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# Physico-chemical characterization of nanocapsule polymeric wall using fluorescent benzazole probes

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**Abstract**

Fluorescent polymers were used to prepare innovative formulations with the objective of verifying the chemical composition of the particle/water interface of nanocapsules at a molecular level. The benzazole dyes distinguish between apolar and polar/protic environments. Comparing the fluorescent behavior of benzoxazole-loaded nanocapsules (entrapped dye) with that of fluorescent-polymeric nanocapsules (chemically bound dye), the results indicated that the latter was exposed to a different environment than that to which the entrapped dye was exposed. The polymer in the nanocapsule suspensions is actually at the oil/water interface, interacting with both inner and outer pseudo-phases at the same time. The polymer is restricted at the particle/water interface forming a wall in nanocapsules. The physico-chemical stability of nanocapsules was studied by fluorescence, light scattering, ζ-potential and potentiometry. After 15, 30, 45 and 60 days of preparation different fluorescent behaviors were observed for the benzimidazole physically entrapped in nanocapsules compared to the benzimidazole chemically bound to the polymer wall. This spectrum presented an isoemissive point indicating that only two species were in equilibrium in the medium. The study showed that the water is increasingly interacting with the polymer in the nanocapsule suspensions.


*Keywords:* Polymeric nanoparticles; Nanocapsules; Benzazole; Fluorescent polymers; ESIPT; Stability study

## 1. Introduction

In the past 20 years, nanocarriers for drug delivery have been extensively studied in the pharmaceutical nanotechnology field, as well as, more recently, in different nanoscience areas including chemistry, physics and biology. Different systems have been proposed comprising inorganic or organic nanodevices. In general, some promising nanocarriers are the liposomes (Fattal et al., 2001; Koynova and MacDonald, 2004), the solid lipid nanoparticles (Müller et al., 2000), the self-assembled lipid superstructures (Barauskas et al., 2005), the polymeric micelles (Kang et al., 2005) and the polymeric nanoparticles (Brannon-Peppas, 1995; Fernández-Urrusuno et al., 1997; Couvreur et al., 2002; Schaffazick et al., 2003; Schaffazick et al., 2005; Teixeira et al., 2005). The nanoencapsulation improves drug efficacy, drug bioavailability or it is proposed to decrease drug side effects (Couvreur et al., 2002; Schaffazick et al., 2003).

Among the different nanocarriers, the polymeric nanoparticles are sub-micrometric drug carriers prepared by *in situ* polymerization or by precipitation of pre-formed polymers. The general term, nanoparticles, includes nanospheres and nanocapsules, which are made of polymer and polymer and oil, respectively. The theoretical models for those nanoparticles are, respectively, a polymeric matrix and a vesicle (Couvreur et al., 2002). The release properties of nanospheres and nanocapsules are frequently compared to each other, as well as those nanoparticles are compared to nanoemulsions (Calvo et al., 1996; Guterres et al., 2000; Pohlmann et al., 2004; Cruz et al., 2006a) that are sub-micrometric emulsions. Overall, the physico-chemical characteristics of those nanocarriers have been identified as critical for the control of the release properties of encapsulated drugs (Leroueil-le Verger et al., 1998; Couvreur et al., 2002; Pohlmann et al., 2004).

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Different raw materials are proposed to prepare nanocapsules, including polyesters or acrylic polymers, as polymers, and triglycerides, benzyl benzoate, large size alcohols or mineral oil, as oil cores (Schaffazick et al., 2003). Several surfactants are used as phospholipids, sorbitan monostearate, polysorbates, dextran, poly(ethyleneglycol) or mixtures of poly(ethyleneglycol) and poly(propyleneglycol) to stabilize the systems (Fessi et al., 1989; Couvreur et al., 2002). Due to the complexity of the systems, any model of the organization of those components at a molecular level can only be proposed for each nanocarrier system after a full physicochemical study (Mosqueira et al., 2000; Müller et al., 2001). For instance, nanocarriers prepared with poly(lactide) or poly(ε-caprolactone), as polymers, and benzyl benzoate, as oil, correspond to nanoemulsions instead of nanocapsules (Guterres et al., 2000). This conclusion was supported by the results of swelling experiments, proposed to verify the solubility of those polymers in this oil, and HPLC, which showed the drug concentration after spray-drying due to losses of benzyl benzoate from the formulation. The organization of the components at a molecular level has been proposed for nanocapsules prepared with poly(ε-caprolactone), caprylic/capric triglyceride, sorbitan monostearate and polysorbate 80 (Müller et al., 2001). DSC curves showed the melting peak of the polymer, the absence of the sorbitan monostearate melting and the decrease of the melting temperature of the triglyceride indicating that, in those nanocapsules, the sorbitan monostearate is dissolved in the oil phase and that the polymer is probably surrounding the core. This model was corroborated by SAXS analyses carried out more recently (Cruz et al., 2006a,b). In another work (Pohlmann et al., 2002), a comparative study has been carried out by dynamic light scattering using nanocapsules prepared with poly(ε-caprolactone), mineral oil, sorbitan monostearate and polysorbate 80 and the respective nanospheres (omitting the oil), nanoemulsion (omitting the polymer) and nanodispersion of surfactants (omitting both the polymer and the oil). In this case, after determining the relative virial diffusion coefficients ($k_D$) for each formulation, those nanocapsules presented low interaction with water, due to the presence of sorbitan monostearate dispersed in the polymer. This model was corroborated by SAXS analyses (Cruz et al., 2006a,b).

