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Selective Release of anti–TB Drugs Complex from Smart Copolymeric Bioactive nano–carriers

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Abstract: Smart nano–copolymeric matrices have been employed to load and release anti tuberculosis (anti – TB) drugs combinated complexes of Ethambutol (EMB), Isoniazid (INH), Rifampicin (RMP) and Pyrazinamide (PZA). Copolymeric nanocarriers were synthesized using a microemulsion polymerization method previously reported. These nanocarriers can show selective swelling–collapse response under changes in local environments such a temperature, pH, solvent composition and electrical stimuli. The employ of these kinds of systems permits a controlled and selective delivery and release on specific human tissues. High Performance Liquid Chromatography technique was used to allow the detection of combinated mixtures of different active principles of anti–TB drugs using an acetonitrile mobile phase at 0.5 mL/min of flow rate whit a Spherisorb ODS2, C_{18} column. The results obtained suggest that the employ of smart nanohydrogels is a novel method in several tuberculosis therapies.

Keywords: Smart copolymers; drug release; anti–TB; Isoniazid.

Introduction

During the past decade the tuberculosis disease has generated a great worldwide focus of alarm but particularly in places where the mortality rate caused by this disease is highly worrying[1]. Tuberculosis (TB) is a chronic bacterial infection caused by *Mycobacterium tuberculosis* histologically characterized by the formation of granules. Typically, the disease is found in the lungs, but can affect almost any organ in the human body. TB is the most common cause of death from an infectious agent worldwide in adults[2]. The discontinuation in the application of anti–TB drugs has several implications. Actually the treatment of this kind of diseases takes a long time to ensure that the infectious agent is eradicated from the host[3]. Isoniazid (INH) is the most powerful mycobactericidal drug available that ensures early

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sputum conversion and helps in decreasing the transmission of TB[4]. Rifampicin (RMP) is a mycobactericidal with sterilizing activities and is crucial for preventing relapses. There are also another two drugs capable to be used to combat this disease: the Ethambutol (EMB) and Pyracinamide (PZA) [5].

Thus, Isoniazid and Rifampicin are keystone drugs in the management of TB[6]. Otherwise, the particularly hepatoxicity, risk factors, morbidity and mortality from INH as well as the adverse reactions to RMP and EMB are well documented in literature[7–12]. The adverse effects of these drugs can be decreased when they are used in a combinated way[13]. One of the most versatile methods, not used enough, to administrate this kind of drugs is the employ of nanoparticles as drug carriers[14].

The synthesis of these vehicles includes the use of very complex methods with high precision and accuracy[15]. Smart nanoparticles can act in a selective manner when they are functionalized through chemical procedures[16]. Smart polymers are macromolecules capable of undergoing rapid, reversible phase transitions from a hydrophilic to a hydrophobic microstructure when triggered by small changes in their immediate environment, such as slight variations in temperature, pH or ionic strength[17].

Smart polymers are becoming increasingly more prevalent as scientists learn about the chemistry and triggers that induce conformational changes in polymer structures and devise ways to take advantage of, and control them[18]. New polymeric materials are being chemically formulated that sense specific environmental changes in biological systems, and adjust in a predictable manner, making them useful tools for drug delivery or other metabolic control mechanisms[19].

The versatility of particulate technologies enables tailoring of the nanoparticles based drug delivery systems with consideration of the target, desired pharmacokinetic profile, and route of administration[20].

Results and Discussion

Characterization of nanohydrogels

By differential scanning calorimetry the glass transition temperature of the nanohidrogel was determined by the criterion of the midpoint and it was 98.2°C value which agrees with previous reports[21] for the copolymer of NIPA–2AAECM–NPAM. The nuclear magnetic resonance (^{1}H NMR) shows that the skeleton of the copolymer contains segments of NIPA, NPAM and 2AAECM and the calculated composition in weight was of 93.4% NIPA, 3.73% NPAM and 2.83% 2AAECM, values which are very close to added to the reactor (90% NIPA, 5% NPAM and 5% 2AAECM). Figure 1 shows the ^{1}H NMR spectrum of a nanohydrogel.

