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Indomethacin uptake into poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane) network: *In vitro* and *in vivo* controlled release study

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ABSTRACT

Networks based on poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane), synthesized through radical dispersion polymerization, were used as template for indomethacin (INN) as model drug. The copolymers were characterized by swelling studies at three pH values (2.4, 5.5 and 7.4) and two temperatures (room temperature 24 °C and physiological temperature 37 °C). Fourier transform infrared (FTIR) spectroscopic analysis was used to sustain the copolymer structures. Scanning electron microscopy (SEM) and thermogravimetric (TG) investigations were used to examine microstructure and appreciate the thermal stability of the polymer samples. The studies of the INN drug release from the copolymer networks were *in vitro* performed. The *in vivo* study results (biocompatibility tests, somatic nociceptive experimental model (tail flick test) and visceral nociceptive experimental model (writhing test)) are also reported in this paper.

1. Introduction

Increasing interest has been devoted to find new compositions and applications of polymeric network based on 2-hydroxyethyl methacrylate (HEMA) especially in medical and biological fields (Son et al., 2006). Thus, poly(HEMA) (PHEMA) becomes one of the well-studied synthetic polymers, because it is nontoxic, biocompatible, swells but does not dissolve in aqueous media, and meets the nutritional and biological requirements of cells. By the bulk polymerization of HEMA, a glassy and transparent polymer is produced, which is hard like poly(methyl methacrylate). But, when immersed in water, PHEMA swells and becomes soft and flexible (Songa et al., 2003). PHEMA-based hydrogels are hydrolytically stable and may be engineered to possess similar water content and elastic module as body tissues. There are numerous studies that aim to modify the properties of PHEMA, to improve its mechanical (Mack et al., 1988) and transport properties (Elvira et al., 2002), temperature responsive characteristics (Flory and Rehner, 1943) as well as the degree of hydration (Brahim et al., 2003). The degree of hydration and/or swelling is one of the important properties that allows for an understanding of the transport of small molecule solutes through the hydrogel matrix. However, hydration also influences the elastic modulus and surface properties such as wettability – and consequently, bioactive substances adsorption – being, thus, strongly correlated with *in vitro* and *in vivo* bioapplications (Guiseppi-Elie, 2010).

The incorporation of spiroacetal groups in polymer structures improves the solubility and the adhesive properties, inducing as well good oxidative and thermal stability, this structural unit presenting good flexibility and tensile strength (Salamone, 1996). More than that, the polymers including spiroacetal rings are considered biodegradable and useful for biomedical applications (Moorea et al., 2005).

In previous studies some polymeric structures based on 2-hydroxyethyl methacrylate and 3,9-divinyl-2,4,8,10-tetraoxaspiro (5.5) undecane (U) acquired through radical polymerization in the presence of different radical initiators as well as surfactants and protective colloids have been realized (Chiriac et al., 2011; Nita et al., 2011, submitted for publication, in press). The studies are justified by the special effects which may be generated by both comonomers – network formation, biodegradability and biocompatibility, gel formation capacity, binding properties, amphilicity, good oxidative and thermal stability, good film formers, acid pH sensitivity.

This paper presents the evaluation of the indomethacin (INN) controlled release from the networks based on poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane)-P(HEMA-co-U)$_{PVA}$. The copolymers were

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Table 1
Copolymerization recipe series (unit, g).

Polymer samples and their codes	HEMA	U	ACPA	SLS	PVA	Water
Poly(2-hydroxyethyl methacrylate) $P(HEMA)_{PVA}$	8	0	0.05	0.267	0.267	100
Poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane): 98.25/1.75 $P(HEMA\text{-}co\text{-}U)_{PVA}$ (98.25/1.75)	7.86	0.14	0.05	0.267	0.267	100
Poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane): 96.5/3.5 $P(HEMA\text{-}co\text{-}U)_{PVA}$ (96.5/3.5)	7.72	0.28	0.05	0.267	0.267	100
Poly(2-hydroxyethyl methacrylate-co-3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane): 90/10 $P(HEMA\text{-}co\text{-}U)_{PVA}$ (90/10)	7.2	0.8	0.05	0.267	0.267	100

HEMA-2: hydroxyethyl methacrylate; U: 3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane; ACPA: 4,4′-azobis(cyanopentanoic acid); SLS: sodium lauryl sulphate; PVA: poly(vinyl alcohol).

synthesized by radical dispersion polymerization, having different ratio between HEMA and U comonomers, and the dispersive system based on sodium lauryl sulphate and poly(vinyl alcohol). The polymeric systems were characterized from the viewpoint of their swelling capacities at different pH and temperature values. Fourier transform infrared (FTIR) spectroscopic analyses confirm the polymer network structures, while scanning electron microscopy (SEM) and thermogravimetric (TG) investigations were used to examine the microstructure and for the thermal stability evaluation of the polymer samples. The INN drug release kinetic from the copolymer networks were *in vitro* performed. The *in vivo* study results, including biocompatibility tests, somatic nociceptive experimental model (tail flick test) and visceral nociceptive experimental model (writhing test), are also presented.