The use of fluorescent dyes to label structures is a versatile method to probe a system (Campo et al., 2000; Rodembusch et al., 2005). The benzazole dyes are able to differentiate chemical environments due to dual emissions (normal and ESIPT). The ESIPT is resulted from an intramolecular proton-transfer mechanism in the excited state (Rodembusch et al., 2005). In the ESIPT mechanism (Fig. 1), the UV light absorption by the enol-*cis* ($E_I$) produces the excited enol-*cis* ($E_I$*), which is quickly converted to an excited keto-tautomer (K*) by an intramolecular proton transfer. The tautomerism occurs because the hydrogen in $E_I$* becomes more acid and the nitrogen more basic than in the $E_I$ conformer. In the K*, an intramolecular hydrogen bond is also formed by the interaction between N–H and C=O (Rodembusch et al., 2005). The K* decays to a keto-tautomer (K) emitting fluorescence, and the initial $E_I$ is regenerated after tautomerism without any photochemical change. Depending on

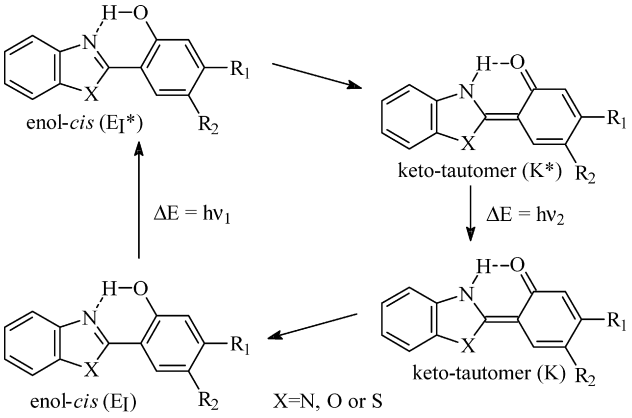


Fig. 1. Excited state intramolecular proton-transfer mechanism (ESIPT).

the chemical environment, $E_I$ can equilibrate with other conformers, like enol-*cis* open conformer ($E_{II}$) (Fig. 2), which can be stabilized by intermolecular hydrogen bond with the solvent. $E_{II}$ is originated from the $E_I$ intramolecular hydrogen bond rupture between the hydrogen of the OH group and the nitrogen at the position 3, followed by rotation of the 2-hydroxyphenyl group. In non-polar solvents additional enol-*trans* conformers ($E_{III}$) in benzoxazoles and benzothiazoles (X = O and S, respectively) and enol-*trans* open ($E_{IV}$) in benzimidazoles (X = NH) could also exist. The conformers ($E_{II}$ to $E_{IV}$) present normal relaxation and can compete with $E_I$, which produces $E_I$* and consequently the excited keto-tautomer (K*), responsible for the ESIPT mechanism (Rodembusch et al., 2005). In this way, the benzazoles are able to differentiate the chemical environments of either polar and non-polar or protic and non-protic solvents.

Fluorescent polymers prepared by radicalar polymerization of methyl methacrylate and benzazoles have been reported (Campo et al., 2000; Rodembusch et al., 2005). Then, we have chosen two of those benzazole-labeled polymers to prepare innovative formulations containing fluorescent-labeled polymeric nanocapsules with the objective of verifying the physicochemical interactions at a molecular level in the particle/water interface of those systems. Our specific goal was to determine the chemical composition of the interface of nanocapsules prepared

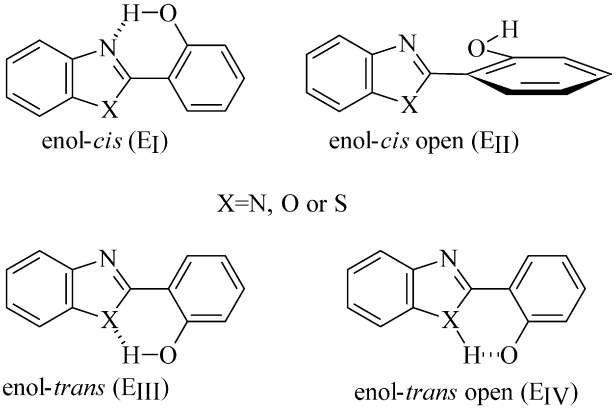


Fig. 2. Structures of enol-*cis* ($E_I$), enol-*cis* open conformer ($E_{II}$), enol-*trans* conformers ($E_{III}$) and enol-*trans* open ($E_{IV}$).

with poly(methyl methacrylate), caprylic/capric triglyceride, sorbitan monostearate and polysorbate 80, as well as to study the physico-chemical stability of those nanocapsules by fluorescence, light scattering, ζ-potential and potentiometry. As far as we know, this is the first use of the benzazole dyes in probing the chemical environment of polymeric nanocapsules.

