimers make them interesting materials for applications in optoelectronic [5], sensing, catalysis and nanomedicine [6–8]. In particular, numerous studies demonstrate the applicability of dendrons and dendrimers as drug nanocarriers in solution [9–12]. The development of this application has been carried out using two main drug-dendrimer association strategies: covalent attachment of the pharmacological active compounds to dendrimer surface, and the incorporation in its interior or surface, sustained by specific and non-specific interactions. When the drug-dendrimer composite reaches the action site, the drug is released due to physical or chemical changes suffered by the complex in response to some stimulus present in the medium. The drug release trigger mechanism can be originated in exogenous and endogenous stimuli. External induced exogenous stimuli can be generated by temperature changes, illumination, electric and magnetic fields application, etc. On the other hand, endogenous stimuli involve local physiological aberrations originated by the pathological conditions, such as pH variations, proteins and/or enzyme concentrations, and temperature [13,14].

Over the past decade, a novel opportunity has been widely investigated in the field of drug delivery; the utilization of surface polymer films and coatings [15]. In these applications, the deposited polymer films can act as a reservoir for the drug, allowing the controlled release of therapeutic molecules [15–18]. Dendrons are presented as very promising candidates for surface mediated drug delivery systems, because their structure holds a focal point that can be modified in order to optimize the junction to the solid substrate, and functionalizable branches that are able to interact with the active drug [19]. In this sense, it is very important to study how structural properties influence the dendrimeric films conformation and stability. Langmuir and Langmuir-Blodgett techniques provide versatile tools for the study of these characteristics [20–22]. These methods allow the generation and characterization of monomolecular films at the air-liquid interface and their transfer to solid substrates [23–25]. Various authors have described Langmuir and Langmuir-Blodgett formation of dendrimer monolayers. The reports include PAMAM structures [26], poly(propyleneimine) dendrimers [27], carbazole [19], poly(aryl ether) dendrimers [28], poly(benzyl ether) dendrons [29–31], linear-dendritic block copolymers [32], among others. The studies are focused on the influence of various factors (such as generation number, focal point, and terminal groups) on the formation and stability of Langmuir monolayers and transferred films [33–36]. However, there are very few studies where Langmuir monolayers of dendrimeric systems are proposed as potential active compound reservoirs, for the development of surface mediated drug delivery systems [21,37].

In previous works [38–41] we have investigated the association of poorly soluble active drugs with dendrimers in water solution and in air-water interface. Also the phase transfer (water-1,2-dichloroethane) characteristics of a series of dendrons was analyzed [42]. We found that the dendrimer-drug interactions increase the drug solubility in water, and that the composites are able to retain these small molecules at the air-water interface in Langmuir monolayers [41]. However, we did not succeed in their controlled and reproducible transfer to a solid substrate, possibly due to the dendrimer-drug mixture dissolution in the aqueous sub-phase during the transfer process. Thus, it was not possible to construct a mixed film as a model of a drug delivery system. In this paper we describe the formation of Langmuir monolayers by Newkome-type dendrons with different structural characteristics (Fig. 1). The systems hold different focal points, branches and terminal groups, which are highlighted by circles of different colors in Fig. 1. Aniline (in **BBA** and **BTA**) and nitrobenzene (in **BBN**) residues were selected as focal points in order to analyze their influence in the monolayer formation and transfer. On the other hand, two kinds of terminal groups were evaluated: tert-butyl (in **BBA** and **BBN**) and methyl ester (in **BTA**), with the aim of analyzing their possible effect in the monolayer characteristics, and in the formation of composite dendron-therapeutic drug Langmuir and Langmuir-Blodgett films. Methyl thio-5-propyl-1H-benzimidazole 2-yl carbamate (Albendazole, ABZ, Fig. 1) was used as model therapeutic agent. ABZ is a drug commonly utilized as anthelmintic [43] that also holds a good potential as antitumor agent [44,45]. However, ABZ is poorly soluble in water [46], and unable to form ordered Langmuir films. Thus, we evaluated the Newkome-type dendrons as platforms capable of interacting with ABZ, and including the drug in thin films at solid surfaces. We present here the interfacial behavior of pure dendrons and ABZ-dendron mixed systems. We were successful in the formation and characterization of pure and composite stable and reproducible monolayers, and their transfer to solid substrates. Also, we conducted a detailed study of structural characteristics of generated surfaces by atomic force microscopy (AFM). Dendron modified surfaces were used as substratum to evaluate mammalian cell adhesion and proliferation, as evidence of their biocompatibility.

2. Materials and methods

2.1. Materials

Newkome-type dendrons were synthesized, purified and characterized using the methodology already described [4,47,48]. Methyl (5-[propylthio]-1H-benzimidazol-2-yl) carbamate was purchased from Sigma-Aldrich. All solvents were obtained from Sintorgan in HPLC quality. Deionized water was obtained from Elga Classic equipment (resistivity of 18 MΩ cm).

