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Novel PLA modification of organic microcontainers based on ring opening polymerization: Synthesis, characterization, biocompatibility and drug loading/release properties

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ABSTRACT

In the current study, poly lactic acid (PLA) modified hollow crosslinked poly(hydroxyethyl methacrylate) (PHEMA) microspheres have been prepared, in order to obtain a stimulus-responsive, biocompatible carrier with sustained drug release properties. The synthetical process consisted of the preparation of poly(methacrylic acid)@poly(hydroxyethyl methacrylate-*co*-*N*,*N*′-methylene bis(acrylamide)) microspheres by a two stage distillation-precipitation polymerization technique using 2,2′-azobisisobutyronitrile as initiator. Following core removal, a PLA coating of the microspheres was formed, after ring opening polymerization of DL-lactide, attributing the initiator's role to the active hydroxyl groups of PHEMA. The anticancer drug daunorubicin (DNR) was selected for the study of loading and release behavior of the coated microspheres. The loading capacity of the PLA modified microspheres was found to be four times higher than that of the parent ones (16% compared to 4%). This coated microspherical carrier exhibited a moderate pH responsive drug release behavior due to the pH dependent water uptake of PHEMA, and PLA hydrolysis. The in vitro cytotoxicity of both the parent and the DNR-loaded or empty modified hollow microspheres has been also examined on MCF-7 breast cancer cells. The results showed that although the empty microspheres were moderately cytotoxic, the DNR-loaded microspheres had more potent anti-tumor effect than the free drug. Therefore, the prepared coated microspheres are interesting drug delivery systems.

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1. Introduction

In recent years, polymeric microcapsules have been widely studied for bioapplications (Duncan, 1992; Gil and Hudson, 2004; Pack et al., 2005), drug delivery being the most prominent one (Duncan, 2006; Farokhzad and Langer, 2009; Moses et al., 2003; Seymour, 1991). Microspheres have received enormous attention due to their structural and morphological advantages (Brigger et al., 2002; Duncan, 2003; Gil and Hudson, 2004; Hu et al., 2007), the variety and the combination of the polymers that can be used, along with the existence of microcavities being the most important ones. In particular, both synthetic and natural polymers have been introduced into this very active field (Donaruma, 1974; Pack et al., 2005; Xue et al., 2011). Additionally, polymeric microspheres having specific properties, including pH (Dufresne et al., 2004; Lee et al., 2005; Li et al., 2008a), temperature (Chon et al., 2009; Yang et al., 2009), magnetic field (Chen et al., 2009; Fang and Zhang, 2009) and redox sensitivity or a combination of them, have been widely used for applications in drug delivery, diagnosis and biosensors (Brigger et al., 2002; Xue et al., 2011) due to their stimuli responsiveness (Allemann et al., 1993; Caruso, 2001; Kim et al., 2003; Kohn et al., 1983; Li et al., 2008b; Siegel et al., 2006; Xu and Asher, 2004).

These characteristics could be further improved by surface modification of the microspheres. The most widely used polymers for surface modification are polyethylene glycol (PEG) (Ogris et al., 1999) and polylactic acid (PLA) (Anderson and Shive, 1997). In contrast to PEG, PLA is a biodegradable polyester belonging to the group of poly a-hydroxy acids. It has been widely used in various biomedical applications (Nakamura et al., 1989; Xu and Asher, 2004) due to its biodegradability, biocompatibility, good mechanical properties and ability to be dissolved in common solvents for processing (Li et al., 2008a; Xu and Asher, 2004; Yang et al., 2007). Many copolymers based on DL-lactide offer various advantages compared to other polymers including the possibility to accurately control the resulting drug release kinetics over periods of days to months and complete biodegradability. The above advantages make PLA polymers attractive in medicine and pharmacy because of their suitability in controlled delivery of medicaments in the body

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(Panyam et al., 2002; Stevanovic et al., 2008). The precise effect of PLA depends on many factors including its molecular weight (Bodmeier et al., 1989; Hakkarainen et al., 1996).

Recently, many groups have reported polymeric microspheres which have as template polystyrene or polymethacrylic acid, cores that can be easily removed, forming in this way hollow microspheres, in which drugs can be encapsulated (Chatzipavlidis et al., 2011; Mandal et al., 2000; Pavlyuchenko et al., 2009; Xu and Asher, 2004; Yang et al., 2010; Zha et al., 2002). Among the most widely used anticancer drugs, daunorubicin hydrochloride (DNR) is a potent antibiotic anthracycline with a therapeutic impact on a broad spectrum of cancers. The major problem of DNR uses is mainly the side effects such as toxicity and especially cardiotoxicity (Kohn et al., 1983; Minotti et al., 2004). In order to minimize these side effects, an array of polymeric delivery systems has been employed.

In this work, we have synthesized and characterized PHEMA hollow microspheres and their PLA modified derivatives. PHEMA has been selected due to its excellent biocompatibility and physicochemical properties similar to those of living tissues (Brahim et al., 2003; Lahooti and Sefton, 2000). Additionally, changes in the pH or/and temperature affect its water uptake capacity (Tomić et al., 2007), controlling in this way its drug release behavior (Chouhan and Bajpai, 2009). On the other hand, PLA is considered suitable for controlled drug delivery and is also biodegradable. Specifically, polymeric microspheres were prepared by a two stage distillation precipitation polymerization in neat acetonitrile. Monodisperse poly(methacrylic acid)@poly(hydroxyethyl methacrylate-*co*-*N*,*N*′-methylene bisacrylamide) (PMAA@P(HEMA-*co*-MBAAm), Scheme 1) core–shell microspheres have been synthesized. Subsequently, hollow microspheres were afforded after removal of the non-crosslinked PMAA core in water (P(HEMA-*co*-MBAAm) microspheres, Scheme 1). The active hydroxyl groups on the microspheres' surface played the initiator's role for ring opening polymerization of DL-lactide resulting to the additional coating of microspheres (P(HEMA-*co*-MBAAm)-PLA microspheres, Scheme 1). There are very few examples reported for the surface functionalization of hollow microspheres, using ring opening polymerization (Kohn et al., 1983). Scanning Electron Microscopy (SEM), Fourier Transform Infra Red Spectroscopy (FT-IR) and Dynamic Light Scattering (DLS) were applied for morphological and structural analysis of both modified and unmodified microspheres. Their DNR loading and release behavior under different pH conditions have been also examined, as well as their cytotoxic effect on MCF-7 breast cancer cells.