## 2. Materials and methods

### 2.1. Materials

*o*-Aminophenol, 1,2-phenylenediamine, 5-aminosalicylic and 4-aminosalicylic acids were acquired from Aldrich® (reagent grade) and used without purification. Polyphosphoric acid (PPA) was purchased from Acros Chemicals®, and sorbitan monostearate, caprylic/capric triglyceride and polysorbate 80 from Delaware®. Methyl methacrylate, acquired from Aldrich®, was purified before polymerization by passing through a column with activated alumina (Merck®). 2,2′-Azo-bis-isobutyrylnitrile (AIBN) was obtained from Merck® and purified before use by recrystallization from methanol and maintained under vacuum. The diethyl β-ethoxymethylenmalonate and the acryloyl chloride were purchased from Acros Chemicals®. Silicagel 60 (Merck®) was used for chromatographic column separations. All the solvents were used as received or purified using classical standard procedures. Spectroscopic grade solvents (Merck®) were used for fluorescence, UV–vis and NMR measurements.

### 2.2. Methods and instruments

$^1$H NMR analyses were performed at room temperature on a Varian® (model VXR-200, 200 MHz) using tetramethylsilane as internal standard and DMSO-$d_6$ (Aldrich) or $CDCl_3$ (Merck) as solvents. The UV–vis and fluorescence analyses were carried out using a Shimadzu® UV-1601 PC spectrometer and a Hitachi spectrofluorometer (model F-4500), respectively. Fluorescence spectrum correction was performed to enable measuring a true spectrum by eliminating instrumental response such as wavelength characteristics of the monochromator or detector using Rhodamine B as standard (quantum counter).

### 2.3. Synthesis of the fluorescent copolymers

The syntheses of the fluorescent copolymers, previously described (Campo et al., 2000; Rodembusch et al., 2005), were based on the radical copolymerization of methyl methacrylate and 2-(5′-amino-2′-hydroxyphenyl)benzazole or 2-(4′-amino-2′-hydroxyphenyl)benzazole derivatives.

Briefly, the 4-aminosalicylic acid reacted with the *o*-aminophenol. The 2-(5′-amino-2′-hydroxyphenyl)benzoxazole was isolated and purified by column chromatography. To a solution of 2-(5′-amino-2′-hydroxyphenyl)benzoxazole in chloroform was added a solution of acryloyl chloride in chloroform. The *N*-acryloylamide derivative (**1**) was obtained with satisfactory yield and its chemical identity was verified by NMR.

In parallel, the 5-aminosalicylic acid was condensed with the 1,2-phenylenediamine. The 2-(4′-amino-2′-hydroxyphenyl) benzimidazole was isolated and purified by column chromatography. To a solution of 2-(4′-amino-2′-hydroxyphenyl)benzimidazole in ethanol was added diethyl β-ethoxymethylenmalonate. The vinylene derivative (**2**) was obtained with satisfactory yield and its chemical identity was verified by NMR.

The copolymers **3** and **4** were respectively prepared by polymerization of *N*-acryloylamide derivative **1** or vinylene derivative **2** with methyl methacrylate in the presence of AIBN. The copolymers were purified by dissolution in chloroform (1 mL) and precipitation into petroleum ether (20 mL), as much as any dye was not detected in the filtrate by UV spectroscopy. The copolymers were characterized by UV spectroscopy as previously described (Campo et al., 2000; Rodembusch et al., 2005).

The polymerization of only methyl methacrylate, without any dye, in the presence of AIBN was also carried out to prepare nanocapsules of poly(methyl methacrylate) (PMMA) containing **1** or **2** entrapped and not chemically bound to the polymer. The PMMA was purified as described above for copolymers **3** and **4**.

### 2.4. Size exclusion chromatography

Size exclusion chromatography (SEC) analyses were carried out using an LDC Analytical Model Constametric 3200. The polymer samples (0.004–0.005 g) were dissolved in tetrahydrofurane, and polystyrene was used as reference.

### 2.5. Preparation of nanocapsules and nanoemulsion

All samples were prepared by interfacial deposition of preformed polymers or spontaneous emulsification (Fessi et al., 1989). For nanocapsules (NC), the organic phase consisted of copolymer **3** or **4** (0.030 g), sorbitan monostearate (0.077 g) and caprylic/capric triglyceride (0.33 mL) dissolved in acetone (27 mL). This organic phase was added into an aqueous solution (53 mL) containing the polysorbate 80 (0.077 g) under moderate magnetic stirring. After 10 min, the mixture was evaporated under reduced pressure to eliminate the acetone and concentrate the water to 10 mL. These nanocapsule suspensions were called NC3 and NC4. Formulations containing respectively equimolar quantities of the derivatives **1** or **2** (15.5 and 24.0 μg) were also prepared using PMMA (0.030 g) as polymer, and they were designated NC1 and NC2. A nanoemulsion (NE1) was prepared with the derivative **1** (15.5 μg) omitting any polymer as described above for nanocapsules. Table 1 shows the type and concentration of polymers and probes in each formulation.