The FTIR spectrum of the nanohydrogels (Figure 2) confirmed the presence of major functional groups of the monomers (NIPA, 2AAECM and NPAM) in the skeleton of the copolymer. We observed bands to 2940 and 3298 cm $^{-1}$ due to the vibration of the amide protons. NH and CH bonds of the aromatic ring of NPAM. The vibration of the NO_2 group from NPAM appears near to 1350 cm^{-1}. There is also a confirmation of the NH group at 1560 cm^{-1} and a confirmation signal of NIPA and aromatic NPAM at 840 cm^{-1}.

The particle size determined by QLS was 45.5 ± 11 nm measured in water milliQ grade, 42.7 ± 9 nm measured in acetone and 57.4 ± 7 nm in buffer solution of pH 2. The average particle size determinated by scanning electron microscopy (analysis was made by visual inspection and counting), was 54 nm. Figure 3 shows a scanning electron micrograph of the nanohydrogels.

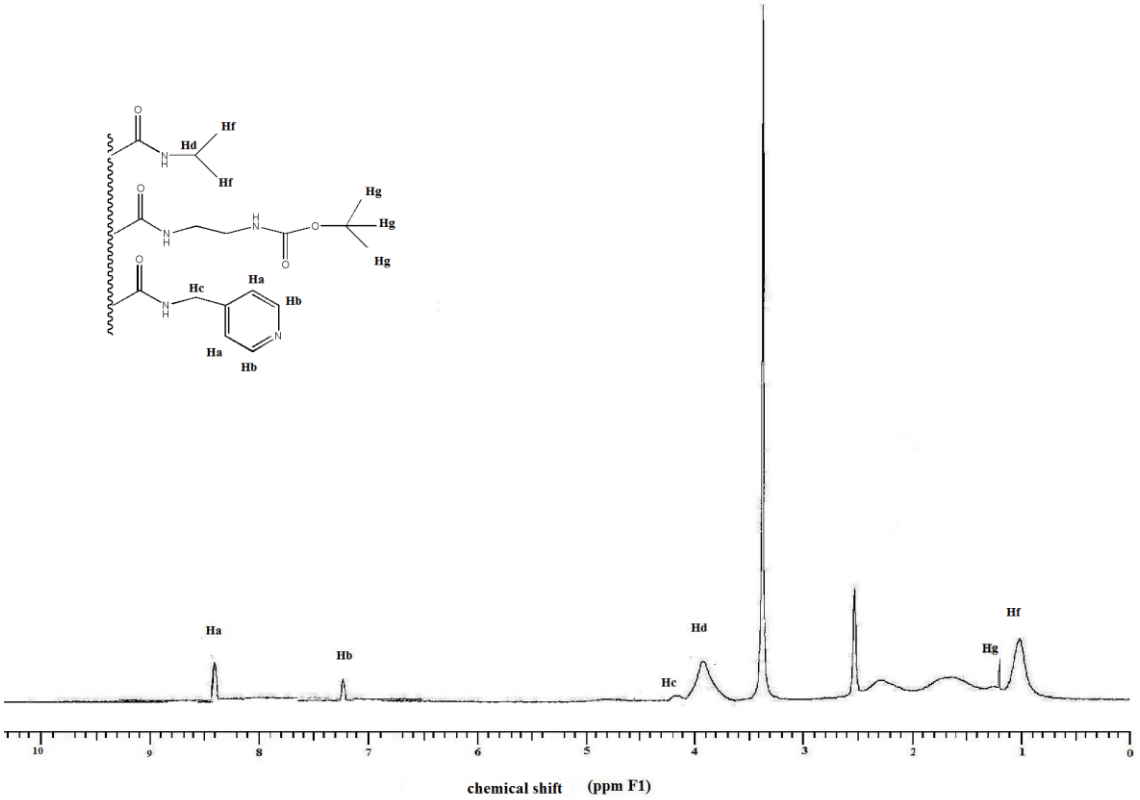

Figure 1. NMR ^{1}H spectrum of the nanohydrogels.