2.2. Langmuir and Langmuir-Blodgett films

Monomolecular monolayers formation and their transference to solid substrates were carried out using a Nima Technology Model 611 Langmuir-Blodgett (L-B) trough. The surface pressure was measured using the Wilhelmy plate method. Deionized water was used as subphase. Blank control isotherms were determined prior dendrons spreading in order to confirm the purity of the surface. Dendron solutions in chloroform (50 μL, 6×10^{-4} M), were carefully spread on the water surface and 10 min were allowed to pass before measurements, in order to permit solvent evaporation. Mixed drug-dendron monolayers were formed from chloroform solutions of both molecules in a molar ratio [ABZ]/[dendron] of 4:1. In all the experiments the subphase temperature was kept constant by thermostatic water circulation in the trough. For monolayers compression and expansion barrier speed was 50 cm²/min. As solid substrates for monolayer deposition we used freshly cleaved hydrophilic mica sheets, which were previously immersed in the subphase. Then the monolayers were formed at the air-water interface as already described and compressed at 50 cm²/min. The monolayers were transferred to the slides by the vertical transfer method at 5 mm/min at constant surface pres-
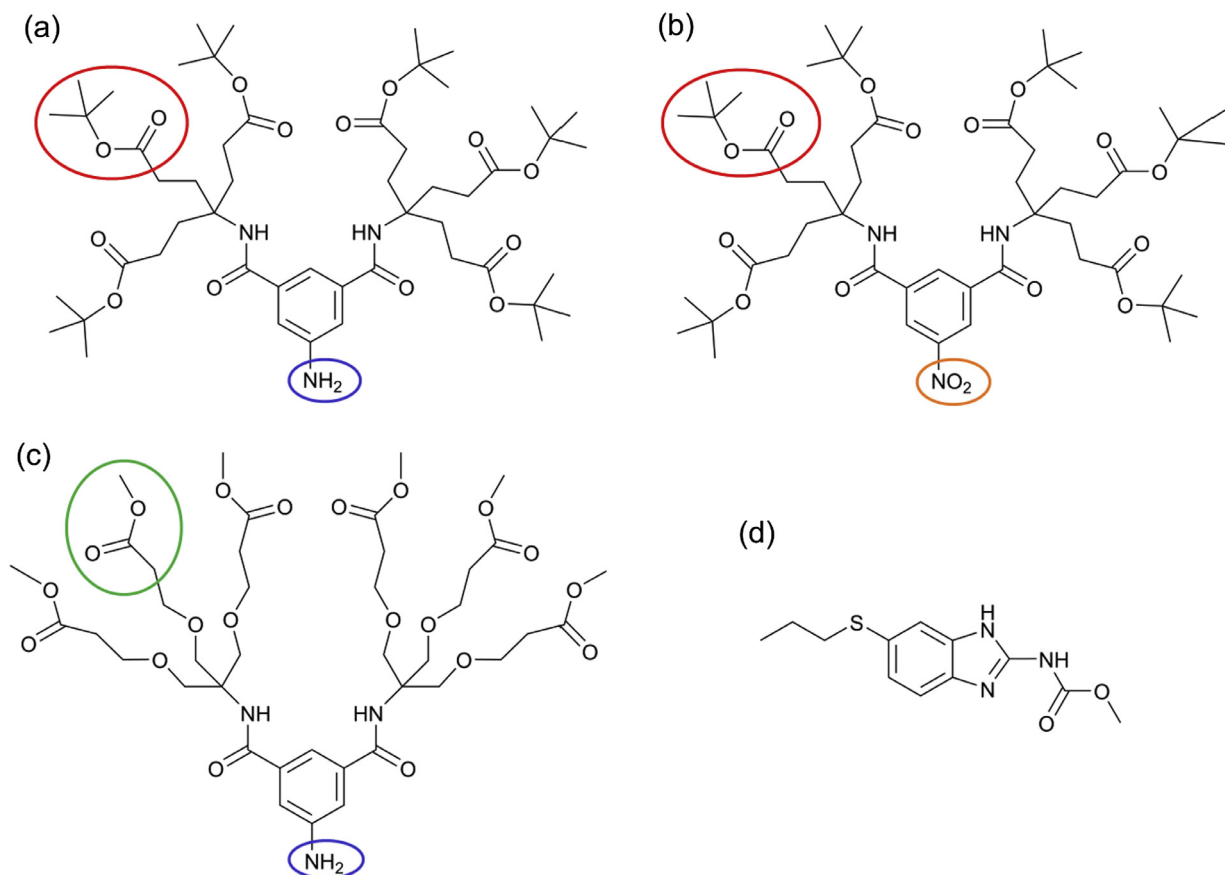
Fig. 1. Chemical structure of Newkome-type dendrons: (a) BBA, (b) BBN and (c) BTA; and the therapeutic agent: (d) methyl (5-[propylthio]-1H-benzimidazol-2-yl) carbamate (ABZ).

sure (4 and 10 mN/m). Films having transfer ratios of 1.0 ± 0.1 were used for AFM characterization experiments. This ratio is defined as A_L/A_S, where A_L is the decrease of area occupied by the monolayer on the liquid surface, at constant surface pressure, and A_S is the coated area of the solid substrate.

2.3. Atomic force microscopy (AFM)

AFM characterization of the Langmuir-Blodgett films was performed using an Agilent 5500 SPM microscope (Agilent Technologies, Inc.) working in Acoustic AC Mode. Commercial silicon cantilever probes, with aluminum backside coating and nominal tip radius of 10 nm (MikroMasch, NSC15/Al BS/15, spring constant ranging 20–75 N/m), were employed just under their fundamental resonance frequencies of about 325 kHz. The experiments were performed in stationary dry-air atmosphere. Images were treated and analyzed using Gwyddion, an open source software for the visualization and analysis of scanning probe microscopy data.

2.4. Biocompatibility analysis

2.4.1. Cell culture

NIH 3T3 cell line, originally derived from mouse fetal fibroblasts, was used to investigate dendron's biocompatibility. Before cell culture, substrate and substrate-dendron film surfaces were carefully immersed three times (3 min each), in antibiotic-antimicotic solution. Afterwards, film surfaces were rinsed thrice by immersion in sterile phosphate buffer solution (PBS) and finally each side was UV-light irradiated for 60 min. Substrate-dendron films and substrate alone were placed individually on the bottom

of a 60 mm culture dish. Then, NIH 3T3 cells were seeded on each surface at a density of 5×10^6 cells in a 60 mm plate. Cell culture was carried out in complete Dulbecco's Modified Eagle Medium (DMEM) with 10% of bovine fetal serum (FBS), antibiotic-antimitotic at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 h of culture, morphology of attached cells on each surface was observed in an inverted microscope (Nikon Ti-S 100, Nikon Corp., Tokyo, Japan) and pictures were taken using a digital camera (Nikon Corp., Tokyo, Japan). All experiments was replicated three times.

2.4.2. Hoechst staining

Cells were cultured on different surfaces as described in the previous section. For Hoechst staining, the slides were rinsed with PBS, fixed overnight in 100% methanol, permeabilized for 10 min with 0.1% Triton X-100, and finally immersed into Hoechst 33258 (Sigma-Aldrich Ltd., St. Louis, MO, USA) solution (1 µg/mL in PBS). Pictures of Hoechst stained cells (filter range: excitation 360 ± 40 nm and fluorescence emission 460 ± 50 nm) were taken with a Nikon digital camera mounted on an inverted microscope Nikon Ti-S 100 equipped with epifluorescence. Hoechst 33258 is a nucleic acid fluorescent commonly used to study cell nuclear morphology and structure and to identify cells in mitosis. The percentage of mitotic cells was calculated as the number of mitotic nuclei multiplied by 100 and divided by the total number of counted nuclei.

2.4.3. Acridine orange staining

Cells were grown on BBN dendron film during 24 h. Then, cells were stained with 1 µg/mL acridine orange (Sigma-Aldrich Ltd.) in

complete medium for 15 min. The cells were washed with PBS and observed with a Nikon Ti-S 100 fluorescence microscope and the images were captured with a digital camera (Nikon Corp., Tokyo, Japan) [49]. Acridine orange is a cationic dye which preferentially stains acidic cellular structures such as DNA, lysosomes and mitochondria, therefore it is useful to study the morphology and distribution of these organelles [50].

3. Results and discussion

3.1. Dendron Langmuir films

The capability of **BBA**, **BBN** and **BTA** dendrons (Fig. 1) to form stable monolayers at the air-water interface was studied by the corresponding surface pressure-Area (π-A) isotherms. Fig. 2 shows the changes in the surface pressure with the area per molecule for the three pure dendrons. In all cases monolayer compression produces an increase in the surface pressure, indicating the dendrons surface activity in the air-water interface. A close examination of the isotherms shows a clear difference between those originated from **BBN** dendron (which holds a nitrobenzene residue as focal point) and those obtained for dendrons with aniline residue (**BBA** and **BTA**, Fig. 1). Clearly, the focal point of the dendrons has a significant effect on the shape of the isotherm. For the three dendrons the π-A curves show, at low pressures, a continuous increase in the compression modulus (defined as $C_s^{-1} = -A(\delta\pi/\delta A)$) which would correspond to gaseous phase (G) and liquid expanded (LE) regions (see inset in Fig. 2).