2. Materials and methods

2.1. Materials

D,L-Lactide was purchased from Acros and was purified with recrystallization from CCl_4 and C_6H_6. Toluene (Merck) and dichloromethane (Calro Erba) were used after having been dried over CaH_2 and distilled under nitrogen. Methacrylic acid (MAA, 99%) and 2-hydroxyethyl methacrylate (HEMA 97%) were purchased from Acros and used after purification with vacuum distillation. Acetonitrile, diethyl ether, hexane and ethyl acetate were purchased from Calro Erba and used as received. Stannous octoate (≥99%) was purchased from Sigma. 2,2′-Azobisisobutyronitrile (AIBN, 98%) and *N*,*N*′-methylenebisacrylamide (MBAAm, 96%) were obtained from Acros and used as received. Daunorubicin.HCl (DNR) was provided by Pharmacia & Up John. Porcine esterase (17 u/mg) was purchased from Sigma. High glucose Dulbecco's modified Eagle Medium (DMEM), Trypsin-EDTA, L-glutamine and penicillin–streptomycin solution were obtained from Biochrom KG, Berlin, Germany, while the heat inactivated fetal bovine serum (FBS) was purchased from Invitrogen Co., Carlsbad, CA, USA. MTT assay kit was purchased from Sigma.

2.2. Methods

2.2.1. Preparation of monodisperse PMAA@P(HEMA-co-MBAAm) core–shell microspheres

Monodisperse PMAA microspheres were synthesized by distillation precipitation polymerization in acetonitrile with AIBN as initiator in the absence of any crosslinker (Bai et al., 2007). In a typical experiment, MAA (2.0 g, 23.3 mmol) and AIBN (0.04 g, 0.24 mmol) were dissolved in 80 mL of acetonitrile in a dried 100 mL two-necked flask attached with a fractionating column, Liebig condenser and a receiver. The flask was immersed in a heating mantle and the reaction mixture was heated from ambient temperature to the boiling state within 20 min and then the solvent was distilled off from the reaction system. The initially homogeneous reaction mixture became opalescent and then deepened in color as a milky white dispersion after boiling for 10 min. The reaction was ended after 40 mL of acetonitrile were distilled from the reaction system within 1 h. The resultant PMAA microspheres were separated and purified by repeated centrifugation, decanting and resuspension in acetonitrile with ultrasonic bathing for three times.

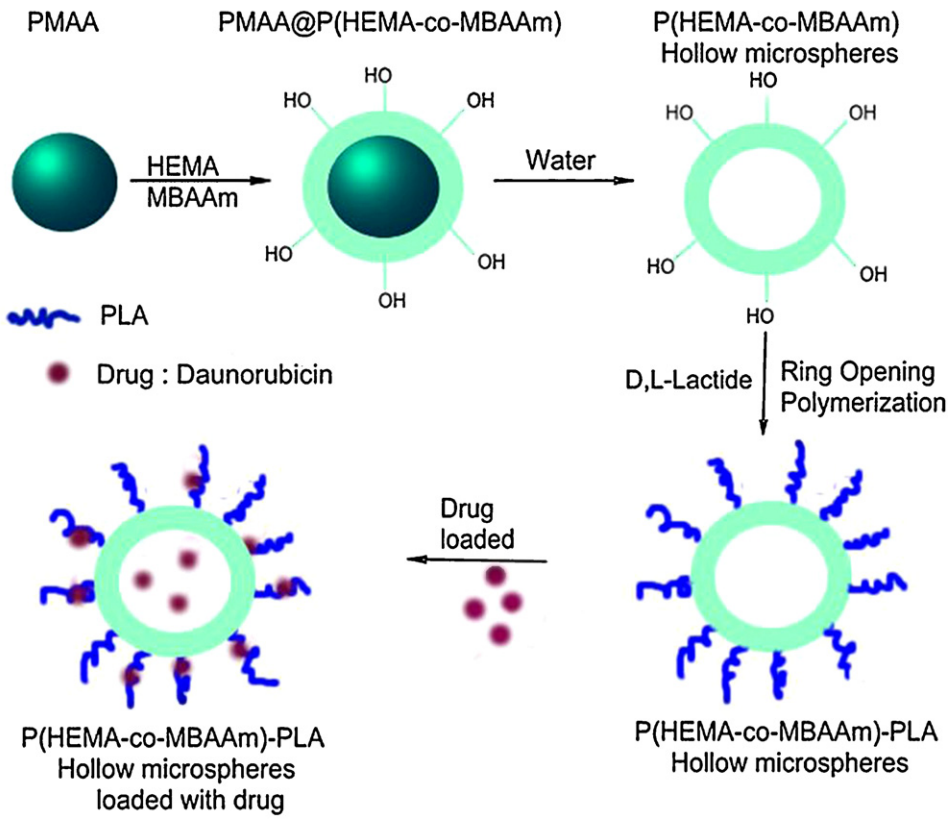
Monodisperse PMAA@P(HEMA-*co*-MBAAm) core–shell microspheres were synthesized in the presence of PMAA as seeds for the second-stage polymerization of HEMA, employing MBAAm as crosslinker and AIBN as initiator. In a typical procedure, PMAA seeds (0.40 g), HEMA (1.18 g, 9.0 mmol) and MBAAm (0.22 g, 1.4 mmol, 13 mol% corresponding to the sum of HEMA and MBAAm monomers) were dispersed in 90 mL of acetonitrile at room temperature in a 250 mL two-necked flask. After homogenization of the suspension by ultrasonication for 10 min, AIBN (0.02 g, 0.1 mmol, 1.4 wt% relative to the monomers) was added to a flask equipped with a fractionating column, Liebig condenser and a receiver. The flask was submerged in a heating mantle and the second stage polymerization mixture was heated from ambient temperature to the boiling state within 30 min. The polymerization continued under the boiling state for further 20 min when the solvent was distilled off from the reaction system. The reaction was ended after 45 mL of acetonitrile were distilled off from the reaction mixture within 1 h. The resultant PMAA@P(HEMA-*co*-MBAAm) core–shell microspheres were purified by five cycles of centrifugation (11,950 × *g* × 5 min), decanting and redispersion in acetonitrile with ultrasonic bathing.