### 2.6. Particle sizes

The particle sizes and polydispersity were determined by photon correlation spectroscopy by analysis of the polarized scattered light at 90° in diluted samples (MilliQ®). Measurements were made at room temperature (20 °C) using a Brookheaven Instruments® standard setup (BI-200M goniometer, BI-9000AT digital correlator and a BI9863 detection system). A coherent Spectra Physics He–Ne laser (35 mW,

Table 1
Type and concentration of polymers and probes in nanocapsules (NC) and nanoemulsion (NE)

| Formulation* | Polymer | | Probe | |
|---|---|---|---|---|
| | Type | Concentration (mg/mL) | Type | Concentration (μg/mL) |
| NC1 | PMMA | 3.0 | **1** | 1.55 |
| NC2 | PMMA | 3.2 | **2** | 2.40 |
| NC3 | **3** | 3.2 | – | – |
| NC4 | **4** | 2.9 | – | – |
| NE1 | – | – | **1** | 1.55 |

* All formulations also contain: oil (33 μL/mL), sorbitan monostearate (7.7 mg/mL), polysorbate 80 (7.7 mg/mL) and water (q.s. 10 mL).

$\lambda_0 = 632.8$ nm) was used as light source. The standard deviations were calculated by the average of three determinations.

### 2.7. pH and ζ-potential measurements

The pH values of samples were determined at 25 °C using a potentiometer DPMH-2 (Digimed). The measurements were made in triplicate. The ζ-potential analyses were carried out after dilution of formulations in 1 mM NaCl using a Zetasizer® nano-ZS ZEN 3600 model (Malvern). The measurements were made in triplicate.

### 2.8. Transmission electron microscopy

Analyses were conducted by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, *Centro de Microscopia—UFRGS*) operating at 80 kV. The diluted suspensions were deposited in Formvar-Carbon support films on specimen grid (Electron Microscopy Sciences), negatively stained with uranyl acetate solution (2% m/v) and observed at magnification of 250,000×.

### 2.9. UV–vis analyses

UV–vis absorption spectra were recorded at room temperature. The copolymers (0.030 g), corresponding to dye concentrations of $10^{-6}$ M, were dissolved in appropriate solvents (3 mL). The spectra were normalized.

### 2.10. Fluorescence measurements

The fluorescence spectra were obtained at room temperature. The solutions were analyzed using rectangular cuvette (Hellma, Quartz, Suprasil®, 10 mm), and the nanoemulsion or the nanocapsule suspensions were analyzed directly using triangular cuvette (Hellma, Quartz, Suprasil®, 10 mm). The spectra of solid polymers were obtained using the solid support at 45°. ESIPT and normal emission areas in the spectra were calculated by spectral deconvolution (Microcal™ Origin® v6.0). The spectra were normalized except for the stability study.

### 2.11. Stability studies

Films of PMMA and copolymer **4** were obtained in order to perform the swelling experiments by evaporation of the chloroform up to constant weights. For experiments, one peace of each film was exactly weighed in a glass flask. Each peace was covered with sufficient caprylic/capric triglyceride or distilled water. The flasks were closed and stored at room temperature. At pre-determined time intervals (2, 4, 6, 15, 30, 45 and 60 days), the films were sieved and the oil or the water removed using absorbing paper, thereafter the films were weighed. The florescent study, ζ-potential, pH and particle size measurements were carried out for NC2 and NC4 formulations, as described above, after 15, 30, 45 and 60 days of storage at room temperature.

## 3. Results and discussion

The polymers were obtained with satisfactory yields. SEC was carried out to characterize the poly(methyl methacrylate) (PMMA) and the copolymers prepared with the *N*-acryloylamide derivative of 2-(5′-amino-2′-hydroxyphenyl)benzoxazole (**1**) and vinylene derivative of 2-(4′-amino-2′-hydroxyphenyl)benzimidazole (**2**), which were called, respectively, **3** and **4**. The results showed Mn values of 170 g mol$^{-1}$, 162 g mol$^{-1}$ and 195 g mol$^{-1}$ and ratios Mw:Mn of 3.5, 5.2 and 3.2, respectively, for PMMA, **3** and **4**. The concentration of dye units attached to each polymer backbone was calculate using the respective molar extinction coefficients in ethyl acetate (Rodembusch et al., 2005), which were determined as $3.81 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ for **1** and $3.20 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ for **2**. The results showed that the copolymer **3** presents 0.28 mmol of dye per mol and **4** has 0.39 mmol of dye per mol of copolymer.

### 3.1. Preparation and physicochemical characterization of nanocapsules and nanoemulsion

All formulations (NC1, NC2, NC3, NC4 and NE1) were obtained as opalescent bluish liquids. The pH values determined for all nanocapsule formulations were in the range of 6.0–6.2, whereas, for NE2 the value was 5.5. The lower pH found for NE1 was probably due to the presence of acidic impurities in the oil, which can reach the oil/water interface since the polymer is absent in this formulation. This interpretation was previously reported for similar nanoemulsion (Calvo et al., 1996). The effective diameters and the polydispersity were in the range of 210–260 nm and 0.08–0.13 for all formulations. The results are in agreement with other works that reported similar formulations (Mosqueira et al., 2000; Cruz et al., 2006a,b). Fig. 3 shows the photomicrographs obtained by TEM for NC3 and NC4.