However, in the case of **BBN** dendrons a plateau is noticeable at ~6.5 mN/m in the π-A curve, possibly due to the coexistence of LE and liquid condensed (LC) phases. This effect is clearly evident in the compression modulus graph, where this transition produces an abrupt drop [51,52]. At higher pressures, the π-A graph for **BBN** shows the region corresponding to the LC phase, before the collapse (this last at ~20 mN/m). On the other hand, π-A compression isotherms for the **BBA** and **BTA** dendrons do not show a clear plateau, indicating that there is not a first order transition between LE and LC phases. Instead, a continuous increase in pressure, as the available surface area per molecule decreases, is observed in both **BBA** and **BTA** dendrons π-A compression isotherms, and no changes are noticeable in the compression modulus. These results allow us to infer that the dendrimeric structures are gradually accommodated in the air-water interface during compression,

showing a second order transition from the gaseous to the condensed phase [51,52]. However, for the three studied dendrons the maximum compression modulus values are similar and around 40 mN/m, which are lower than those generally obtained for amphiphilic molecules with linear non-polar chains [52]. In **BBN**, **BBA** and **BTA** Langmuir films, under compression, the dendrimeric ramifications can intertwine each other, generating low compression modulus. This fact is corroborated by the area occupied by molecule in the condensed phase, which is lower than the expected for the three dendrons (vide infra).

It should be noted that between **BBN** and **BBA** dendrons the only structural difference resides in the focal point (nitrobenzene and aniline groups, respectively, Fig. 1). Thus, the marked differences already described in the π-A compression isotherms can be attributed to the different physicochemical properties of the nitro and amino groups. We propose that, even at low pressures (G and LE regions in π-A isotherms), **BBN** dendrons are able to form domains in the air-water interface, where the nitro groups of two molecules strongly interact with each other [53,54]. As the pressure increases, the **BBN** molecules adopt a more ordered and compact arrangement, with the polar groups in the subphase and non-polar groups toward the air phase, resulting in an aligned monolayer of dendrons, and probably in an anisotropic film (see below the AFM characterization of the L-B films). Similar results were found by Lee et al. [20], who studied the molecular assembly of amphiphilic dendrimer structures at the air-water interface.

Unlike **BBN**, **BBA** and **BTA** dendrons do not form domains at low pressure. This difference in behavior of dendrons at the air-water interface can be explained by the nature of the focal points. For **BBA** and **BTA**, the interaction between the aniline group and the aqueous subphase, through the formation of hydrogen bonds, is highly favored in comparison with the nitro compound (alpha and beta Kamlet-Taft solvent parameters 0.26 and 0.50 for aniline, and 0.05 and 0.30 for nitrobenzene) [55,56]. Thus, the aniline containing dendrons have less possibility to form domains, when they are spread in air-water interface. At low pressures, the molecules would occupy all the available area, with the focal point interacting with the subphase (through hydrogen bonds). When the surface pressure increases, dendrons gradually approach each other, adopting a more compact configuration, with branches pointing to the air phase. Thus, **BBA** and **BTA** dendrons display a continuous transition from gas to condensed phase in the monomolecular films, without a phase transition plateau.

Furthermore, as it can be observed in Fig. 2, all dendrons hold collapse pressure values (20, 25 and 27 mN/m for **BBN**, **BBA** and **BTA** respectively) that are compatible with stable monolayers formation that can resist these compressions [57]. The collapse pressure values are consistent with our suggestion about the differences in the molecular interactions established between water subphase and the focal points. Dendrons with aniline residue, which have higher capability to establish hydrogen bonds with water, showed larger collapse pressure than the dendrons with nitrobenzene focal group, regardless of the chemical nature of the branching and end groups. **BBA** and **BTA** possess different branches and peripheral groups (tert-butyl and methyl ester, respectively) but they share the same focal point (aniline), and exhibit similar collapse pressures. On the other hand, **BBN** and **BBA** share the same branches but exhibit different collapse pressure, being **BBN** (nitrobenzene focal group) the dendron which holds the lowest value, in agreement with the weaker interaction with the water subphase. Thus, this fact indicates that the interactions between the subphase and the focal groups are predominant over the effects generated by the characteristics of the dendrimeric branches and end groups in the collapse pressure values.

The molecular areas obtained extrapolating the slope of the condensed phase of the π-A isotherms to zero-pressure (A_0) were
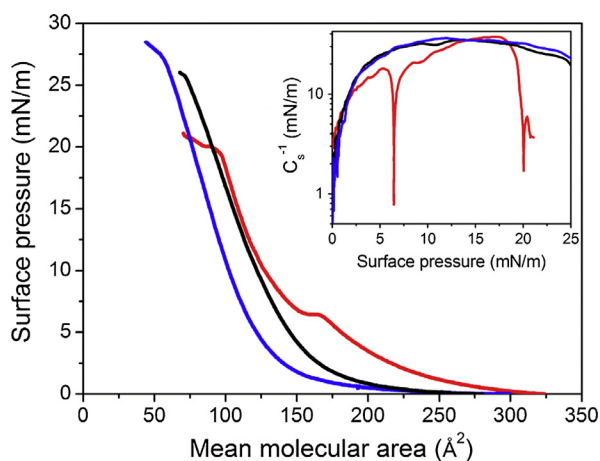
Fig. 2. Surface pressure-area isotherms of BBN (red), BTA (blue) and BBA (black) at the air-water interface at 25 °C. The inset shows Compression Modulus (C_s^{-1}) versus Surface pressure graph for the dendrons monolayers of BBN (red), BTA (blue) and BBA (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

161, 150 and 126 Å^2 from **BBN**, **BBA** and **BTA** dendrons, respectively. In a two dimensional solid phase, dendrons are well oriented and in a relatively close packing. Thus, A$_0$ parameter provides quantitative information on the dimensions and shape of the dendrimer in the interface. The area values found are smaller than the geometric projection obtained by semi empirical calculations (AM1 method at HyperChem package, Table 1). This suggests that, in the compressed monolayer, the dendrimeric ramifications intertwine each other, decreasing the effective area occupied by each dendron.

This effect could produce hysteresis in sequential Langmuir isotherms cycles. Thus, the reversibility of the Langmuir monolayer formation was analyzed using successive compression-expansion cycles, and the time stability through isobaric measurements of the films deformation. The study of both properties provides additional information about the interactions established between the dendrimeric molecules, their ability to remain stable in the formation of a monolayer, and the capacity to return to the two dimensional gaseous state once unzipped the film. Stability of Langmuir films is one of the main requirements to achieve good transfer ratios, and to get films with suitable quality on solid substrates [19]. Fig. 3a–c shows the successive compression-expansion cycles for the different dendrons. In general, all monolayers possess a reversible behavior at low pressure (Supplementary Material, Fig. S1), because in this region the interactions between the molecules are very weak and the compressibility is high. However, at higher surface pressures, before the point of collapse, the second compression isotherm moves to smaller areas (Fig. 3a–c), indicating possible loss of material during compression.

