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Polymeric/inorganic multifunctional nanoparticles for simultaneous drug delivery and visualization

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ABSTRACT

Nanoparticles consisting of different biocompatible materials are attracting a lot of interest in the biomedical area as useful tools for drug delivery, photo-therapy and contrast enhancement agents in MRI, fluorescence and confocal microscopy. This work mainly focuses on the synthesis of polymeric/inorganic multifunctional nanoparticles (PIMN) based on biocompatible di-block copolymer poly(L,L-lactide-co-ethylene glycol) (PLLA-PEG) via an emulsion-evaporation method. Besides containing a hydrophobic drug (Indomethacin), these polymeric nanoparticles incorporate different visualization agents such as superparamagnetic iron oxide nanoparticles (SPION) and fluorescent Quantum Dots (QDs) that are used as contrast agents for Magnetic Resonance Imaging (MRI) and fluorescence microscopy together. Gold Nanorods are also incorporated in such nanostructures to allow simultaneous visualization and photodynamic therapy. MRI studies are performed with different loading of SPION into PIMN, showing an enhancement in T_2 contrast superior to commercial contrast agents. Core-shell QDs absorption and emission spectra are recorded before and after their loading into PIMN. With these polymeric/inorganic multifunctional nanoparticles, both MRI visualization and confocal fluorescence microscopy studies can be performed. Gold nanorods are also synthesized and incorporated into PIMN without changing their longitudinal absorption peak usable for lased excitation and phototherapy. In-vitro cytotoxicity studies have also been performed to confirm the low cytotoxicity of PIMN for further in-vivo studies.

INTRODUCTION

Great challenges in the medical field today, such as early diagnosis of pathologies and effective personalized treatment of patients, are driving the development of novel materials with peculiar properties, among all nanostructured materials. Nanoparticles, in particular, have been developed for advanced applications in the biomedical area, i.e. for drug delivery [1], diagnostic tools improvement [2], biomolecules separations [3] and enhanced contrast imaging of different types [4]. Nowadays the focus is shifted into combining and integrating different functionalities into a single entity at a nanometer scale to allow simultaneous diagnostic, drug delivery, imaging and so on [5]. For applications in biology and medical diagnostics and therapy the superparamagnetic (no magnetic remanence and no coercivity) iron oxide nanoparticles (SPION) are desired. These iron oxides based nanoparticles with a narrow size distribution and

superparamagnetic character are FDA approved materials for biological applications and can be used as T_2 MRI contrast agent or hyperthermia treatments[6]. Semiconductor nanoparticles (QDs) are also widely used in biology and medicine due to their superior fluorescent brightness and photostability compared to conventional fluorophores [7]. Gold nanorods have also been synthesized with different sizes and aspect ratios and their biomedical applications have been exploited, especially for photothermal therapy due to their peculiar properties of absorbing light at specific wavelength and generate heat locally [8]. Surface modification of inorganic nanoparticles is a necessity in order to avoid agglomeration but also to increase the biocompatibility and the retention time in the blood stream. For this purpose, different biocompatible polymers, such as PLLA, PLGA and PEG, have been used to coat inorganic nanoparticles as well as to form micellar structures that can be loaded with a desired drug to be delivered [9].

In this work, we report the preparation of polymeric/inorganic multifunctional nanoparticles (PIMN) that can be loaded with a model drug and can carry different visualization agents, such as SPION for MRI T2 contrast imaging and/or QDs for fluorescent visualization. Gold nanorods can also be loaded in PIMN in order to allow phototherapy.

EXPERIMENTS

Materials and Methods

L(-)-lactide (Purac), poly(ethylene glycol) methyl ether (mPEG with M_W 2000 Da), stannous 2-ethyl hexanoate, diethyl ether, toluene anhydrous (99.8%), indomethacin, 1-octadecene (90%), Se powder (99.8%, 100 mesh), S powder (99.8%, 100 mesh), oleic acid (90%), hexane, iron (III) chloride hexahydrate (99.99%), trioctylphosphine (90%), sodium oleate, ethanol (99.9%), dioctyl ether (99%) and methylene chloride are from Sigma Aldrich. Chloroform from Prolabo; dichloromethane, poly(vinyl alcohol) and CdO powder from Fluka were used. MilliQ pure water with a resistivity of 18 MΩ was used.