2.2.2. Synthesis of hollow P(HEMA-co-MBAAm) microspheres

PMAA@P(HEMA-*co*-MBAAm) core–shell microspheres were dialyzed in deionized water at room temperature for selective dissolution of PMAA cores. The dialysis process to obtain P(HEMA-*co*-MBAAm) microcontainers lasted two days for complete removal of PMAA core. The final product was dried under vacuum at room temperature till constant weight.

2.2.3. Synthesis of PLA coated microspheres

The coating of microspheres' surface was carried out by ring opening polymerization via active hydroxylic groups which are located on the microspheres surface and play the initiator role. D,L-Lactide and stannous octoate ($Sn(Oct)_2$) were used as monomer and catalyst, respectively. Synthesized microspheres (100 mg) were dispersed in 5 mL toluene. DL-Lactide (48 mg, 0.33 mmol) and $Sn(Oct)_2$ (5.5 μL, 0.0169 μmol) were added under nitrogen and the mixture was stirred at 150 °C for 24 h under anhydrous conditions. After the reaction period, the mixture was evaporated to a minimum volume and the product was precipitated by excess diethyl



Scheme 1. Schematic presentation of the steps followed for the fabrication of PLA modified microspheres.

ether twice and the isolated product was further purified with centrifugation (7650 × *g* × 5 min).

2.3. Characterizations

FT-IR spectra were obtained on Perkin Elmer Precisely Spectrum 100 Spectrometer. Particle size measurements were carried out with DLS (Malvern Instruments Series, Nano-ZS with multipurpose titrator). The concentration of the sample was kept at 0.1 mg/mL. In the data presented in this study, each measurement represents the average value of 10 measurements, with 20 s integration time. An ultrasonic bath was used for sonication (Elma Sonic, S. 30H). Scanning Electron Microscopy (SEM) images were obtained with a FEI Inspect nanoscope with Tungsten filament operating at 25 kV.

2.4. Ex vitro stability study

Stability of the microspheres in FBS-containing PBS (10%, v/v) was studied at 37 °C by measuring particle size change as a function of time. Synthesized microspheres (1 mg) were added in 100 mL of 10% FBS-containing PBS solution. Size measurements were taken at different time intervals (within a period of 12 h).

2.5. Enzymatic degradation of the PLA coated microspheres

PLA coated microspheres (10 mg) were dispersed in PBS solution (20 mL) and 100 μL of Triton X-100 were added. Porcine esterase (10 μL, 5 u/mL in 0.1 M Tris buffer, pH 8.0) was added and the mixture was incubated at 37 °C under stirring. At given time intervals, aliquots were withdrawn and analyzed with DLS.

2.6. Drug loading of hollow microspheres

DNR (0.2 mg) was dissolved in 10 mL of isotonic solution (0.9 wt% NaCl) and then 8.0 mg of hollow microspheres were added (Li et al., 2008c; Yang et al., 2009; Zhang et al., 2006). The resulting suspension remained under gentle agitation for five days in order to complete the drug loading into the microspheres. Unloaded DNR was removed after centrifugation. The loaded amount of DNR into microspheres was determined by UV spectroscopy (Helios Thermo Electron Corporation) and calculated by the difference of DNR concentration between the original DNR solution and the resulting supernatant after loading. Calculations were based on a standard curve of DNR in NaCl (0.9 wt%) solution. The drug concentration was monitored by measuring the absorbance at 480 nm. The encapsulation efficiency (EE) and the loading capacity (LC) were calculated according to the following formulas:

$$\text{EE}\ (\%) = (\text{weight of loaded drug/weight of drug in feed}) \times 100$$
$$\text{LC}\ (\%) = (\text{weight of loaded drug/weight of microspheres}) \times 100$$

2.7. In vitro release study of DNR from PLA coated hollow microspheres

The release behavior of DNR-loaded hollow microspheres was investigated under acidic (100 mM acetate buffer, pH = 4.5) and slightly basic (100 mM phosphate buffer, pH = 7.4) pH conditions at 37 °C. Drug loaded hollow microspheres were suspended in the respective buffer. At given time intervals, aliquots were withdrawn and centrifuged. The DNR concentration was determined in the supernatant by measuring the absorbance at 480 nm (Li et al., 2008d; Yang et al., 2009).

2.8. Cell culture

Human breast adenocarcinoma cells (MCF-7) were maintained in high glucose DMEM, supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.9. In vitro cytotoxicity studies

Cytotoxicity was evaluated by the MTT assay (Mosmann, 1983). Briefly, MCF-7 cells were seeded at a density of 8×10^3 cells per well in 96-well, flat-bottomed microplates and grown in 100 μL of completed growth medium 24 h prior to the incubation with the microspheres. Stock solutions of microspheres and DNR or DNR-containing microspheres were dissolved in 10% EtOH-containing ddH_2O at a concentration of 1 mg/mL and 300 μM, respectively. Subsequently, fresh serum free medium (100 μL), containing the appropriate concentration of DNR (0.02–60 μM) or microspheres (0.667–202 μg/mL), was added to each well to a final total volume of 200 μL. After a 24 and 48 h incubation, cells were washed once with PBS and the medium was replaced with 100 μL per well of MTT solution (1 mg/mL diluted in phenol red and serum-free growth medium). Cells were further incubated for 4 h at 37 °C and 5% CO_2. Then, the MTT formazan crystals were solubilized in 2-propanol, and the absorbance was measured at 540 nm (reference filter 620 nm) using a microplate reader (Sirio S, SEAC Radim group). Measurements were converted to percent viability by comparison to control experiments in which microspheres had not been added. Results are expressed as the mean value of the absorption at 540 nm ± standard deviation ($n = 3$). Cytotoxicity experiments were repeated twice.