This phenomenon is attributable to the loss of the monolayer-forming molecules in the subphase, multilayer formation, or adhesion of molecules to Wilhelmy system [19]. In addition, a significant hysteresis is observed, may be due to aggregate formation as a result of interactions between the dendrons branches. Furthermore, in order to analyze the stability of the monolayers at the air-water interface, the occupied area per dendron molecule (A) was followed in time at constant surface pressures (π_0 5 and 15 mN/m) and plotted relative to the initial area (A$_i$, see inserts Fig. 3a–c). At low pressure, a 10% decrease in the occupied areas is observed after one hour for the three dendron monolayers. On the other hand, at surface pressures where the condensed phase is formed, stability of **BTA** monolayer is remarkably reduced, meanwhile **BBN** and **BBA** condensed phases exhibit a similar behavior than that observed in expanded liquid region. Thus, it is probable that the hydrophilic/lipophilic balance in these last dendrons permits the formation of stable monolayers at the air-water interface. In addition, the high stability and reproducibility of both, **BBN** and **BBA** Langmuir monolayers allow us to infer that it is possible to attempt the formation of Langmuir-Blodgett films on solid substrate.

The interactions between molecules present at the air-water interface are mainly determined by the physical and chemical properties of the amphiphilic compound. In general, when the molecule has a significant hydrophobic portion, the Van der Waals forces between the hydrocarbon chains are largely responsible for the shape of the isotherm [58]. However, the geometry, size and
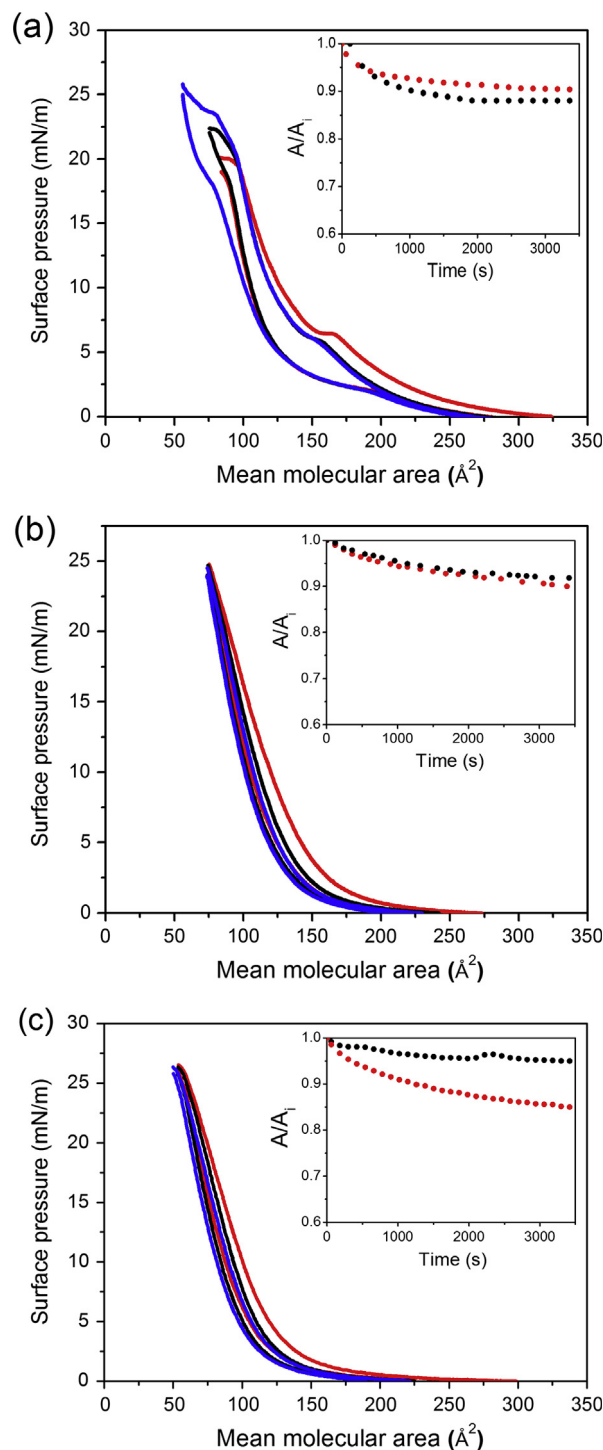
Fig. 3. Compression-expansion cycles of the dendrons Langmuir monolayers on the water subphase at high surface pressures and 25 °C. (a) BBN, (b) BBA and (c) BTA. It shows first cycle (red), second cycle (black) and third cycle (blue). The insets show A/A$_i$ versus time graphs for the dendrons monolayers recorded at 5 mN/m (black) and 15 mN/m (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
(a) Dendrons theoretical projected areas (AM1). (b) Experimental molecular areas extrapolated at zero surface pressure of dendrons. (c) Experimental molecular areas extrapolated at zero surface pressure of dendron-ABZ monolayers.

	BBN	BBA	BTA
(a) Theoretical molecular area of dendron (Å^2)	178	193	136
(b) A$_0$ for dendron monolayer (Å^2)	161	150	126
(c) A$_0$ for dendron-ABZ monolayer (Å^2)	184	170	151

shape of the molecule, the polarity of the focal point and its interaction with the subphase, among other factors, also hold great influence in the monolayer arrangement at each surface pressure. Generally, for those compounds that exhibit a phase transition of first order characterized by the existence of a constant pressure region in the compression isotherms, it is found that the compression isotherm shape is affected by changes in the temperature [59].

It is known that a decrease in the subphase temperature benefits the phase coexistence, which is also evidenced as an increment in the plateau length [58]. In addition, a premature appearance of the transition is generally observed at low temperatures, while the collapse point is shifted toward higher pressures. All these characteristics are observed for **BBN** isotherms in Fig. 4a, where the isotherms for this dendron at 5, 25 and 50 °C are shown. As the temperature increased, the collapse pressure diminished, and the shape and position of the isotherm was affected. At 5 and 25 °C, isotherms exhibit a plateau, which is wider and appears at lower surface pressures at 5 °C. Instead, at 50 °C, the plateau disap-

pears indicating that at this temperature, the kinetic energy of the molecules is high enough to avoid domains formation, and consequently no plateau is observed due to the phase transition. On the other hand, in the case of **BBA** and **BTA** dendrons, the isotherm shape remains almost the same (Fig. 4b and c) in agreement with the second order transition observed for the Langmuir compression isotherms of these dendrons.