The emulsion system to form PIMN was induced by applying ultrasounds with a tip sonicator with energy up to 140 J. Dynamic Light Scattering (DLS) analysis was performed on a liquid suspension of PIMN with a Beckman Coulter Delsa™Nano S. TEM analysis was performed by placing one drop of nanoparticle suspension on a carbon coated copper grid and images were recorded at an acceleration voltage of 200kV with a JEOL FEG-HRTEM 2100 F. Drug release experiments have been performed using a Franz-cell set-up at 37ºC with a membrane with 14kDa cutoff separating the donor chamber (with nanoparticle suspension) and the receiving chamber (containing 0.1 M PBS buffer). A 4.7 T MR scanner with bore diameter of 155 mm (PharmaScan, Bruker BioSpin, Germany) was used to record T_2 weighted MRI images of the samples and to measure the r_2 and r_1 relaxivity. The cell toxicity was tested by monitoring the cell viability with FACS analysis. The cells (PC12 cell lines) were incubated at 37 °C for 24 h with different concentrations (1, 10 and 100 μg/mL) PIMN containing SPION and QDs only.

Synthesis procedures

The polymerization of PLLA-mPEG was carried out via ring-opening polymerization: typically, 0.8 g of L-lactide, 2.0 g of mPEG and 0.04 g of stannous(II)-ethylhexanoate were dissolved into 20 ml of anhydrous toluene under protected atmosphere. The reaction was carried out at 110 °C for 12 hours with constant magnetic stirring. Upon completion of the reaction, polymer was collected by precipitating against an excess volume of cold diethyl ether and

filtered before drying in vacuum overnight [1]. Iron oxide nanoparticles (SPION) were prepared by thermal decomposition of iron oleate in a high boiling point solvent (1-octadecene) through an earlier reported two-step method[4,10]. Quantum dots (QD) consisting of a CdSe core and a ZnS shell were prepared as described in literature. [11] Gold nanorods were synthesized by reducing gold salt in the presence of CTAB surfactant [8] and transferred into dichloromethane by an established procedure [12].

Multifunctional polymeric/inorganic nanoparticles (PIMN) were synthesized through an oil-in-water (o/w) nanoemulsion. The oil phase consisted of a solution of 4% w/v of PLLA-mPEG and indomethacin (IMC) as model drug in dichloromethane and SPION or QDs or NR or a combination of them. The second phase was consisting of 0.6% w/v aqueous solution of PVA. The o/w emulsion was prepared under the strong external hydrodynamic forces generated by sonication for 3 min in an ice bath. Finally, the emulsion was formed with a slight milky color and added into 5 ml of water with moderate shaking for 12 h to evaporate the organic solvent. PIMN were collected by centrifugation at 25 krpm for 20 minutes and re-suspended in ultrapure water. The procedure was repeated three times. The sample was then freeze-dried and stored in the freezer for further use.

RESULTS AND DISCUSSION

PLLA-mPEG copolymer was characterized by NMR and thermal analysis (TGA and DSC). ^{1}H NMR spectra obtained (in $CDCl_3$): d =1.58 (dm, J=7.0 Hz, 3H, PLLA), 3.65 (m, 2H, PEG), 5.16 (q, J=7.0 Hz, 1H, PLLA) ppm. With thermo-gravimetric analysis (TGA, data not shown), two degradation steps were clearly visible, corresponding to the PLLA (22% mass, T= 200°C ca) and mPEG (78% mass, T=400°C ca) segments respectively. These results are well in agreement with the initial feeding ratio between L-lactide monomers and mPEG polymer as well as with previously reported data [13]. The detailed characterization of PLLA-mPEG copolymer confirmed its di-block structure as well as the ratio between the two polymeric segments. Freeze-dried PIMN have been re-suspended in water and then characterized by DLS in order to measure their hydrodynamic size and its distribution (data not shown). The average hydrodynamic diameter is 96 ±23 nm for PIMN loaded with SPION and QDs and 146 ±45 nm for PIMN that also contain IMC model drug with a monomodal distribution in both cases.