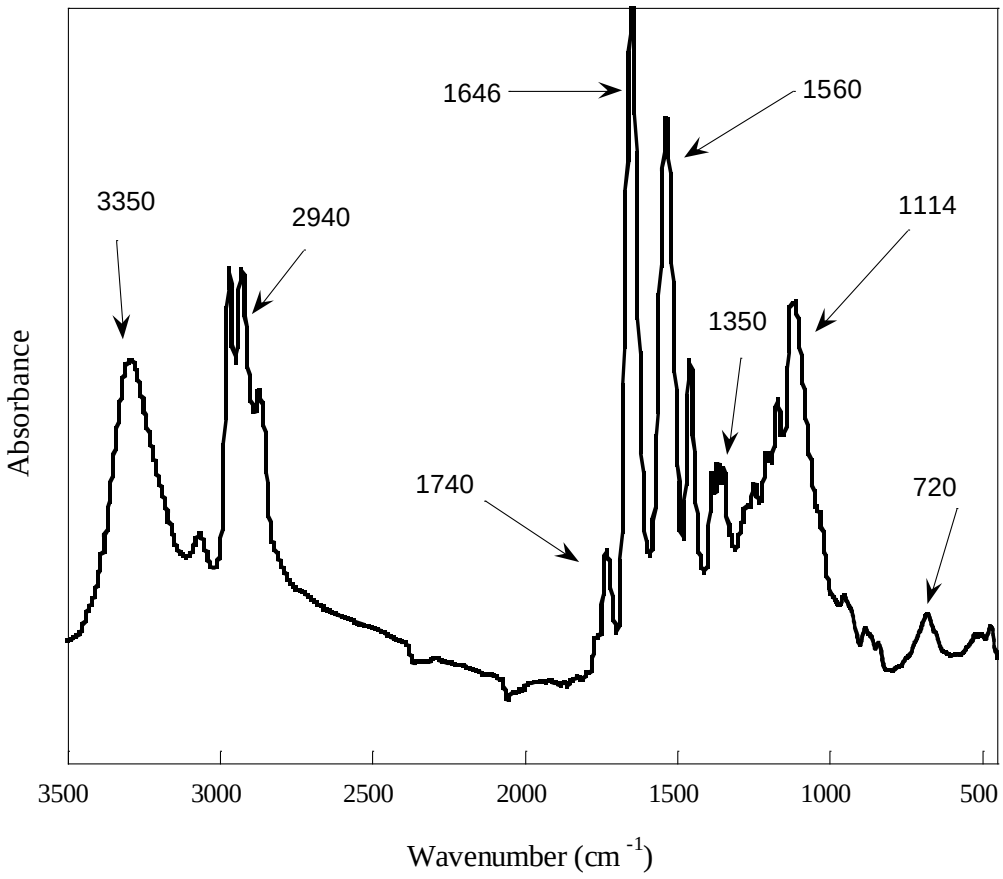

Figure 2. FTIR analysis of smart nanohydrogels (NIPA–co–NPAM–co–2AAECM).