### 3.2. Fluorescence study

The first fluorescence study were carried out in order to determine either the behavior of the derivative **1** dissolved in the caprylic/capric triglyceride, which was used as oil in the formulations, or the response of the dye bound to the copolymer

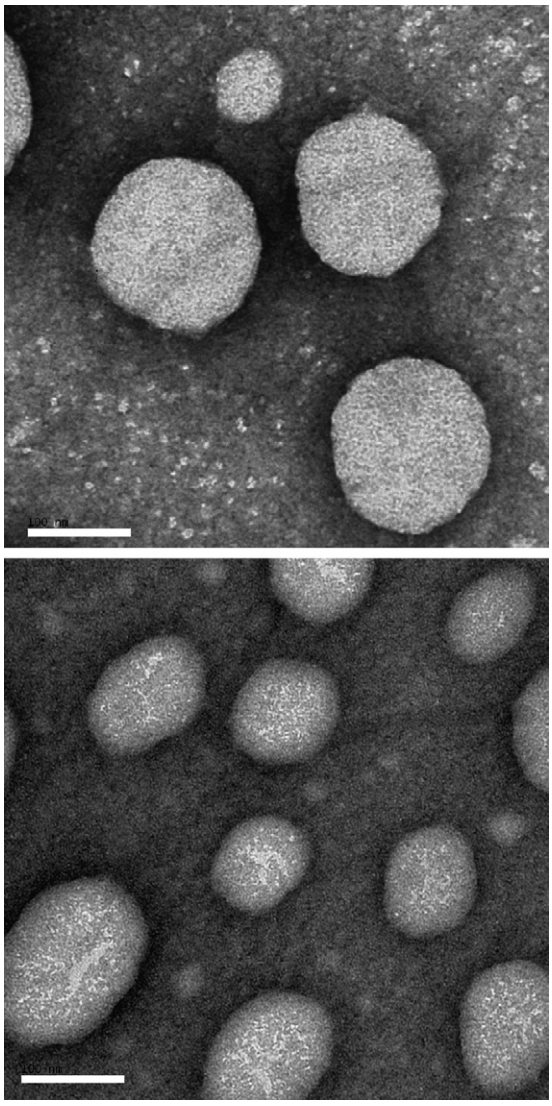

Fig. 3. TEM images (bar = 100 nm) of NC3 (top) and NC4 (bottom).

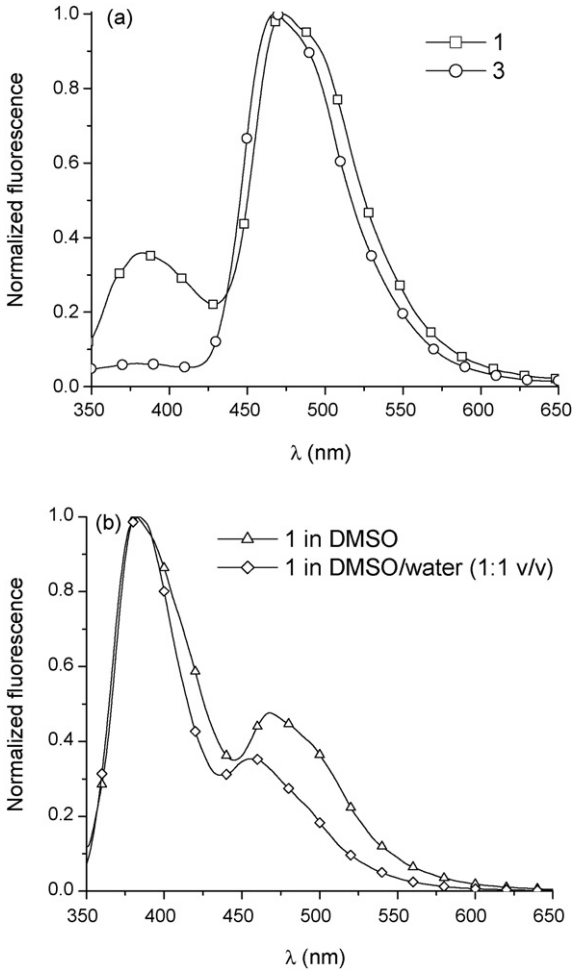


Fig. 4. Fluorescence spectra (a) derivative **1** dissolved in the caprylic/capric triglyceride and copolymer **3** as a solid and (b) derivative **1** dissolved in dimethylsulfoxide (DMSO) or in a DMSO/water solution (1:1 v/v).

**3**. The copolymer was analyzed as a solid or dissolved in polar and polar/protic solvents (Fig. 4a). The spectrum recorded for the derivative **1** in oil showed a small normal emission at 382 nm and a predominant ESIPT emission at 472 nm (relative intensities of 0.36 and 1, respectively). In the same way, the spectrum of the copolymer **3** in the solid state presented a predominant ESIPT emission at 468 nm and a negligible emission at 380 nm (relative intensity 0.06). On the other hand, after dissolving the dye **1** in dimethylsulfoxide (DMSO) or in a DMSO/water solution (1:1 v/v) the spectra showed inversions of intensities for the normal and the ESIPT emissions (Fig. 4b). For the polar and the polar/protic environments the normal emissions presented maxima at 382 and 384 nm while the ESIPT emission maxima were 468 and 456 nm (relative intensities 0.47 and 0.35, respectively). The results corroborated with previous reports (Campo et al., 2000; Rodembusch et al., 2005).