To confirm the size and morphology of the prepared multifunctional nanoparticles, electron microscopy studies have been performed. SEM analysis (Figure 1 (a)) of freeze-dried PIMN confirm their spherical morphology and average size.

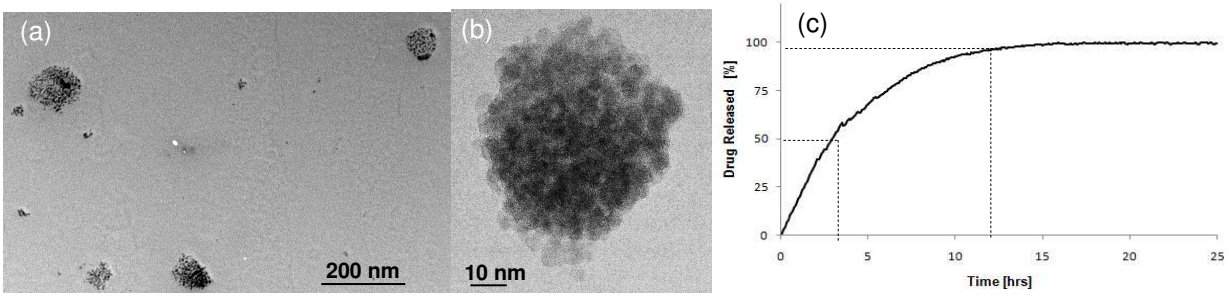

Figure 1. (a) SEM image of freeze-dried PIMN. (b) TEM micrograph of PIMN loaded with SPION and QDs. (c) Drug release profiles of IMC from PIMN loaded with SPION, QDs and IMC model drug (50 and 95% release level are highlighted).

By TEM analysis (Figure 1 (b)) it is possible to study the internal loading of PIMN. The polymeric part is not clearly visible owing to its low electron density, while both QDs and SPION give a high contrast in the image (Figure 1 (b)), and it is possible to observe that they are confined in a circular area that confirm that they are loaded in the inner core of PIMN due to their interaction with the hydrophobic part of PLLA-mPEG (both SPION and QDs are dispersed in the organic phase).

The released IMC was able to pass the membrane, while nanoparticles were retained in the donor chamber. UV spectroscopy was used, after a concentration calibration, to determine the real time concentration of IMC released by monitoring the intensity of the IMC peak at 319 nm. As it can be seen from Figure 1 (c), the time for the release of half of the IMC loaded in PIMN (containing also QDs and SPION) is ca 3 hours and the time to release 95% of loaded IMC is ca 11 hours. This release profile shows that it is possible to have a sustained release of a model drug loaded into the PIMN. According to the literature [14], the mechanism responsible for drug release can be ascribed either to diffusion due to concentration gradients or dissolution of the polymeric nanoparticles. Comparing the obtained release profile with the theoretical ones, it seems that the drug diffusion is the most probable mechanism for the release of IMC from PIMN.

The prepared PIMN containing QDs and SPION were also tested as a T_2 contrast agent for Magnetic Resonance Imaging (MRI). T_2 weighted MRI phantom images of PIMN at different Fe concentrations (30 ppm, 10 ppm, 4 ppm and 1 ppm) were recorded at 4.7 T (data not shown). The test tubes containing PIMN are darker due to the T_2 contrast agent effect compared to bright signal from water samples. The higher the concentration PIMN, the more pronounced is the contrast effect. In order to obtain more quantitative data regarding the contrast properties of the prepared nanoparticles, the relaxivity values r_1 and r_2 were measured at 4.7 T and are reported in Figure 2.