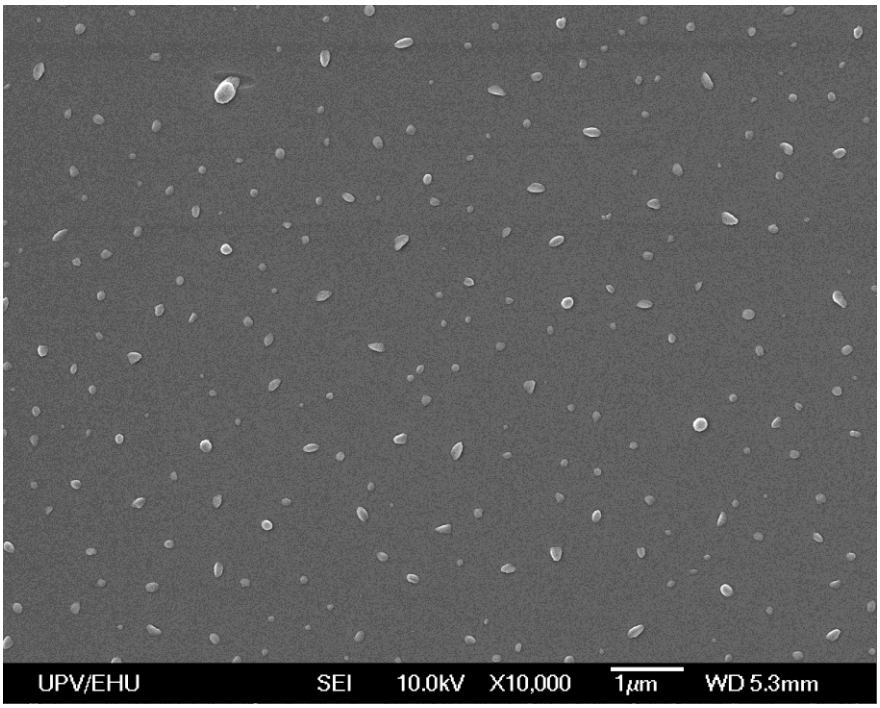

Figure 3. SEM photomicrography of the nanogels.

Release of ATD's from drug-loaded nanohydrogels

The monitoring of drug released from the nanohydrogels was made by high performance liquid chromatography (HPLC), following the EMB signal since it was the drug that produced a retention time of 3.5 min that differed clearly from those of the other three drugs (4.8, 7.2 and 4.6 minutes for INH, RIF and PZA, respectively), and that it did not overlap with the peaks of the buffer that showed after four minutes.

Table 1. Releasing measurements of ATD's from smart nanocarriers by HPLC.

Releasing times	**Retention time min**	**Area μv·s**	**Concentration ppm EMB**
0	0	0	0
30	3.4	137659.7	0.5506388
60	3.3	171578.5	0.686314
90	3.4	295534.0	1.182136
120	3.4	1014428.5	4.057714
180	3.5	1821406.0	7.285624
240	3.5	2215885.5	8.863542
300	3.5	2078689.0	8.314756
360	3.5	2122477.5	8.48991
420	3.5	2230548.0	8.922192
480	3.6	2520820.0	10.08328
540	3.6	3011615.5	12.046462
600	3.6	2945778.5	11.783114
1800	3.7	3181426.0	12.725704
3600	3.7	3134261.5	12.537046

In the study by UV–Vis spectrophotometry EMB located had the highest absorbance at 197 nm, then the measurements on the HPLC detector were made at 200 nm. In Table 1 can be seen the follow–up EMB released at each time interval.

The drug release media (buffer pH 2) intended to ensure that the degree of swelling of nanohydrogels were maximum; thus, facilitating the removal of the drug to the environment.

Conclusion

This study reports for the first time the use of nanohydrogels from a copolymer of acrylamide (NIPA–2AAECM–NPAM) sensitive to pH as a carrier for four first–line TB drugs in a simultaneously way and their subsequent quantification by high resolution liquid chromatography. However, future studies would be required for the quantification of loading and release of the four drugs (INH, RIF, PZA and EMB) simultaneously did this study only for the EMB for ease of experiments and analysis. But even hypothesized that the other three drugs showed a comparable effectiveness, the EMB load and release given the simultaneous conditions of the nanohydrogels with the complex of the four antituberculosis is the preferment analytic procedure.

It is important to note that although these release of drugs tests were supplemented with calculations of their kinetics; it is clear that there was an advance to verify the release of at least one of the four ATD's and its quantification in function of time showed a trend of rapid growth in the first minutes and then moderate to reach the first hour of liberation. Furthermore, the size of nanoparticles used in this study, as it turned out, have an average of 57.4 ± 7 nm at pH 2, favor their use as vehicles for oral drug delivery, taking into account that particles of less than 400 nm are taken intact from the intestine[23] . Even this particle size achieved by this novel technique of microemulsion synthesis, is very close to the required diameter (15–30 nm in the terminal vascular system) for these nanohydrogels can be injected via bloodstream without posing a problem of vascular obstruction[24], which is of great interest because it may release the drugs directly into the bloodstream throughout the body arrived in a very short time. These results suggest that the use of smart nanohydrogels could be an alternative to serve as vehicles for ATD's complexes.