3.2. Generation and characterization of Langmuir-Blodgett dendrimeric monolayer

The transfer of monolayers to a solid substrate and their study by AFM permit a detailed morphological characterization of the films, allowing a better understanding of the molecular organization in the Langmuir monolayer. Fig. 5 shows AFM topography and phase images of **BBN** and **BBA** dendron monolayers on mica. In the case of **BBN** dendron, the topographic images (Fig. 5a and c) clearly exhibit a striped pattern. Such arrangement is obtained when the transfers are carried out at both, 4 (Fig. 5a) and 10 mN/m (Fig. 5c) surface pressures. We choose these two surface pressures for the monolayers transfers due to the fact that they clearly belong to different phases for **BBN** dendron, before and after the transition plateau in the pressure-area isotherm (see Fig. 2). Also, the same striped array is also distinguished in the phase images (Fig. 5b and d). The phase shift differences observed can be explained in terms of variations of the dissipated energy and/or composition [60].

An analysis of heights and surface roughness of the topography images in the observed nanostrip arrangement suggests that the high regions correspond to the **BBN** dendron film and the valleys to the mica substrate. Although a specific interaction between the **BBN** monolayer and the mica (a negative charged surface) cannot be discarded as the origin of this arrangement, we suggest that the dendron-dendron interactions are the responsible for the observed striped structures in **BBN** Langmuir-Blodgett films. Duan and Liu [33,61] proposed for related molecular structures that the presence of a nanostrip type arrangement in L-B films is originated in a very orderly packing of the molecules at the interface, where π-π interactions and hydrogen bond are responsible for the conformation. In BBN dendrimeric systems these both types of interactions can be established between nitrobenzene groups present in the dendron focal point, as it has already been pointed out. The intermolecular interactions induce the development of monolayers with preferential orientation in a given axis, generating dendron rows, consequently, anisotropic films are observed as strip-type arrangements, when they are analyzed by AFM.

For the dendron **BBA**, the AFM images of the films were very different from those obtained for **BBN** (Fig. 5e and f). In the case of **BBA** dendron, clear rounded areas are observed on a dark background in the topographic image, and the fund also has small brighter higher spots. Comparing the topography and phase images, the rounded islands and small dots clearly are dendrons on mica, with a height similar to those calculated for the **BBA** dendron size. The morphologic facts observed in **BBA** L-B films can be related to the **BBA** chemical structure. Unlike **BBN** (whose L-B films show a molecular preferential arrangement), in **BBA** L-B films, it would seem that there is not a favored orientation of a molecule relative to another. Consequently, we observed very dense islands of deposited material on the mica substrate arranged symmetrically, without any preferential growth toward a given direction. The structural difference between **BBN** and **BBA** is that the last one holds an aniline group as focal point, instead nitrobenzene in **BBN**. As it is known [54] nitrobenzene groups possess intermolecular specific interactions that could originate the ordered topographic pattern observed in **BBN** L-B films AFM images. Such strong specific interactions are absent between **BBA** dendron mole-
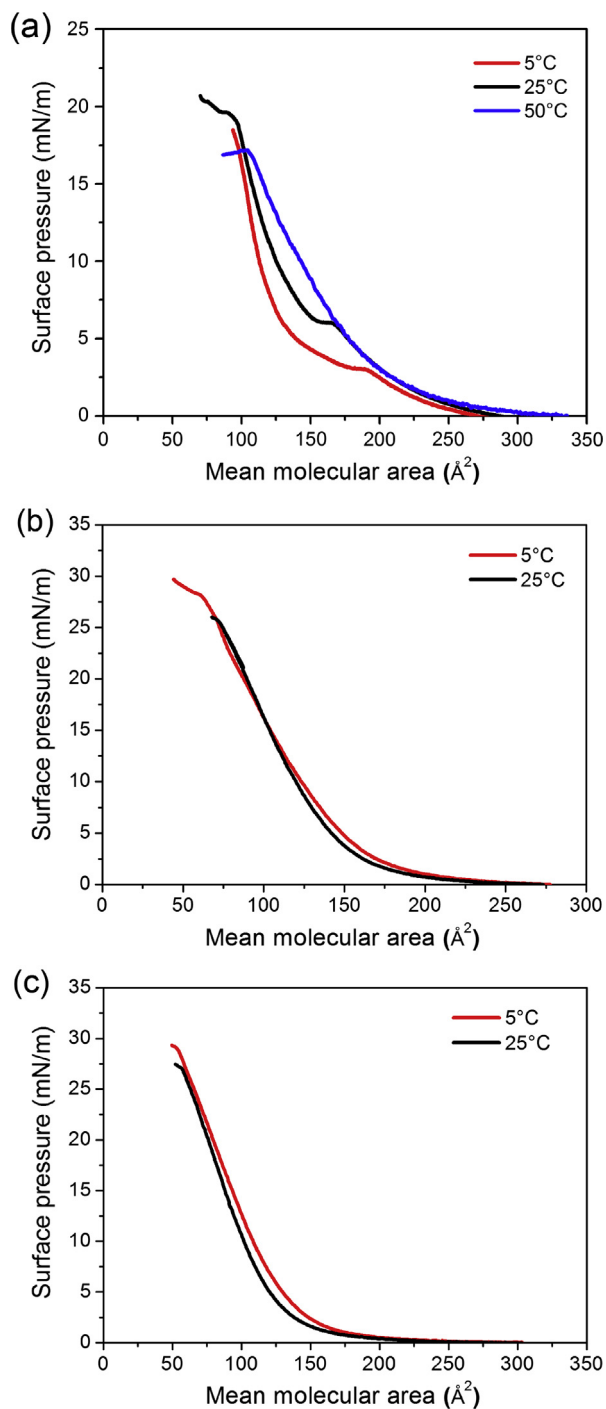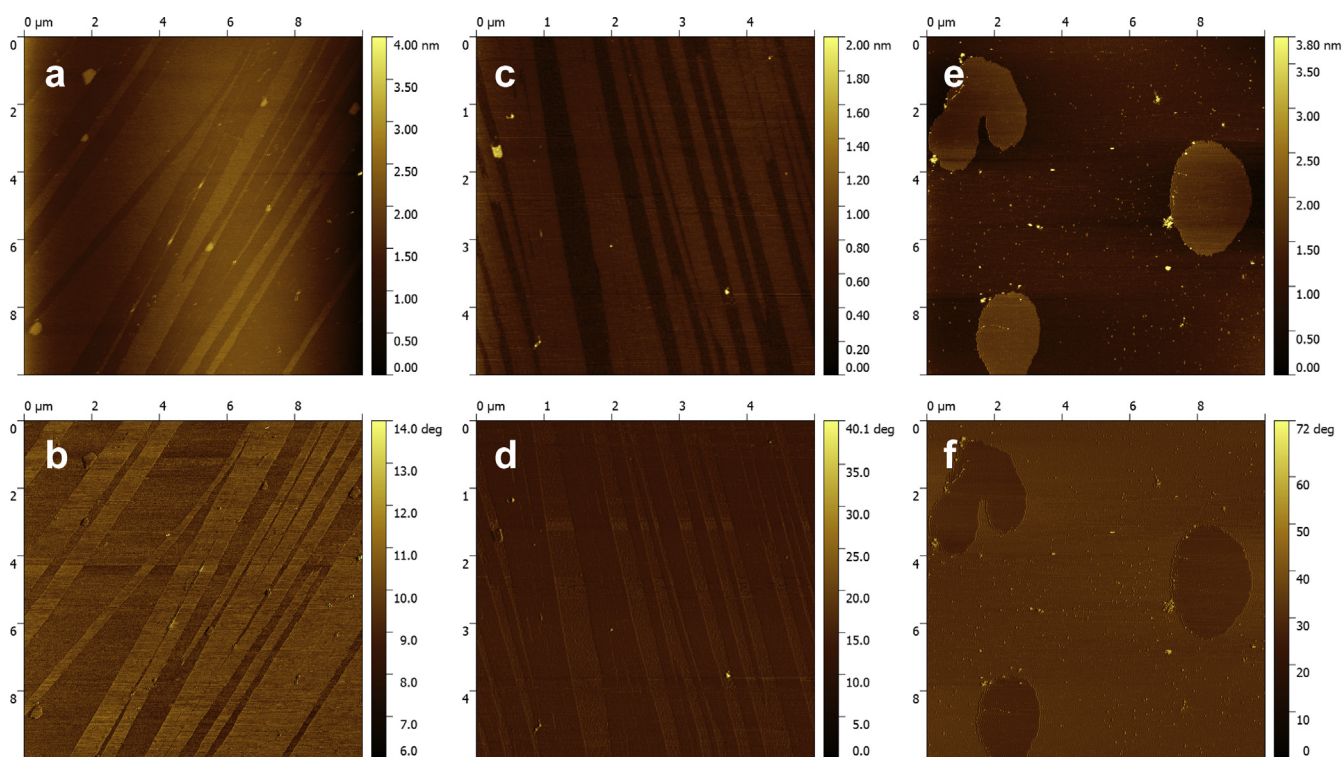
Fig. 4. Surface pressure-area isotherms of (a) BBN, (b) BBA and (c) BTA monolayers at 5 °C (red), 25 °C (black) and 50 °C (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Topographic (top) and phase (bottom) AFM images of Langmuir-Blodgett films transferred at 25 °C on mica: (a and b) BBN monolayer transferred at 4 mN/m. (c and d) BBN monolayer transferred at 10 mN/m. (e and f) BBA monolayer transferred at 10 mN/m.