2. Experimental part

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA) (from Fluka, purity >96%) was purified by passing it through an inhibitor removal column. The inhibitor–remover replacement packing (for removing hydroquinone and hydroquinone monomethyl ether) was purchased from Aldrich.

3,9-Divinyl-2,4,8,10-tetraoxaspiro[5.5]undecane (U, from Aldrich, 98%), sodium lauryl sulphate (SDS, from Sigma, c > 95 wt%), poly(vinyl alcohol) (PVA) from Oriental Chemical Industry (M_w = 120,000 Da, hydrolysis degree = 88%), the radical initiator 4,4′-azobis(cyanopentanoic acid) (ACPA, from Fluka, 98%), as well as indomethacin (from Fluka, with purity higher than 99%), were used without further purification.

In all experiments twice distilled water (purified with an Ultra Clear TWF UV System) was used, which contained no foreign ions.

2.2. Preparation, procedures and sample characterization

2.2.1. Polymeric network synthesis

Polymerization recipes are presented in Table 1. The reactions were conducted under nitrogen atmosphere, in a constant temperature bath at 80 °C, with a mechanical stirring rate of 180 rpm, for 480 min.

After synthesis the polymeric particles were precipitated from water solution three times with methanol and finally freeze-dried by lyophilisation for 24 h.

2.2.2. The INN loading polymer matrices

In order to evaluate the copolymer capacity as template network the $P(HEMA\text{-}co\text{-}U)_{PVA}$ samples were loaded with indomethacin (INN). The drug powder (about 10 wt% from the copolymer weight) was dissolved in ethanol/phosphate buffer solution (pH = 7.4). The obtained drug concentration in the initial loading solution was $c = 1.9005 \times 10^{-5}$ mol/ml. Approximately same polymeric mass compound was immersed in this solution and let to swell till equilibrium. The swollen drug-loaded samples were then dried at ambient temperature for several days until the prepared particles had constant mass. The particles were then freeze-dried to obtain the resultant drug-loaded micelles to use them in the release experiments.

2.3. FT-IR spectra

The polymer composition, respectively the presence of both comonomers, as well as the bioactive compound structure were confirmed by FT-IR spectroscopy (spectra recorded on a Vertex Brucker Spectrometer in an absorption mode ranging from 4000 cm^{-1} to 400 cm^{-1}) (Fig. 1a and b). Spectra were acquired at 4 cm^{-1} resolution as an average of 64 scans.

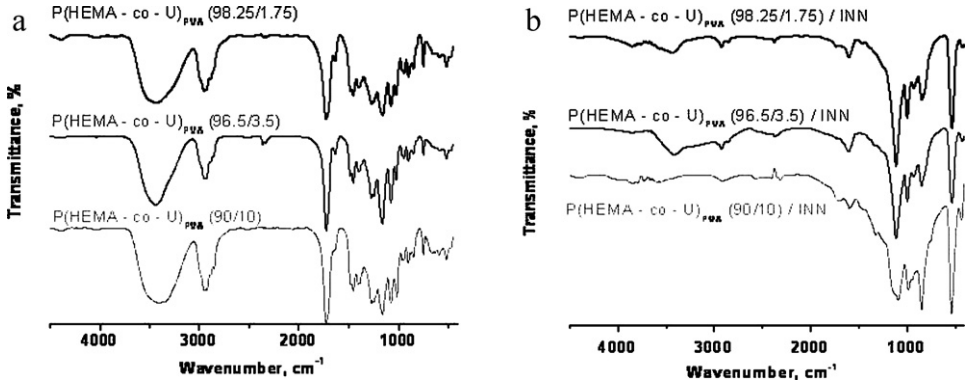

Fig. 1. The FTIR spectra of the copolymers synthesized according to recipes from Table 1 (a) and of the copolymer samples loaded with INN (b).