The fluorescence emission spectra of the formulations NC1, NC3 and NE1 showed different behaviors for the fluorescent dye (Fig. 5). In the case of NC1 and NE1, in which the dye added

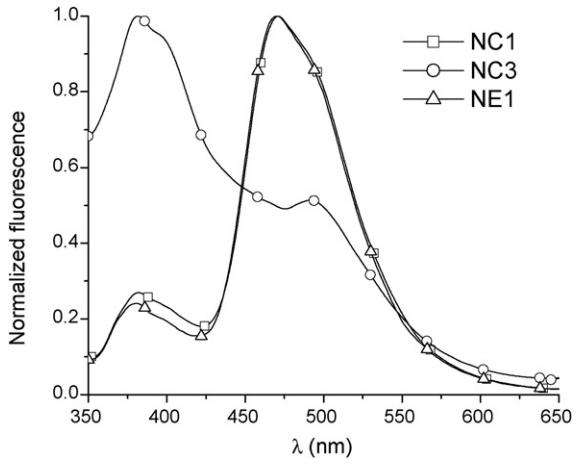


Fig. 5. Fluorescence spectra of derivative **1**-loaded nanocapsules (NC1), derivative **1**-loaded nanoemulsion (NE1) and nanocapsules prepared with copolymer **3** (NC3).

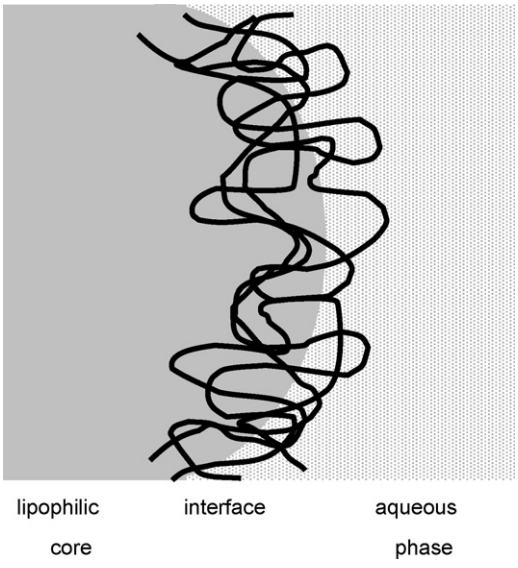


Fig. 6. Schematic model for the nanocapsule interface.

was the derivative **1** (physically entrapped) the ESIPT emission was predominant, presenting maxima at 472 and 471 nm, respectively. In those spectra, the normal emissions were observed at 382 nm and 381 nm with relative intensities of 0.26 and 0.24. The results showed that the derivative **1** is dissolved in the lipophilic core of the nanocapsules and in the oily phase of the nanoemulsion.

On the other hand, the spectrum obtained for NC3 (Fig. 5), prepared with the copolymer **3**, demonstrated that the normal emission is predominant presenting its maximum at 380 nm, whereas, an ESIPT emission is observed at 491 nm with relative intensity of 0.51. In this case, the results indicated that the dye chemically bound to the polymer (NC3) was exposed to a different environment than that to which the derivative **1** was exposed in the NC1 or NE1 formulations. At this point, the first interpretation suggests that the nanocapsules have a polymeric wall, which is placed at the oil/water interface. The second consideration is that the lipophilic phase and the aqueous phase are present at this interface. Those statements are supported by the data since the normal and the ESIPT emission intensities were inverted. The spectra of the dye **1** in NC1 and in NE1 compared to that of copolymer **3** in NC3 showed that the dye in NC3 cannot avoid the water. In addition, the presence of an ESIPT emission at 491 nm suggests that a molecular interaction occurs between the lipophilic core and the copolymer **3** in the nanocapsules. The presence of water at the interface led to the disruption of the intramolecular hydrogen bond in the dye, shifting the conformational equilibrium in the ground state from the conformer that originates the ESIPT fluorescence emission to those conformers which are responsible for the normal fluorescence emission. At a molecular level, the interface in those nanocapsules (Fig. 6) is composed by the lipophilic phase, the polymer and the water. The structural analysis of nanocapsules has been previously studied by field gradient NMR using ethanol as probe (Mayer et al., 2002). In this case the ethanol can render compatible the inner and the outer phases of the system because this solvent is soluble either in water or in the oil of the nanocapsules. The model for poly(*n*-butyl cianoacrylate)-nanocapsule suspensions consisted of an external phase containing water, ethanol and surfactant, separated from the inner phase, containing the medium chain triglyceride and ethanol, by a polymeric wall, on which the oil and the surfactant were adsorbed.

Even though the dynamic light scattering analyses previously reported (Pohlmann et al., 2002) showed a negative virial diffusion coefficient indicating that the nanocapsules are less probable to interact with water at a nanometric level; the fluorescence study performed with NC1, NC3 and NE1 showed that the oil and the water are simultaneously interacting with the polymer. In fact, the polymer in the nanocapsule suspensions is actually at the oil/water interface, interacting with both inner and outer pseudo-phases at the same time. Briefly, the results indicate that the polymer forms a wall in nanocapsules. This model, which considers that the polymer is restrict at the particle/water interface in nanocapsule formulations, can explain the different release rates after varying the polymer concentration in formulations. The increase in the polymer concentration causes an augmentation of the sustained half-life of lipophilic substances encapsulated within nanocapsules (Romero-Cano and Vincent, 2002; Cruz et al., 2006b).