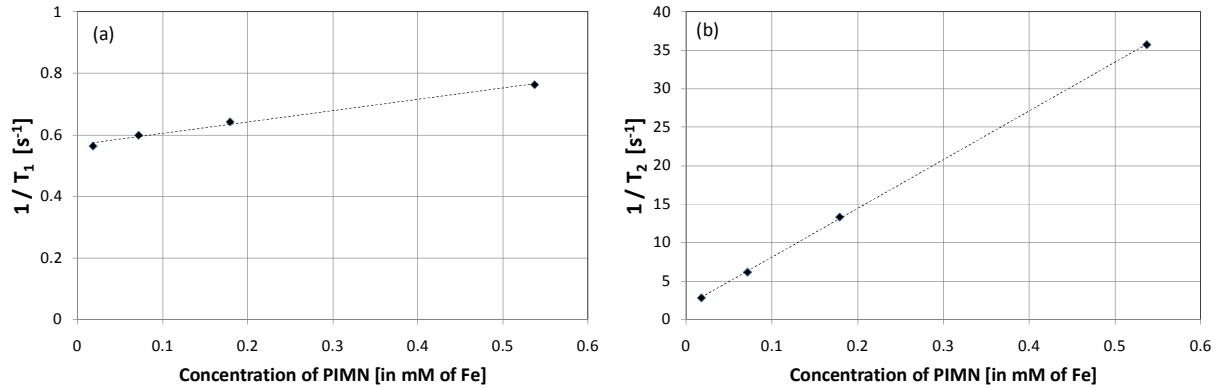

Figure 2. Plots showing (a) $1/T_1$ relaxivity and (b) $1/T_2$ relaxivity for PIMN.

The values of $1/T_2$ (related to T_2 contrast) is increasing linearly as a function of iron concentration, due to excellent magnetic propertied of SPION encapsulated in PIMN. A similar trend (but with a much lower slope) can be observed also for $1/T_1$ as a function of iron concentration. To further compare the efficiency of the nanoparticles prepared, we also calculated the relaxivities ratio r_2/r_1 that is commonly used to estimate the performance of a contrast agent for MRI. At 4.7 T the r_1 value is 0.37 $s^{-1}mM^{-1}$ and r_2 is 63 $s^{-1}mM^{-1}$, giving r_2/r_1 ratio of 170.3, which indicate that the prepared nanoparticles are an excellent T_2 contrast agent.

Extra relaxivity experiments were also performed at 20 and 60 MHz to compare our nanoparticles with commercial T2 contrast agents and the data are summarized in Table I.

Table I. The relaxivity values of PIMN, Resovist® (Schering AG, Germany) and Feridex® (Advanced Magnetics, Massachusetts) reported values measured at 20 MHz and 60 in water.

	$r_1(s^{-1}mM^{-1})$		$r_2(s^{-1}mM^{-1})$		r_2/r_1	
	20 MHz	60 MHz	20 MHz	60 MHz	20 MHz	60 MHz
synthesized PIMN	2.8±0.1	1.3±0.1	26.8±0.6	39.3±0.5	9.6±0.5	30.2±2.7
Resovist[15]	24.9	10.9	177	190	7.10	17.4
Feridex[16]	40	9.9	160	158	4	15.9

It is to be noticed that r_2/r_1 ratios for the synthesized PIMN are twice as high as those of Resovist and Feridex at 60 MHz MHz (1.41 T) and at 20 MHz (0.47 T) which indicates a higher T_2 contrast in case of PIMN. In order to estimate the fluorescent properties of PIMN, samples loaded with SPION and QDs with various sizes were exposed to UV-light and the emission spectra were recorded both for QDs suspended in chloroform and for PIMN (data not shown). Different QDs samples had a different emission peak, according to their size: green emission at 524 nm, yellow at 568 nm, orange at 608 nm and red at 640 nm. There was no significant shift in the position of the emission peak for the different samples, meaning that QDs are not affected by the encapsulation procedure to form the multifunctional nanoparticles.