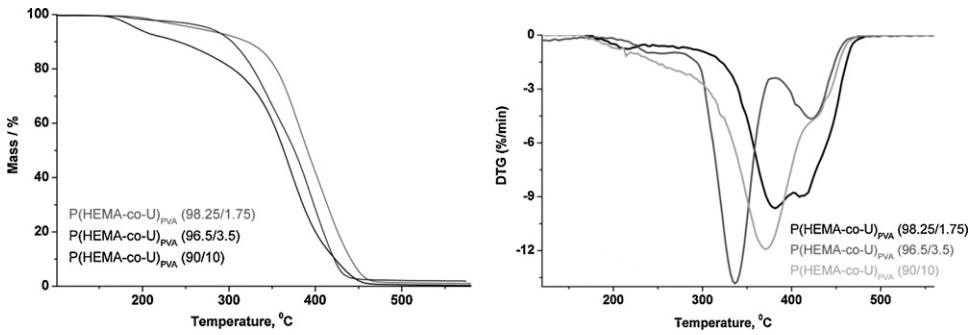

Fig. 2. TG and DTG curves which characterize the thermal decomposition of P(HEMA-U)PVA copolymers.

Thus, from the polymer matrix spectra, ν (O—H) stretching vibration of HEMA was registered in the 3400–3500 cm^{-1} range as broad absorptions, and a strong band between 2950 cm^{-1} and 2970 cm^{-1} indicated the ν (C—H). Another strong band at ~1730 cm^{-1} was attributed to ν (C=O) group; the peak from ~2940 cm^{-1} was ascribed to ν (C—H) stretching of $-CH_3$, and other peak from ~1270 cm^{-1} to ν (C—O) stretching vibration. The spiroacetal moiety presence was confirmed by some new strong bands in the 1000–1200 cm^{-1} region (attributed to ether C—O—C stretching) and at ~1715 cm^{-1} (owing to C=O stretching conjugated ether).

The loading process of the INN onto the polymeric matrix was also confirmed by FTIR and near chemical imaging. The FT-IR spectra of the biocompounds based on polymeric systems loaded with INN drug illustrate many distinct aspects (Fig. 1b). The uptake of the INN by the polymeric template is confirmed through the shifting of the major peaks of the polymers. Also, the peaks from 1609 cm^{-1}, and about 1740 cm^{-1} are attributed to the CO— groups of INN, responsible for the asymmetric stretch in the drug and actively participate in the formation of polymorphic structure. The other absorption bands at 532 cm^{-1}, 844 cm^{-1}, 927 cm^{-1} are attributed again to the drug (Greco and Bogner, 2010).

2.4. Thermal analysis

The thermal decomposition behaviour of the copolymer networks was registered on a Jupiter STA 449 F1 (Netzsch) simultaneous TGA/DSC device calibrated with high purity chemicals, respectively with standard indium, tin, zinc and aluminium. The samples were previously maintained in a controlled humidity atmosphere, in the presence of $CaCl_2$ inorganic salt. 7.5–8 mg polymer sample mass were heated in open Al_2O_3 crucible, under 50 ml/min nitrogen flow rate. Runs were performed in the dynamic mode in nitrogen from room temperature up to 600 °C, at a 10 °C/min heating rate.

The thermal behaviour evaluation of the polymer samples is presented in Fig. 2. As can be observed the results agree with other data from the literature (Salamone, 1996; Pielichowski and Njuguna, 2005; Tomic et al., 2010). The study evidences a decomposition process of P(HEMA-co-U)$_{PVA}$ in two or three stages related to the U comonomer amount. Also, the maximum temperature decomposition value of the main stage depends on the U amount.

The spiroacetal moieties inclusion, group which generates the 3D network structure formation, leads to more stable polymer systems, the thermal stability of the copolymers being increased with the comonomer content.

Improved thermal stability was also proved by the residual mass monitoring during thermal pyrolysis.

2.5. SEM visualization studies

SEM visualization studies were performed on copolymer samples fixed by means of colloidal copper supports. The samples were covered by sputtering with a thin layer of gold (EMITECH K 550×). The coated surface was examined by using an environmental scanning electron microscope (ESEM) type Quanta 200 operating at 30 kV with secondary electrons in high vacuum mode.

2.6. Measurement of particle dimension

Measurement of particle dimension of the samples (polymeric template loaded or not with drug) was done with a Mastersizer 2000 system (version 5.31, Malvern Instruments, UK). The system is constituted of an optical bank which uses laser light He–Ne 632 nm/2 mW, a dispersion unity of the sample Hydro 2000A type equipped with stirrer, recirculating pump, ultrasonic and software to record and process results on the computer. The measurement domain is between 0.020 and 2000 μm.