3. Results and discussion

3.1. Preparation and characterization of the parent and PLA coated hollow PHEMA microspheres

Monodisperse PMAA@P(HEMA-*co*-MBAAm) microspheres were prepared by distillation–precipitation polymerization of HEMA and MBAAm in the presence of PMAA microspheres (Chatzipavlidis et al., 2011; Bai et al., 2007) as seeds (Scheme 1). The hydrogen-bonding interaction between the carboxyl group on the surface of PMAA core and the hydroxyl and amide groups of HEMA and MBAAm, respectively, control the second-stage polymerization without modification of the PMAA surface in the absence of any stabilizer or surfactant (Liu et al., 2009). The molar ratio between the HEMA monomer and the MBAAm crosslinker remained constant during the experiments. When the monomers/template weight ratio was 2.5, microspheres with an average shell size (35 nm) were formed. When the monomers/template weight ratio was 3.0, the microspheres had an average shell size of 50 nm. Finally, when the monomers/template weight ratio was 3.5 the synthesized microspheres exhibited an average shell size of 90 nm.

Selective removal of the non-crosslinked PMAA core with water, resulted in the formation of hollow P(HEMA-*co*-MBAAm) microspheres (Scheme 1). Fig. 1 shows SEM images of the resultant hollow PHEMA microspheres, synthesized from the abovementioned different monomers/template weight ratios during the second stage distillation precipitation polymerization. At a monomers/template weight ratio equal to 2.5, the formation of the shell was incomplete, leading to hemisphere-like particles (Fig. 1A), while at a ratio equal to 3.0, the shell was very thin (Fig. 1B). Nearly monodispersed microspheres, with smooth surface were synthesized when the monomers/template weight ratio was 3.5 (Fig. 1C). Therefore, the last ratio was selected for further PLA modification.

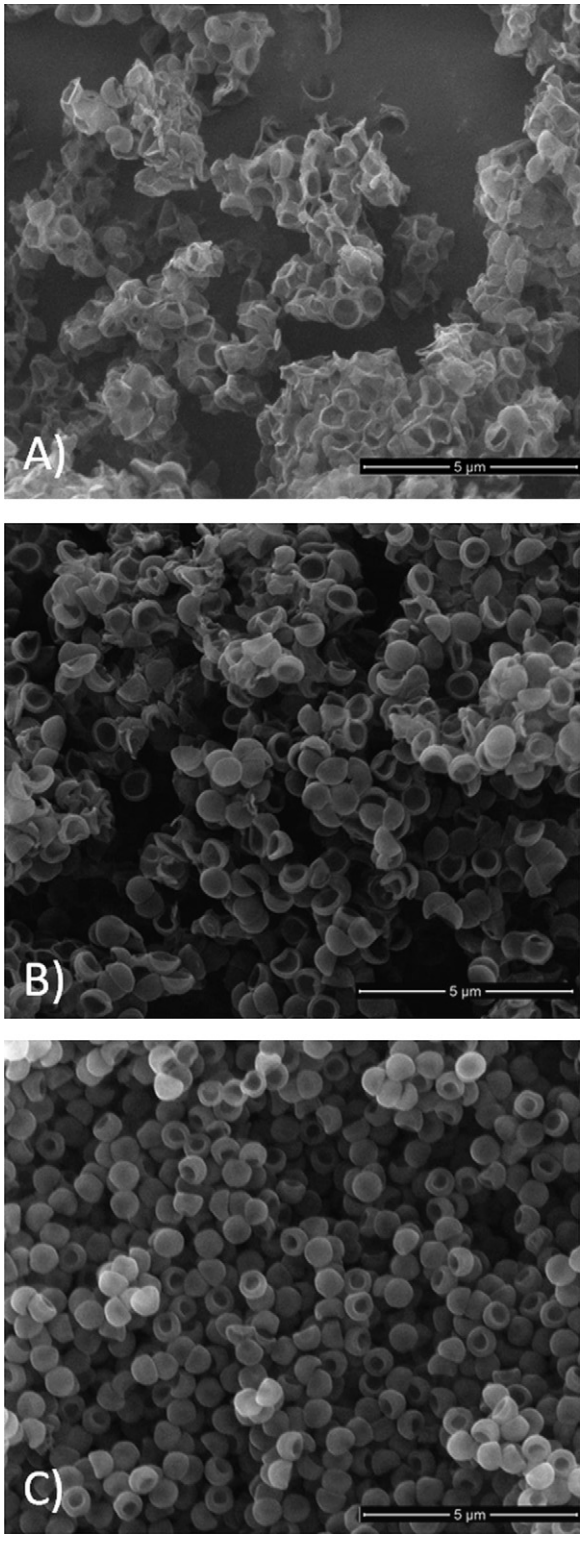


Fig. 1. SEM images of P(HEMA-*co*-MBAAm) microspheres synthesized from different monomers/template weight ratio. (A) 2.5, (B) 3.0 and (C) 3.5.

Polylactic acid modified hollow microspheres were synthesized via ring opening polymerization (Chouhan and Bajpai, 2009; Efthimiadou et al., 2011; Ignatius and Claes, 1996; Kim et al., 2003) for the first time. During the polymerization in toluene, the

solubility of the coated microspheres was improved due to the success of the surface modification. The modification of the microspheres was achieved by using three different percentages of the catalyst relatively to the monomer used. Actually, while constant amount of hollow microspheres (0.1 g) and D,L-lactide monomer (0.048 g) was used, the amount of the catalyst ($Sn(Oct)_2$) used varied (4, 8 and 16% relative to the monomer used).

Fig. 2 illustrates the SEM micrographs of the three different resulting microspheres. It can be observed (Fig. 2) that the morphology of the resulting microspheres varied according to the amount of the catalyst used. Higher or lower modification extent could be achieved according to the catalysts' percentage used. This behavior can be attributed to the fact that the activated centers on the surface increased or decreased accordingly to the catalyst amount used. Actually, at low catalyst ratio (4%) (Fig. 2A) PLA coating was incomplete, while it became optimal when 8% ratio was used (Fig. 2B). At the higher ratio of 16% (Fig. 2C) the morphology of microspheres was non-uniform.