Besides loading SPION, QDs and IMC into PIMN, gold nanorods have also been loaded into PIMN together with SPION for photo-thermal applications. TEM was used to analyze the morphology and size of gold nanorods and a typical micrograph is reported in Figure 3 (a). The average length was calculated to be 40 ±7 nm and the aspect ratio to be 4.5 ±1 nm. The absorption spectra of the prepared nanorods and PIMN loaded with SPION or SPION and gold nanorods were recorded (Figure 3 (b)). Two peaks are clearly seen for gold nanorods at 525 nm and 836 nm, corresponding to the transversal and longitudinal absorption due to the elongated morphology of the nanorods. For PIMN loaded with SPION and gold nanorods, a peak can be seen at 836 nm, superimposed on the background due to SPION absorption below 400 nm.

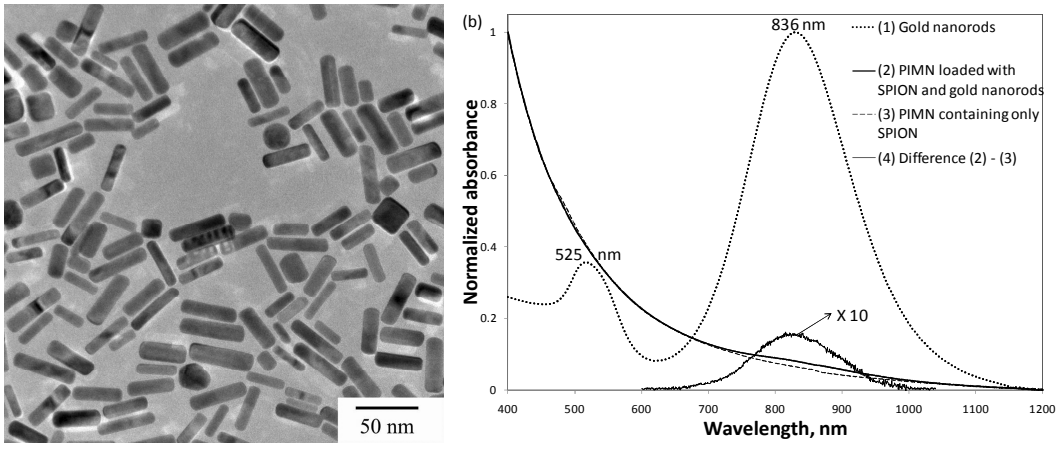

Figure 3. (a) TEM micrograph of Gold nanorods and (b) absorption spectra for (1)gold nanorods in dichloromethane, (2) PIMN with SPION and gold nanorods, (3) PIMN with only SPION and (4) their difference magnified 10 times.

In order to highlight the peak at 836 nm for PIMN with gold nanorods, a spectrum obtained for PIMN with only SPION is subtracted and magnified 10 times. The absorption peak for PIMN is then clearly visible at around 836 nm, confirming that the rods are loaded inside PIMN. The preliminary cytotoxicity tests revealed that the particles were not affecting the viability and

proliferation in the time interval studied. Further tests for confirming these results are undertaken and results will be reported elsewhere.

CONCLUSIONS

In the present study, the synthesis and the characterization PIMN for drug delivery system have been discussed. The emulsion/evaporation technique used for the synthesis is a very good and versatile tool to prepare PIMN with relatively narrow size distribution and with different loading of drug and inorganic nanoparticles for multimodal visualization. The prepared multifunctional nanoparticles can release a model drug (IMC) over a period of several hours, mainly due to diffusion mechanisms. SPION show a very narrow size distribution and they are responsible for very high T_2 contrast signal of PIMN, with better performance compared to commercially available contrast agent (Resovist and Feridex). PIMN containing QDs and SPION show very bright emission (due to QDs) when excited with UV light and they are therefore very promising for confocal microscopy imaging to track nanoparticles in cell cultures. Gold nanorods can also be loaded into PIMN without changing the absorption properties. Preliminary cell tests of the polymeric/inorganic multifunctional particles showed no significant cytotoxicity.

According to their properties and characteristics, such nanoparticles have been demonstrated to be suitable for their designed application, as they simultaneously release drug while contain visualization agent for MRI imaging and fluorescent confocal microscopy.

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