### 3.3. Stability studies

The physico-chemical stability of the nanocapsules has been studied by fluorescence, light scattering, ζ-potential and potentiometry during 60 days. The benzimidazole dye **2** was chosen for this study due to the intramolecular higher stability of its hydrogen bond compared to the benzoxazole dye **1**. Formulation NC2 was prepared using the dye **2** and PMMA (dye physically entrapped) and NC4 was prepared employing copolymer **4** (dye chemically bound to the polymer). Previously, swelling experiments were performed for copolymer **4** and PMMA to verify the macroscopic responses of films of those polymers after contact with water or caprylic/capric triglyceride. The results showed that films of PMMA or copolymer **4** presented constant masses (standard deviations: oil, 10%; water 1%) after 60 days at room temperature, indicating that no swelling or dissolution occurred.

The fluorescence spectrum of NC2 (Fig. 7), just after preparation, showed exclusively an ESIPT emission at 506 nm, corroborating with the result obtained for the dye **2** dissolved in the oil, while in the spectrum of NC4, beyond an intense ESIPT emission at 506 nm, a small band at 402 nm (normal emission) was detected.

The fluorescence spectra obtained for NC2 and for NC4 after 15, 30, 45 and 60 days of preparation showed different behaviors for the dye physically entrapped in NC2 compared to the dye chemically bound in NC4. In the case of NC2 (Fig. 8a), the spectra demonstrated an only emission at 504 nm, which relative intensity slightly decreased in 30 days from 1 to 0.80. From this point to 60 days a progressive increase in the relative intensity was observed for the same band from 0.80 to 0.95. In addition, the study carried out with NC4 showed that the ESIPT emission maximum at 506 nm presented a decrease in relative intensity from 1 to 0.3, while the intensity of the normal emission

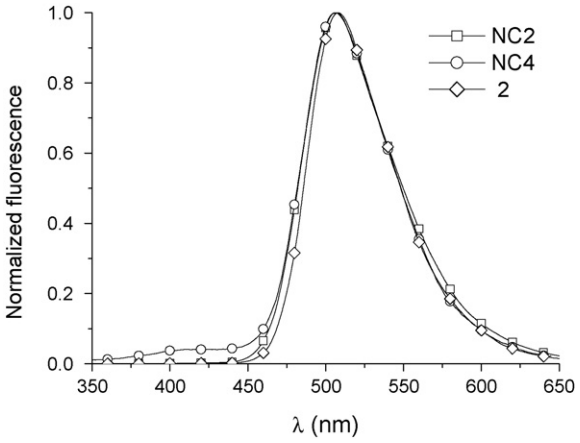


Fig. 7. Fluorescence spectra of the derivative **2** dissolved in caprylic/capric triglyceride, derivative **2**-loaded nanocapsules (NC2) and nanocapsules prepared with copolymer **4** (NC4).

at 402 nm increased from 1 to 1.9. Furthermore, an isoemissive point at 470 nm was observed for NC4 in function of time (Fig. 8b). An isoemissive point indicates that only two species are in equilibrium in the medium (Mukherjee et al., 2005). In general, when the intramolecular hydrogen bond of benzazoles is disrupted by the presence of protic solvents, the intensity of the

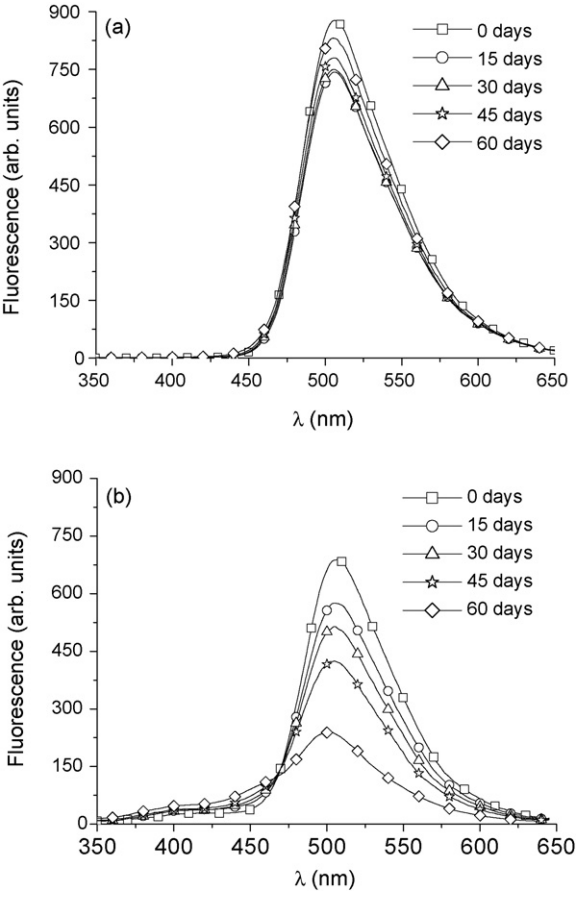


Fig. 8. Fluorescence spectra of the (a) derivative **2**-loaded nanocapsules (NC2) and (b) nanocapsules prepared with copolymer **4** (NC4) after 0, 15, 30, 45 and 60 days of preparation.