The successive formation of core–shell, hollow PMAA@P(HEMA-*co*-MBAAm) and PLA-modified microspheres was confirmed by FT-IR spectroscopy (Fig. 3) (Yang et al., 2010). In Fig. 3A the peak at 1695 cm^{-1} is assigned to the characteristic stretching vibration of the carbonyl group of the PMAA segment and the peak at 1532 cm^{-1} is assigned to the vibration of the amide bond of PMBAAm parts. All of the core–shell microspheres with PHEMA as copolymer had a broad peak at 3500 cm^{-1} due to the vibration of hydroxylic stretching. The characteristic peak of esteric bond of PHEMA segment is overlapping with the two different carbonyl groups of PMAA and PHEMA (Liu et al., 2009). In the spectrum of hollow spheres (Fig. 3C) the observed decrease of the strong peak at 1695 cm^{-1} is due to PMAA template removal. After modification of microspheres with PLA a new strong peak at 1532 cm^{-1} appears (Fig. 3D). This peak increment is attributed to symmetric and asymmetric deformation vibration of C—H in CH_2 groups in the polylactic segment. It is worth noticing that the bands at 1710 and 1698 cm^{-1} are attributed to the overlapping peaks of the two different carbonyl groups of PHEMA and PLA which are introduced on their surface (Bai et al., 2006; Pamula et al., 2001; Zhang et al., 2006). In all obtained spectra two bands appeared in the range 2800–3000 cm^{-1} related to stretching vibrations of hydrocarbon groups, i.e. CH_2 and CH_3 characteristic of HEMA, lactic and methacrylic groups (Bai et al., 2006; Feldstein et al., 2009).

The hydrodynamic diameter of PLA coated microspheres compared to that of the parent microspheres is depicted in Fig. 4. The results show that the hydrodynamic diameter of microspheres increased from 644 to 904 nm due to the PLA modification. Furthermore, the unmodified microspheres exhibit narrow polydispersity index (~0.10) whereas the modified ones demonstrate higher polydispersity (~0.22).

Further biological evaluation was conducted on the moderately PLA modified microspheres (Fig. 2B). Our choice was based not only on the morphological characteristics of the fabricated microspheres, but also on bibliographic data supporting that the molecular weight of PLA controls the degradation rate of the polymeric drug delivery systems, controlling in that way their release behavior. High molecular weight PLA is degraded slowly, being therefore, the limiting factor on the ideal drug release time (Bodmeier et al., 1989; Vert et al., 1992).

3.2. Stability studies

The stability of PLA coated microspheres was investigated in PBS solution containing 10% FBS at 37 °C by monitoring particle size changes as a function of time. As observed in Fig. 5, the size of microspheres increases within the first 2 h of incubation due to aggregates formation, most probably originating from PLA interaction with plasma proteins (Lück et al., 1998). The size remains relatively stable for another 6 h, and afterwards decreases once again, being stabilized at the initial one. The last phenomenon can be attributed to PLA hydrolysis, resulting in this way to partial aggregates dissolution. Degradation of PLA is known to occur in aqueous environment through simple hydrolysis of ester bonds, autocatalyzed by carboxylic groups (Hakkarainen et al., 1996). SEM micrographs drawn at the end of the incubation period

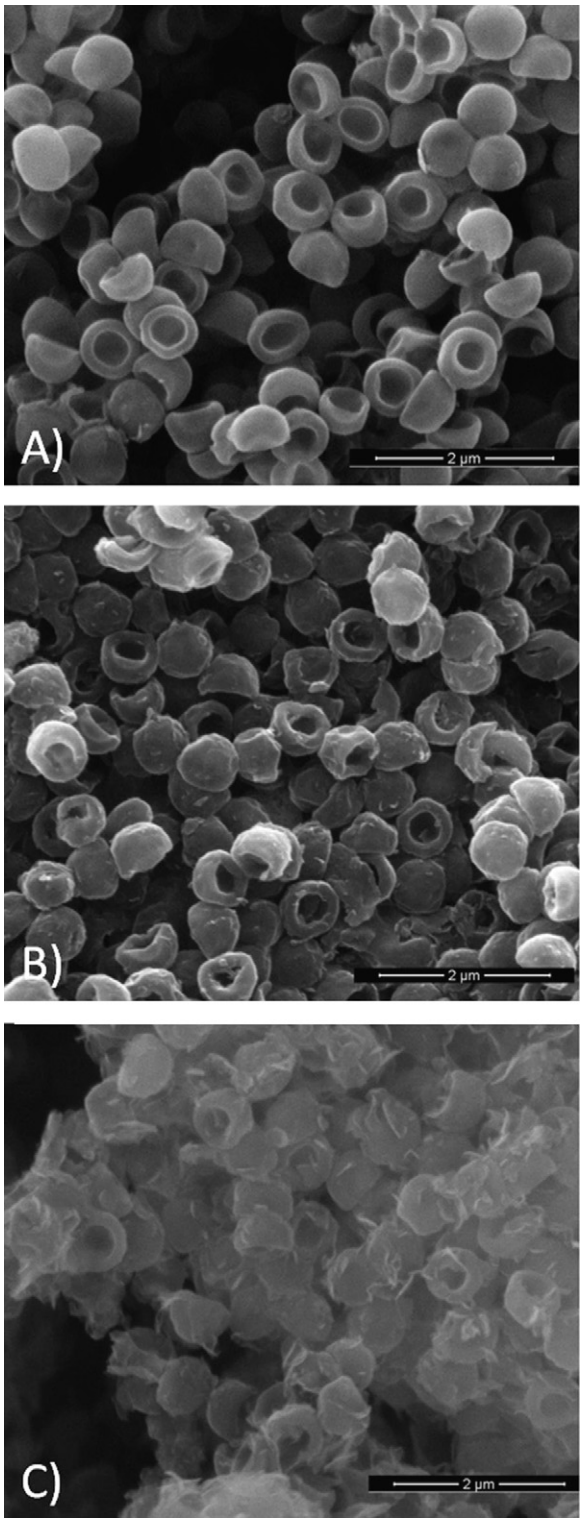


Fig. 2. SEM images of PLA modified PHEMA microspheres with different amount of catalyst ($Sn(Oct)_2$) related to the monomer. (A) 4, (B) 8 and (C) 16%.