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Experimental Section

Materials

Isoniazid (INH) ≥ 99%, rifampicin (RIF) ≥ 97%, Pyrazinamide (PZA) ≥ 99% and ethambutol (EMB) > 99% were obtained from Sigma Chemicals Co. Acetonitrile HPLC grade (LAB–SCAN Analytical Sciences), solution buffer pH 0.02 and pH 2.00 ± 7.00 ± 0.02 (20°C) ST (Panreac), concentrated buffer solution pH 2.00 (Titrisol®, Merck Co.). N–Isopropylacrylamide (NIPA) (Aldrich 97%), 4–aminomethyl pyridine (4AMP) (Aldrich 98%), ethylenediamine (ED) (Aldrich 99%), acryloil chloride (Aldrich 96%), di–tert–butyl

dicarbonate (BOC) (Aldrich 97%) and Dichloromethane (Aldrich 98%) were used without further purification. Chloroform (for analysis, Merck > 99%). Diethyl ether (for analysis 99.7%), ethyl acetate (99.8%) and potassium nitrate (> 99%) were provided by Panreac. Polyoxyethylene Sorbitol hexaoleate (Atlas G–1086) (99%) and sorbitan sesquiolate (Arlacel 83) (98%) both obtained from Uniquema QC Laboratory, were used as received. Sodium chloride (NaCl) (Probus 98%), anhydrous sodium sulphate (Scharlau 99%), sodium disulfide (Merck 98%) were used as received. The monomers N (4–methyl pyridine) acrylamide (NPAM) and tert–butyl 2 acrylamidoethyl carbamate (2AAECM) were synthesized by nucleophilic substitution reaction from BOC and modified 4AMP, respectively. The crosslinking agent N, N–methylene bisacrylamide (NMBA) (> 98%) was supplied by Fluka. The C_{18} column with pore size 5.0 μm and 4.6 mm·250 mm was provided by SunFire–Waters Co. (USA). This column was used with prior conditioning buffer solution pH 2.00.

Methods

Synthesis of smart nano–particles of NIPA–NPAM 2AAECM

Copolymeric nanoparticles of NIPA–2AAECM–NPAM were synthesized using a microemulsion polymerization process using a reported method[14,16]. The microemulsion solution was introduced in a mechanical stirred reactor mantained at 25 ± 1°C and operated at 131 rpm and nitrogen was bubbled to maintain an inert atmosphere during the reaction. The monomers N–(4–methyl pyridine) acrylamide (NPAM) and tert–butyl 2–acrylamidoethyl carbamate (2AAECM) are not commercial products; they were synthesized by a nucleophilic substitution reaction from the precursors, modified 4AMP and BOC, respectively. To obtain NPAM monomer, 4AMP reagent was previously prepared and reacted with acryloil chloride at – 5°C under vigorous stirring to produce a nucleophilic substitution by the amino functional group. The 2AAECM synthesis procedure involves several steps: the first was to obtain a di–tert–butyl dicarbonate (BOC) modified by reaction with ethylenediamine at –19°C using dichloromethane as a reaction medium and when all the BOC was added the reaction was maintained for 16 hours at 25°C. The dichloromethane was evaporated and the water insoluble diprotected amine formed as a secondary product was separated by adding water. Then, the diprotected amine was separated by filtration and the resulting solution was saturated with NaCl and extracted with ethyl acetate. The resulting solution was dried by adding anhydrous sodium sulphate and the product was obtained by rotoevaporation. This product was reacted with acryloil chloride to produce the monomer (2AAECM).

Characterization of nanoparticles of NIPA–2AAECM–NPAM

Differential scanning calorimetry (DSC). A TA Instruments calorimeter (DSC 2920) was used to measure the glass transition temperature (T_g) of the nanohydrogels. Thermal analysis for nanohydrogels of poly (NIPA–co–NPAM–co–2AAECM) was performed from 0 to 200°C at a heating rate of 10°C/minute under nitrogen flow (100 mL/min). The criterion of the midpoint was used to assign the T_g.

Nuclear Magnetic Resonance (NMR). Characterization was performed to obtain NMR proton spectrum (^1H) for nanohydrogels in deuterated water (D_2O) as a solvent, an instrument Bruker ACE (250 MHz) was employed at 20°C. The chemical shifts (δ) were measured in ppm relative to deuterated water (δ = 7.26).