cules in the L-B films, generating the round islands on the solid substrate observed by AFM. This fact reinforces our hypothesis about the role played by the nitrobenzene group in the molecular arrangement of **BBN** dendron in Langmuir and Langmuir-Blodgett monolayers.

On the other hand, we were not able to obtain acceptable transfer ratios of **BTA** dendrons to mica substrate. In addition, the AFM images of the molecules on mica do not present any regular feature. However, we observed an effective reduction of the monolayer area when the transfer was intended, indicating a solubilization of the **BTA** dendrimeric molecules in the water subphase. These findings are consistent with the presence of ether groups in the dendron arms, and the non-presence of tert-butyl as terminal groups, in comparison with the other two dendrons. Our results allow us to conclude that both, dendron-dendron and dendron-subphase interactions play a crucial role in the formation of Langmuir-Blodgett films, determining the organization of the molecular monolayers.

3.3. Dendron-ABZ composite Langmuir films

As we already pointed out, dendron layers hold promise as surface mediated drug delivery systems. Thus, the interactions and conformation in carrier-therapeutic drug composite layers must be analyzed. Therefore, the formation and characteristics of Langmuir and Langmuir-Blodgett composite films of Newkome-type dendrons and the carbamate **ABZ**, a poorly soluble anticancer agent, was studied. The formation of **ABZ**-dendron mixed films was studied by the technique of co-spreading, which consists of spreading a solution containing both molecules on water surface. Several drug/dendron molar ratios were tested, and up to 4:1 ratio formed stable and reproducible monolayers in the air-water interface. It is important to point out that the formation of Langmuir films of **ABZ** was intended, but we found that this molecule is not capable of forming an ordered film on the

water-air interface, remaining as aggregated islands in the water surface.

Fig. 6a-c shows the compression isotherms obtained for dendron-**ABZ** composite films, along with isotherms of pure dendron monolayers for comparison. The surface pressures are expressed as function of the occupied area by a dendron molecule, therefore the shift of dendron-**ABZ** isotherm regarding pure dendron isotherm exposed the presence of **ABZ** molecules at the interface. Indeed, Fig. 6a-c shows a displacement of the isotherms of mixed films to larger areas, practically without changes in the shape of the curve or in the value of the collapse pressure. In all cases, the obtained limit areas of the dendrons in presence of **ABZ** are superior to those obtained in pure dendron isotherms (Table 1). These results indicate that in the presence of **ABZ**, the dendrons retain their ability to form monolayers and that the drug molecules are located in the air-water interface, forming a mixed film with the dendron molecules. Furthermore, considering the molecular areas obtained from the pure and mixed monolayers isotherms, and the **ABZ**/dendron concentration relationship in the spreading solutions, the area occupied by **ABZ** molecule was calculated, assuming that all drug molecules are located in the interface. The values obtained were practically the same in all cases, about 5 Å^2, in agreement with the projected area estimated by AM1 method for one **ABZ** molecule (4–7 Å^2). These results allow us to infer that it is possible the generation of homogeneous and ordered drug-dendrimer composite monolayers, where the active compound is incorporated as a guest in all dendrimeric films.