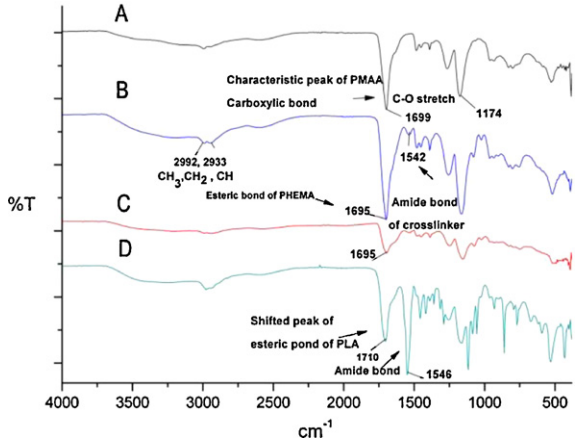


Fig. 3. FT-IR spectra of (A) PMMA, (B) core–shell PMAA@P(HEMA-*co*-MBAAm), (C) hollow P(HEMA-*co*-MBAAm) and (D) PLA modified hollow P(HEMA-*co*-MBAAm) microspheres.

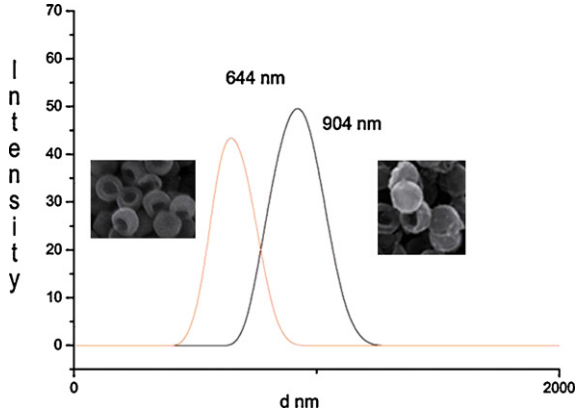


Fig. 4. DLS measurements of microspheres before and after their PLA modification.

revealed the existence of aggregates which were formed, most probably, due to PLA degradation, subsequent aggregation of the degraded polymers and final deformation of the microspheres due to hydrophobic interactions (data not shown).

The stability of the PLA coated microspheres after treatment with porcine esterase at 37 °C is depicted in Fig. 6. According to the results, in the presence of esterase the PLA modified microspheres

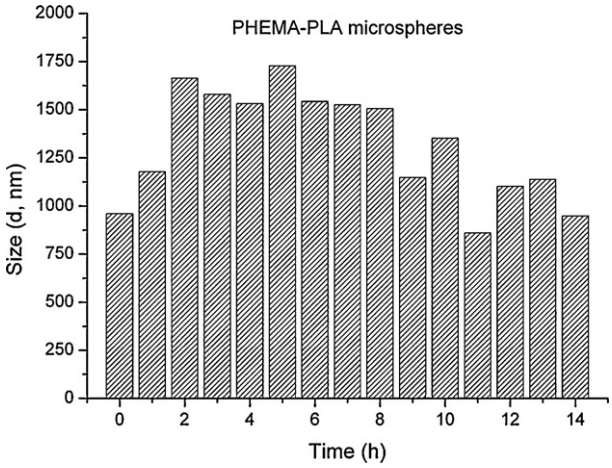


Fig. 5. Microspheres size (diameter, nm) during incubation at 37 °C in 10% FBS-containing PBS at various time intervals as determined by DLS.

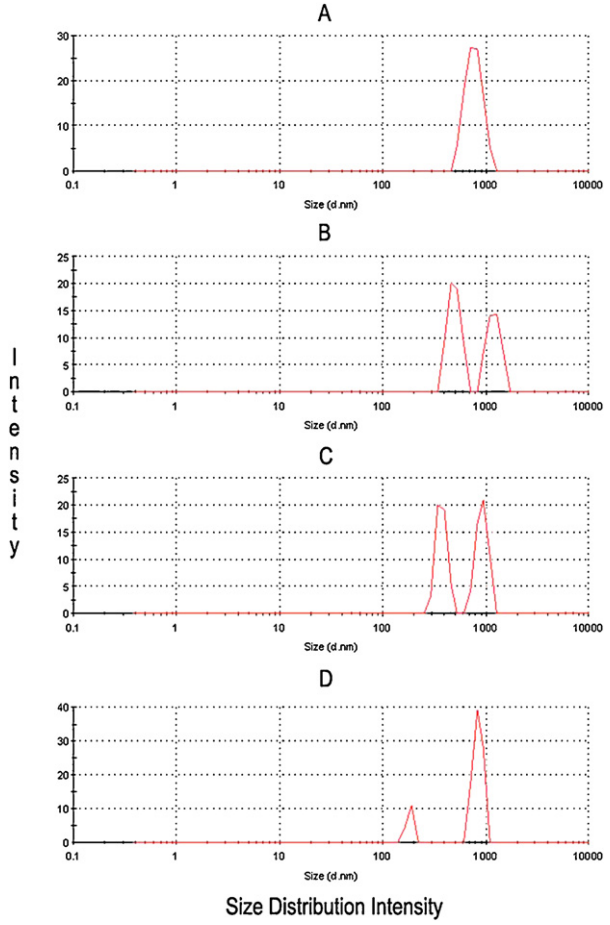


Fig. 6. Size distribution of PLA modified microspheres as a function of incubation time with porcine esterase at 37 °C. Before enzyme addition (A), and after 3 (B), 6 (C) and 12 (D) hours of incubation in the presence of esterase.