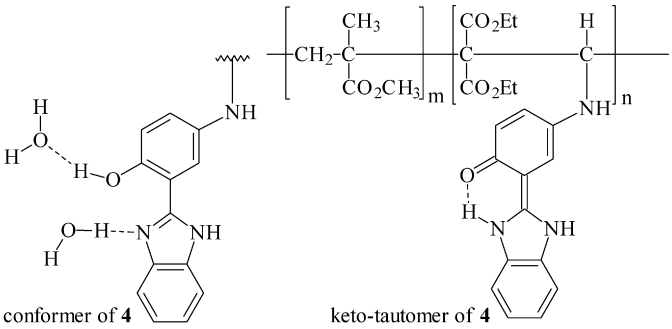


Fig. 9. Schematic model of the polymer wall structure. A conformer of **4** (for example, $E_{II}$) is present simultaneously with the keto-tautomer of **4**.

normal emission increases quite appreciably and the intensity of the ESIPT emission decreases (Sarkar et al., 1995; Sarkar et al., 1997). Considering the relative force of the hydrogen bond in the dye **2** compared to other benzazoles (Rodembusch et al., 2005) a slight increase in the normal emission was expected due to the presence of protic solvents. In the case of NC4, the presence of water could shift the conformational equilibrium between the dye conformers in the NC4. To confirm this hypothesis a solution of the dye **2** in dimethylsulfoxide was treated with increasing quantities of water. The spectra showed the ESIPT emission shifting from 407 to 397 nm and decreasing in intensity (1–0.5) until the proportion DMSO:water of 60:40 (v/v). Beyond this proportion the dye **2** precipitates in the medium. In parallel after 60 days, the sample NC4, which was used to the fluorescence study, was extracted with chloroform. The fluorescence spectrum of the extracted copolymer **4** in chloroform showed an intense emission in 506 nm and no normal emission near 400 nm.

Taking these results into account, the dye chemically bound to the polymeric wall (NC4) presents a conformational equilibrium between two species, the enol-*cis* ($E_I$) and one of the other conformers ($E_{II}$, $E_{III}$ or $E_{IV}$). In this case, one of the conformers of **4** (for example, $E_{II}$) and the conformer $E_I$ (Fig. 9) are simultaneously present in the nanocapsules, both being excited originating the respective excited species, consequently, originating the excited keto-tautomer (K*) from the excited enol-*cis* $E_I$*.

In addition, the study showed that the water is increasingly interacting with the polymer in nanocapsule suspensions. For NC2, the variation of the ESIPT emission intensity indicates that the dye **2** was dissolved in the oil and simultaneously dispersed in the polymer just after preparation, and it diffuses to the lipophilic core in function of time probably due to the increase of the water in the polymeric wall. In the same sense, for NC4 the presence of water increases in function of time in the polymeric wall, but since the dye is chemically bound to the polymer and the polymer is restrict at the particle/water interface, it cannot avoid the water and it experiences a different environment compared to the dye **2** in NC2.

The pH, the ζ-potential and the particle size were also determined during 60 days. The particle sizes for NC2 and NC4 formulations were constant, during 60 days, between 206 and 255 nm and 209 and 247 nm, respectively. The ζ-potential values determined for NC2 and NC4 ranged between $-17.7 \pm 0.9$ and

$-14.2 \pm 1.1$ mV and $-19.1 \pm 1.7$ and $-18.5 \pm 1.0$ mV, respectively. Poly(alkyl methacrylate) and other acrylic derivatives normally present negative ζ-potentials (Lopes et al., 2000). The pH values for NC2 and NC4 decreased from 6.25 and 6.06 to 4.69 and 4.45, respectively. These results are in accordance with the proposition that the water interacts with the polymer because the hydrolysis of the carboxylate groups is dependent on the presence of water. The hydrolysis gives carboxylic groups which are responsible for the pH decrease because of their ionization and consequent release of protons. For NC2 and NC4 despite the pH values decreased in function of time, the ζ-potentials were constant during 60 days. These results can be explained by equilibrium of charges close to the surface of the particles as previously suggested by other authors working on cationic nanoemulsions (Rabinovich-Guilatt et al., 2004).

In conclusion, the fluorescent dyes entrapped in nanocapsules or chemically bound to the polymer of nanocapsules are able to distinguish their chemical environments. The nanocapsule organization at a molecular level has been proposed after analyzing the fluorescent behaviors of benzazole probes. The fluorescence studies showed that the polymer in the nanocapsules forms a wall at the oil/water interface, as well as it is interacting with both oil and water at a molecular level. In this way, swelling experiments, which give the macroscopic response of the polymer solubility either in the oil or in the water, can indicate that nanocapsules presenting a polymeric wall are formed when the polymer does not swell or dissolve in those media. Furthermore, the stability study showed that the water is increasingly interacting with the polymer in nanocapsule suspensions in function of time.

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