Fourier Transform infrared Spectrophotometry (FTIR). The spectra of the nanohydrogels were collected using attenuated total reflectance (ATR) with a Smart Orbit accessory attached to a spectrophotometer Fourier Transform Infrared FTIR (Nicolet 6700). All spectra were obtained from an average of 100 scans with 4 cm^{-1} of resolution.

Quasielastic Light Scattering (QLS). Approximately 5 mg samples of nanohydrogels were dispersed in 10 mL of water and acetone, and kept under constant agitation for at least five days. Then was measured the particle size. The measurements were made at an angle of 90°, the detection time for each sample was 2 minutes and the measurements were repeated at least five times and the average of the values was reported.

Scanning Electron Microscopy (SEM). SEM micrographs were taken using a JEOL electron microscope at 10.0 kV JSM7000F equipped with a field emission microscope (FEM). For these experiments a layer of gold was deposited on the specimen. The spherical shape of the nanoparticles synthesized was confirmed by these measures.

Loading the NIPA–2AAECM nanoparticles with anti TB drugs complex

First, TB drugs or ATDS (Anti tubercular Drugs) (INH, RIF, PZA and EMB) were dissolved in 10 mL of a buffer solution of pH 2, at a concentration of 10,000 ppm and maintained under constant agitation for five hours. The concentration of each drug was in the proportion that each of the 900 mg tablets used in tuberculosis treatment [24,25] (8.33, 16.67, 44.44 and 30.56% wt. for INH, RIF, PZA and EMB, respectively). NIPA–co–2AAECM–co– NPAM hydrogels (approx. 1.5 g) were suspended in 50 mL of a buffer solution of pH 2 and maintained under constant agitation for five hours. Then the solution of the drug compounds was added drop by drop to the suspension of nanohydrogels under continuous stirring. This suspension was left under constant agitation for about 15 hours, then a few drops of NaOH aqueous solution (50% w/w) were added to reach pH 7 ± 0.2 and the nano hydrogels suspension was left to sediment for four hours and the supernatant was separated by centrifugation at 4500 for 10 minutes and stored for subsequent calculations. An aqueous buffer of pH 7 was added to the precipitate and centrifuged under the same conditions (4,500 rpm for 10 minutes). As in the previous step the resulting supernatant was stored. The precipitate (loaded nanohydrogels) obtained at the end of the second centrifugation was placed in a Petri dish and allowed to dry in an oven vent at 50°C for twenty–four hours to remove any residual solvent. Loaded nanohydrogels were stored in a glass jar and left it on the stove vent at 50°C.

In–vitro release studies

The release of the nanohydrogels loaded with ATD's was conducted by adding 15 to 20 mg of loaded nanohydrogels in 10 mL of a buffer solution of pH 2 at 37 ± 1°C under continuous stirring. Time zero was taken when the loaded nanohydrogels were added to the experiment solution. Aliquots were taken at zero time, 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600 s, at 30 min and at 60 min. These aliquots were stored in plastic vials until HPLC injection.

Quantification of EMB by HPLC. To determine the wavelength of maximum absorbance of EMB a scanning spectrophotometer UV–Vis (Cintra GBC model 303) was used to determine the wavelength of maximum absorbance of EMB, which was 197 nm. Then standards were prepared with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm of EMB in solutions with pH 2. Each standard was injected into the chromatograph separately using the

same elution conditions: carrier flow of 0.5 mL/min, column temperature 50°C and the detection wavelength was set at 200 nm. The mobile phase was acetonitrile (HPLC grade) in all cases. Table 1 shows the calibration values for the standards of EMB. In a similar way, quantification of the other drugs by HPLC was carried out. For calibration curves, peak area data of each standard (in microvolts·s) at the corresponding concentration (in ppm) were taken. Linear regression from the experimental points was obtained, as well as the experimental equation. The release curves can be observed in Figures 4A and 4B.