Furthermore, in order to evaluate the effect caused by the presence of the active compound on the reversibility and stability of Langmuir films, these properties were studied by performing similar experiments conducted with pure dendrons, but in **ABZ**-dendron composite monolayers. The study also allowed identifying the most suitable conditions for the preparation of Langmuir-Blodgett films on solid substrate. Successive compression-expansion cycles show that the isotherms are very reversible at
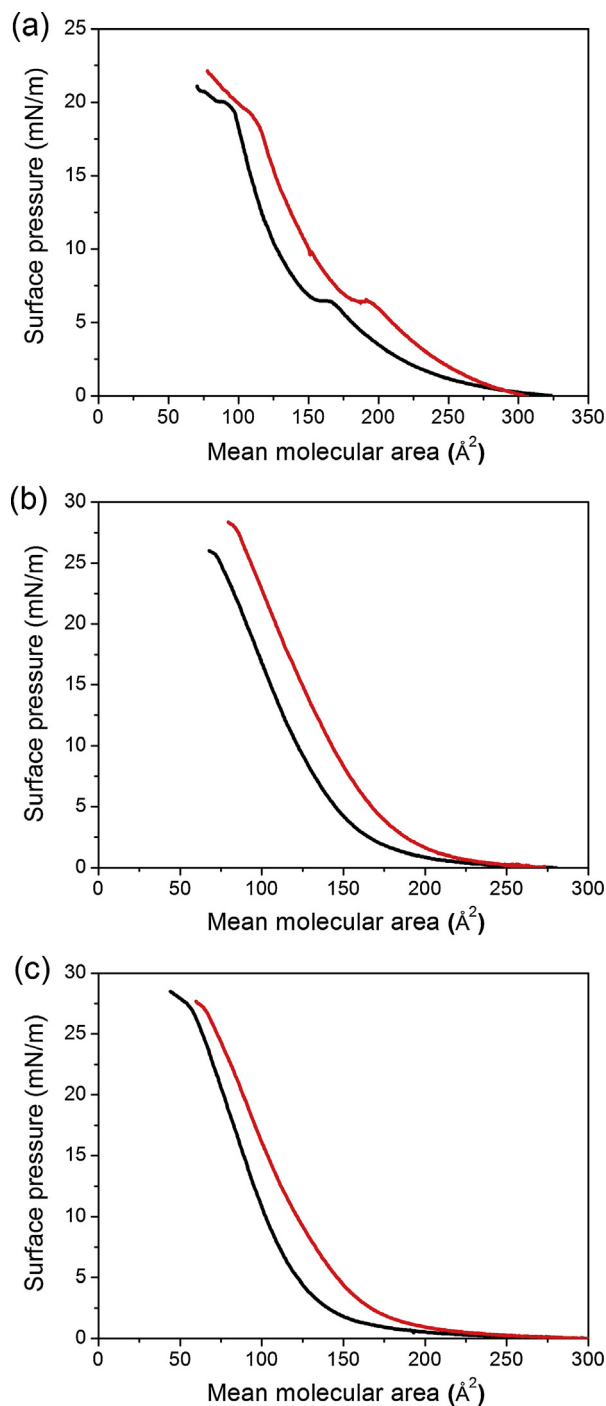
Fig. 6. Surface pressure-area isotherms of (a) BBN, (b) BBA and (c) BTA pure dendrons monolayers (black) and dendron-ABZ monolayers (red) at the air-water interface at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

low pressures (Supplementary Material, Fig. S2), and no hysteresis is observed in any case, even for **BBN-ABZ** isotherm, which exhibit certain hysteresis when **ABZ** is not present. However, at high pressure (near collapse) the monolayer formation is less reversible, similar to the results obtained with pure dendrons films (Supplementary Material, Fig. S2). To determine the time stability of **ABZ**-dendron composite monolayers, the deformation (A/A$_i$) curve at constant pressure (π_0) was monitored. The results show that the stability of the dendrimeric monolayers is not affected by the presence of **ABZ** compound (Supplementary Material, Fig. S3). Thus,

based on these results, it can be concluded that the film transference to a solid substrate to generate mixed Langmuir-Blodgett films should be performed in similar conditions to those used for the transference of pure dendron monolayers.

3.4. Generation and characterization of Langmuir-Blodgett dendron-ABZ monolayer

Acceptable (1.0 ± 0.1) and reproducible transfer ratios from water to solid (mica) substrate were only obtained with **BBN-ABZ** composite monolayers. This result reinforces the already observed relevance for the formation of Langmuir-Blodgett films of the presence of nitrobenzene group in the dendron focal point. Fig. 7 shows topography and phase AFM images of **BBN-ABZ** composite monolayer transferred at 10 mN/m on mica. It is observed the same "strip" type pattern than that observed in **BBN** dendron film on mica. This suggests that the presence of the drug in the monolayer does not alter the packing order of dendrons in the film. Moreover, the phase image shows light areas, evidencing the presence of different composition and/or energy dissipation properties relative to the rest of the strips. It could be thought that these areas correspond to a different dendron conformation or packing.

Therefore, the successful transfer and formation of Langmuir-Blodgett film of dendrimer-drug composite makes this arrangement an interesting model to study surface mediated drug delivery in biological systems, where dendrimer coating hosts active therapeutic compounds. To that end, we evaluated biocompatibility of dendron Langmuir-Blodgett films using an in vitro mammalian cell culture assay.

3.5. Biocompatibility analysis

Before introduction of a composite film, as surface mediated drug delivery system, in the in vivo biological setting (human or animal), numerous biocompatibility and biosafety testing are required. Here, we tested if **BBN** dendron films are able to support murine NIH 3T3 cell normal attachment and growth as a preliminary test of biocompatibility. Cells were seeded on cell culture polystyrene (control), mica or dendron films. Cells attached normally to both mica and mica-dendron surfaces demonstrating that the presence of **BBN** dendrons did not interfere with this process. Furthermore, attached cells showed typical fibroblast morphology when observed at 400× magnification under a bright field microscope (Fig. 8a). Morphology of acidic organelles, stained with the fluorescent dye acridine orange, was similar to those observed in control cells (Fig. 8c). Cells not only adhered to mica and dendron film but also underwent mitosis. This was evident upon microscopic inspection by the presence of mitotic cells (mica: 6.0 ± 0.8%, dendron film: 4.8 ± 0.4%; Fig. 8b) and progressive cell coverage with time in culture. Nuclear staining (Hoechst) reveals normal nuclear morphology with lax chromatin and mitotic images (Fig. 8b). Neither microbial nor fungal contamination was evident during cell culture, which indicates that these materials can be appropriately prepared to support in vitro cell culture. Culture and differentiation of mammalian progenitor cells on phosphoserine-tethered poly(epsilon-lysine) dendron films have been previously described [62]. These results indicate that dendron film systems are gaining ground in other areas such as cell biology and tissue engineering [63].
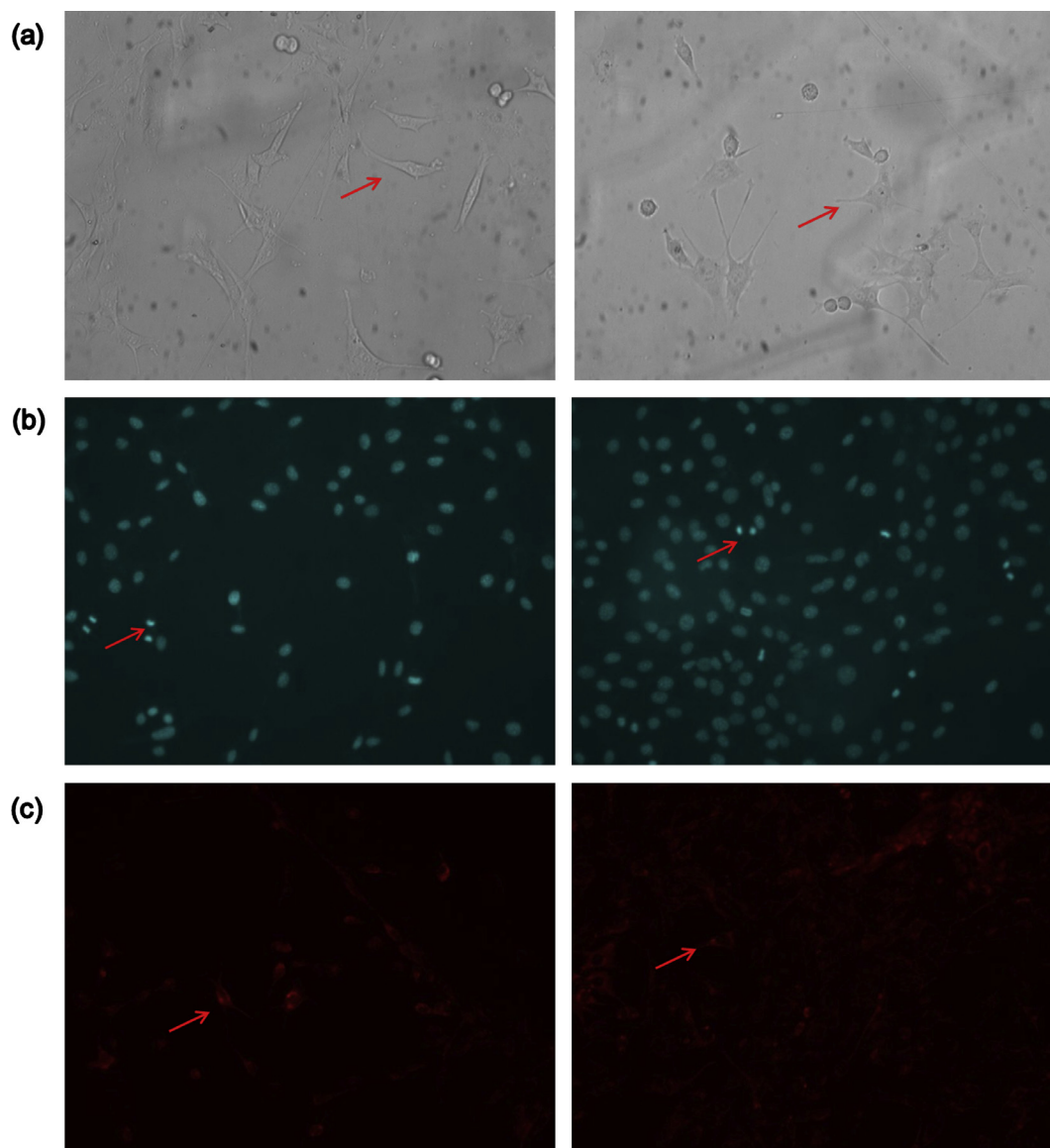
4. Conclusions