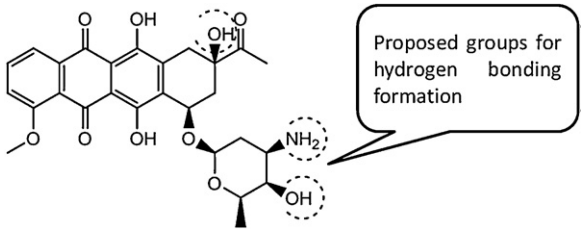
are degraded. Actually, after a 3 h incubation (Fig. 6B) the initial population (776 nm) disappears, and two new populations are observed. The first one (496 nm) is formed due to PLA degradation while the second one due to aggregates formation (1202 nm), the second population being less intense than the first one. Subsequently, after 6 h incubation (Fig. 6C), while the sizes of the two populations further decreases (373, 924 nm), the intensity of the two populations becomes equal. Finally, and after a 12 h incubation (Fig. 6D), both populations' size decreases (183, 846 nm) pointing to extensive degradation.

3.3. Loading and release behavior of PLA modified PHEMA microspheres

The DNR loading capacity of PLA modified microspheres after 120 h treatment in an isotonic solution has been determined, and the encapsulation efficiency and loading capacity were found to be 67% and 16%, respectively (Table 1). In comparison to the respective values for the parent microspheres (13.7% and 4%, respectively) (Table 1), we can conclude that PLA modification resulted in a 5 and a 4-fold increase in the EE and LC, respectively. The results

Table 1
Loading of DNR in PHEMA and PLA modified PHEMA microspheres.

Hollow microspheres	EE (%)	LC (%)
PHEMA	13.7	4.0
PHEMA-PLA	67.0	16.0



Scheme 2. Chemical structure of DNR and its potent groups for hydrogen bonding formation.

above confirm the positive contribution of the PLA modification of the microspheres on their drug loading capacity. Therefore, it can be concluded that PLA also interacts with the drug. It has been proposed by Yang et al. (2009) that encapsulation of doxorubicin, exerting a relative structure to that of DNR, into PMAA microspheres can be attributed to hydrogen bonding formation. In our case, both hydroxyl and carboxyl groups, deriving from PHEMA and PLA polymers, respectively, can form hydrogen bonds with the relevant moieties of DNR (Scheme 2).

DNR release from the PLA modified microspheres was evaluated at acidic (4.5) and slightly basic pH (7.4) (Fig. 7). Under acidic conditions DNR showed an initial burst release (~40%) within a 12-h release period. Afterwards, the drug was released progressively and after 24 h the released amount was 48%, while it reached a plateau at 56% within 48 h period. On the other hand, under slightly basic conditions there was a log phase up to the 12-h release period. The release rate gradually increased up to 69% within the next 72 h, when the plateau was reached.

The dependence of the releasing behavior of the drug from the hollow modified microspheres, on different pH values may be originated from both (1) the difference of the hydrogen bonding strength between the remaining hydroxylic groups of PHEMA and carboxylic groups of PLA and the characteristic groups of DNR (Scheme 2) (Yang et al., 2009) and (2) the hydrolysis rate of PLA. Therefore, two phenomena exist that act in parallel, both contributing to the initial burst of DNR release observed at acidic pH. On the one hand, hydrogen bonding between DNR and the polymers is weaker at acidic pH (Yang et al., 2009) than at slightly basic, and on the other hand, PLA hydrolysis is also higher at the same pH. After hydrolysis of PLA (48 h), the remaining DNR loaded in the resulting PHEMA microspheres, exerts a slower release at low pH (pH = 4.5) most probably, due to aggregates formation and subsequent drug entrapment. At slightly basic pH, the hydrogen bonding strength dominates and the release rate is slower. The observed inversion

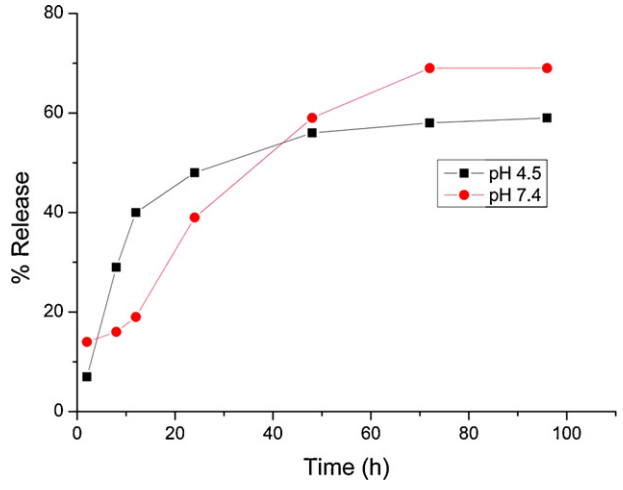


Fig. 7. Release study of DNR under acidic (pH 4.5) and basic (pH 7.4) pH conditions.

at the release rate, after a 24-h incubation, can be attributed to the lack of aggregation due to PLA hydrolysis. These results point to a moderately pH sensitive and sustained drug release that can be optimized by varying the degree of PLA modification.

3.4. Cytotoxicity studies

The cytotoxicity of the PLA modified PHEMA microspheres both DNR loaded and unloaded, along with that of the parent PHEMA was examined by MTT assay. The aim of this biological evaluation was to determine whether DNR, once loaded in the modified microspheres, retains its antineoplastic activity or not. We thus, compared the cytotoxicity on MCF-7 human breast carcinoma cells of pure DNR (0.01, 0.1, 1, 10 and 30 μM) and DNR-loaded microspheres with the same amount of drug. In parallel, we have also examined the cytotoxicity of the polymers used, in order to exclude their potent contribution to the cytotoxic effect on MCF-7 cells. Therefore, we have examined their cytotoxicity based on the corresponding quantities that contain the reference DNR amount (0.034, 0.337, 3.367, 33.67 and 101 μg/mL, respectively). Cytotoxicity was estimated after both 24 and 48 h incubation with the different substances.