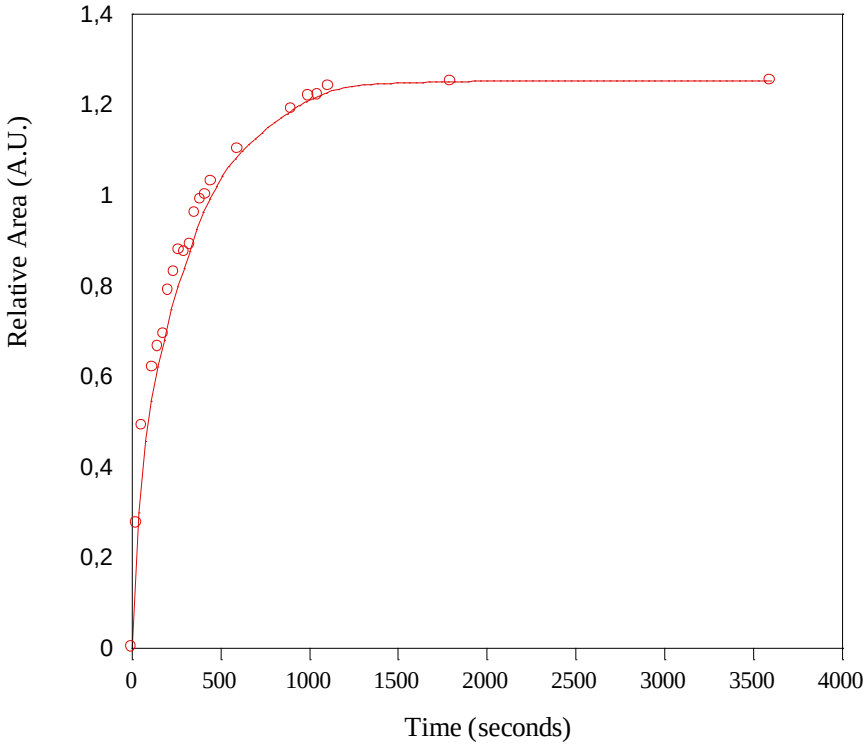

Figure 4A. Release kinetics for EMB from smart nanocarriers. Area vs. time.

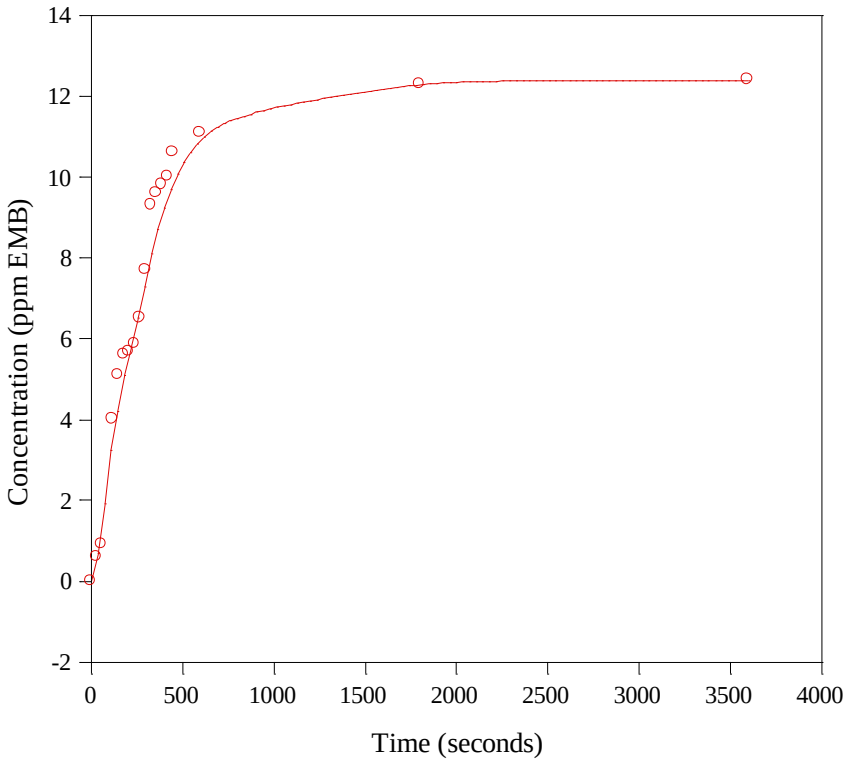

Figure 4B. Release kinetics for EMB from smart nanocarriers. Concentration vs. time.

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