Composite dendron-active drug Langmuir and Langmuir-Blodgett monolayers, at air-water and air-solid substrate interfaces, were successfully generated using a Newkome amphiphilic
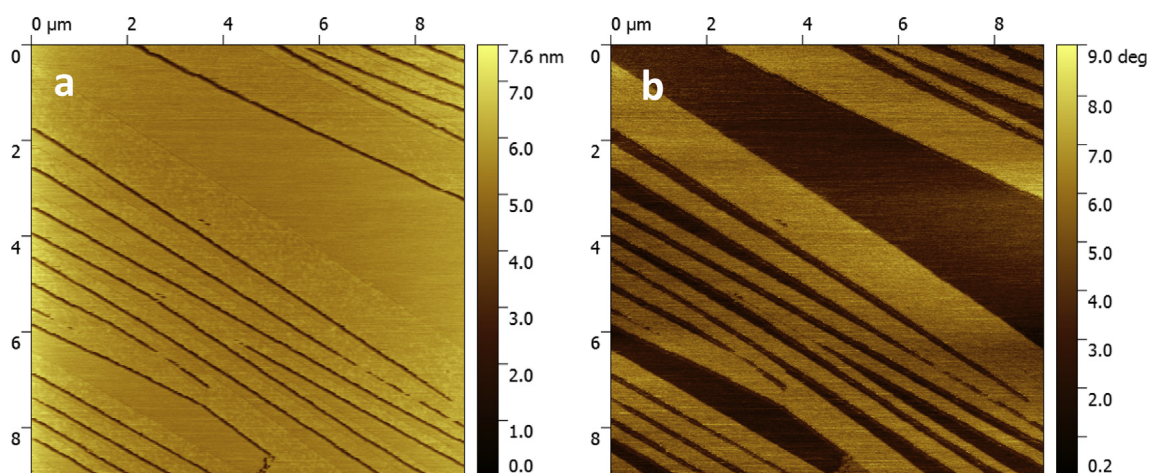
Fig. 7. Topographic (left) and phase (right) AFM images of BBN-ABZ monolayer transferred at 10 mN/m.

Fig. 8. NIH 3T3 cells after 24 h culture on either mica (left) or BBN Langmuir-Blodgett (right) surfaces. (a) Phase contrast images (magnification 200×). Arrows indicated culture cells with typical fibroblast morphology. (b) Fluorescence images (magnification 200×) of cell stained with Hoechst. Arrows indicate cells undergoing mitotic division. (c) Fluorescence images (magnification 200×) of cells stained with the fluorescent dye acridine orange. Arrows show culture cells with typical fibroblast morphology and normal acidic organelle staining. Bacterial and/or fungal contamination is not evident.

dendron and a poorly soluble anticancer drug. Although there is a large number of studies involving dendrimer/drug [9,10] and dendron/drug [11,12] associations for the development of drug delivery systems, as far as we know there is not antecedent about the incorporation of an active compound in the Langmuir monolayer formed by amphiphilic dendrons before this report. A series of works demonstrated that dendrimers [26,27,29,31] and dendrons [19,32,37] holding the necessary hydrophilic/hydrophobic balance are able to form ordered Langmuir films. In this work we show that, using a dendron with nitrobenzene focal group and tert-butyl terminated branches, up to four therapeutic active compound molecules (albendazole) by each dendron can be homogenously incorporated in Langmuir and Langmuir-Blodgett films. The active compound forms amorphous aggregates when it is spread at air-water interface, but in the dendron environment is homogenously incorporated to the Langmuir films, and can be transferred to a solid substrate forming composite films. Thus, this study shows that this kind of composite layers can be used as a model for the study and development of surface mediated drug delivery systems [15]. Indeed, stable and reproducible monolayers were obtained and transferred to a solid substrate. The films were characterized by atomic force microscopy, and a nanostrip type arrangement, originated in a very orderly packing of the molecules at the interface, was observed. This particular organization is attributed to the presence of nitrobenzene groups in the dendron focal point, whose intermolecular interactions induce the development of anisotropic arrangements. Also, dendron monolayer biocompatibility was investigated through the analysis of cell attachment and proliferation, showing that the system possesses no conspicuous cytotoxic effects. Therefore, we believe that this work could inspire the design and study of composites between dendrimeric compounds and active drugs in Langmuir and Langmuir Blodgett films, for future developments in the area of surface mediated drug delivery systems and other possible biological applications.

Acknowledgments

Appendix A. Supplementary material

Compression-expansion cycles of the dendrons Langmuir monolayers at low surface pressures (Fig. S1), Compression-expansion cycles of the dendrons-ABZ composite Langmuir monolayers (Fig. S2), A/A_i relationship in time for dendrons and dendron-ABZ monolayers recorded at 10 mN/m (Fig. S3). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2017.02.036.

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