Our results (Fig. 8) demonstrated that both the parent PHEMA microspheres and the PLA modified ones exert low cytotoxicity at the low concentrations and incubation time examined (approximately 80% cell viability at concentrations up to 3.367 μg/mL and at 24 h incubation time). At higher concentrations of the polymers (up to 101 μg/mL) the viability further lowers to 50%. The toxicity of the two polymers is similar, and only at the highest concentration tested differs. Actually, the PLA modified microspheres exert a relatively higher toxicity at a 101 μg/mL concentration (Fig. 8A).

As shown in Fig. 8A, the effect of drug concentration on MCF-7 cell viability is dose dependent. At drug concentrations up to 1 μM no cytotoxicity is observed, while at higher concentrations (10 and 30 μM) the cytotoxicity increases. On the other hand, the DNR-loaded microspheres exhibit much higher cytotoxicity than free DNR, especially at the lower concentration range. Actually, when the concentration of DNR is low, the difference in anti-tumor effect between the free DNR and DNR-loaded microspheres is significant. With increasing concentration of DNR, the difference diminishes (at 10 μM) and finally extincts (at 30 μM). Comparatively, at a DNR concentration equal to 1 μM, the viabilities of the cells incubated with either empty microspheres, DNR-loaded microspheres or free DNR are 78.7 ± 4.4%, 43.9 ± 2.2% or 115.6 ± 2.6%, respectively.

Incubation time increment (Fig. 8B) resulted to a concomitant increase of free DNR cytotoxicity along with an additional cytotoxic effect on the cells of the maximum concentration of the empty microspherical vector. It can also be observed that while for a 24 h incubation period the cytotoxic effect of the two empty spheres is comparable, when the same experiment was conducted with a 48 h incubation period, the PLA modified microspheres exerted higher cytotoxicity. This is probably due to the presence of the fast degrading PLA polymer (Müller et al., 1996).

In previous studies it was found that nanoparticles are usually internalized inside the cells by the endocytosis pathway and then localized in acid endocytic compartments (Kakizawa and Kataoka, 2002; Tang et al., 2007). Encapsulation of anti-tumor drug in nanoparticles can increase its internalization by cells into lysosomes and enhance their cytotoxicity (Kakizawa and Kataoka, 2002; Tang et al., 2007). In the current study, this enhanced cytotoxicity may be explained by the enhanced cellular uptake of microspheres. It should also be noted that microspheres without DNR exhibited low cytotoxicity (within 80–60% of cell survival) within the lower to higher polymer concentration range, respectively, confirming that the enhanced cytotoxicity was not due to the effect of the microspheres. Similar results have been presented

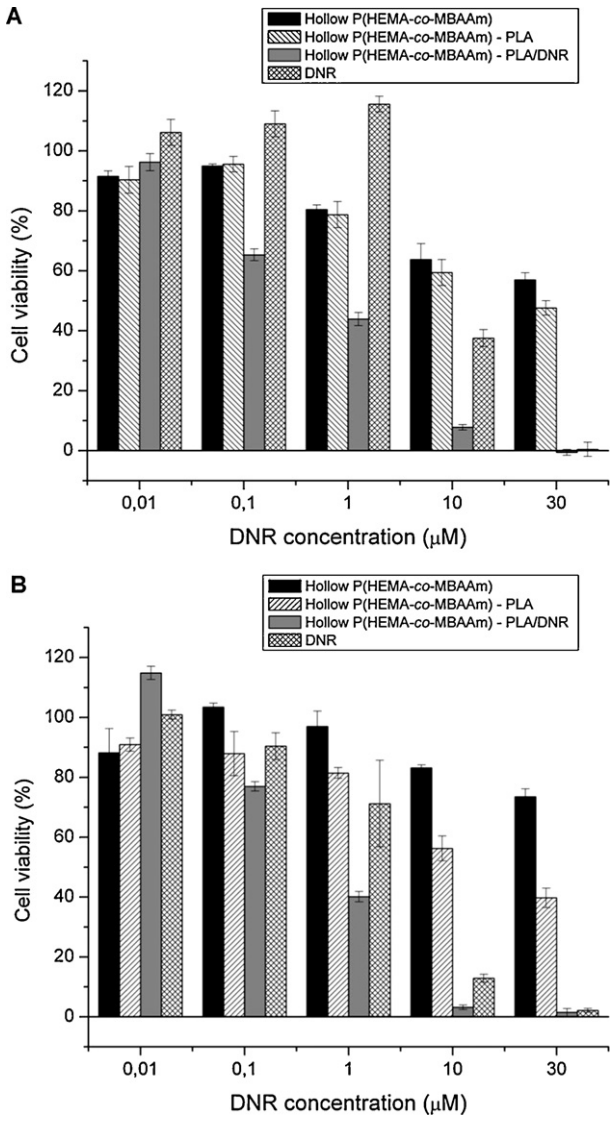


Fig. 8. Cytotoxicity on MCF-7 cells as determined by the MTT assay. Cells were treated with various DNR concentrations or microspheres at equal drug concentration for 24 h (A) and 48 h (B) at 37 °C. Cell viability (%) was calculated relatively to the non-treated cells. Data are represented as the mean ± SD ($n = 3$).

recently (Fan et al., 2011; Gautier et al., 2011; Lu et al., 2011) revealing the importance of the successful design and synthesis of drug delivery vectors.

4. Conclusions

In the present work we have successfully modified the PHEMA microspheres via ring opening polymerization with PLA. The modified microspheres were characterized through IR spectroscopy and their size diameter, as well as morphology were studied, both, by DLS and SEM techniques. The PLA modification has a beneficial effect on the DNR loading capacity of the microspheres. The loading capacity increased due to the concomitant increase of the hydrogen bonds formed between the hydroxylic and carboxylic groups of the polymers used and the drug. Drug release can be partially controlled by changing the pH of the medium. Two phenomena depending on the pH act in parallel, the hydrogen bonding strength between the hydroxylic groups of PHEMA and carboxylic groups of PLA and the characteristic groups of DNR, and the hydrolysis rate of PLA. Therefore, by tuning the composition of the microspheres drug controlled release can be achieved. Finally, the DNR-loaded PLA modified microspheres had more potent anti-tumor effect than the free drug. Thus, the modified microspheres could be further optimized for development of an efficient drug delivery system.

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