2.7. Zeta potential ζ

Zeta potential ζ (as well as the conductivity) was estimated with the Zetasizer equipment (model Nano ZS (Malvern Instruments, UK)) and was calculated from the electrophoretic mobility (μ) using the Smoluchowski relationship:

$$\xi = \frac{\eta\mu}{\varepsilon} \text{ with the condition } k\alpha \gg 1$$

where η is the viscosity, ε is the dielectric constant of the medium, k, α are Debye–Huckel parameter and particle radius respectively. Zeta potential is used to determine the type of interaction between the active substance and the carrier; *i.e.* whether the drug is encapsulated within the body of the particle or simply adsorbed on the surface. This is important because adsorbed drug may not be protected from enzymatic degradation, or may be released very rapidly after administration. The average of five measurements is presented as the mean value for the zeta potential. Also, the difference between the measurements and their average is less than 2.5%.

2.8. Swelling studies

Dynamic swelling measurements were performed in a wide range of pH buffer solutions (pH = 2.4, 5.5 and 7.4), important for biomedical studies (simulated physiological fluids), and at two temperatures (room temperature 24 °C and physiological temperature 37 °C). The swelled polymeric sample was removed from the solvent medium at regular intervals, dried superficially with filter paper, weighed and replaced in the same bath. The measurements were continued until constant weight was reached for the studied sample. The amount of absorbed solution was monitored gravimetrically. The degree of swelling (q) was calculated according with the following relation:

$$q = \frac{M_t - M_0}{M_0} \times 100 \quad (1)$$

where M_t is the weight of the swelled polymer sample at time t and M_0 is the weight of the copolymer sample before swelling. All the swelling experiments were performed in triplicate.

2.9. Near infrared chemical imaging evaluation

The evaluation of the drug distribution homogeneity into the polymeric matrices was made by near infrared chemical imaging (NIR-CI) technique and correspondingly statistical analysis program of NIR-CI device. NIR-CI data were collected by a SisuCHEMA device which employs SPECIM's hyperspectral imaging technology in the NIR (1000–2500 nm) range and the EVINCE as powerful chemometrics and the image processing software package. EVINCE allows to process the image data and to explore the spectral and spatial information as well as classifying and quantifying the image content. The system allows building the spectral calibrations and prediction models for the specific chemical imaging applications. The contrast in the chemical images is compared by methods using the intensity of a single wavelength, the peak-height ratio of two wavelengths, the correlation coefficient with a reference spectrum and the principal component analysis (PCA). The correlation coefficient method was also compared with the partial least squares (PLS-DA) regression for further homogeneity investigations.

2.10. In vitro controlled release of INN

The release studies of the INN have been carried out *in vitro* by using the USP paddle (apparatus II) method with a dissolution tester (ERWEKA Dissolution Testers) at 50 rpm, pH = 7.4 and 37 °C temperature. Thus, the dried samples (approx. 200 mg of microcapsules from each formulation) loaded with INN were immersed in the phosphate buffer solution. 700 ml dissolution medium was used as chamber volume containing potassium dihydrogen phosphate (KH_2PO_4, 0.2 M), monohydrogen phosphate (K_2HPO_4, 0.2 M) and water. Time was recorded as soon as the microcapsules were put into the dissolution vessels. 2 ml sample solution was withdrawn from each vessel at appropriate time intervals (1, 3, 5, 10, 15, 20, 25, 30, 40, 60, 90, 120, 150, 180, 240, 360 and after that at each 120 min until 1600 min) for the drug release analysis. Other 2 ml of fresh phosphate buffer solution previously heated at 37 °C was immediately added to the dissolution medium for compensating the sampling. The dissolution study was carried out for three samples from each formulation. The amount of the loaded INN was determined spectrophotometrically (Perkin Elmer spectrophotometer) at 319 nm.

2.10.1. In vivo studies

All the *in vivo experiments* were performed in accordance with the recommendations and policies of the International Association for the Study of Pain (Zimmerman, 1983) and according to the "GR. T. Popa" University of Medicine and Pharmacy guidelines for handling and use of experimental animals. Each animal was used only once and was sacrificed immediately after the experiment.

2.10.2. In vivo biocompatibility

Biocompatibility testing was realized by determination of the hemodynamic, immune and biochemical profile of animals treated with INN and with the new forms that incorporate the drug. Following parameters were determined: complete blood count, GPT (glutamic pyruvic transaminase), GOT (glutamic oxaloacetic transaminase), LDH (lactic dehydrogenase), phagocytic capacity of neutrophils in peripheral blood, opsonic serum capacity, phagocitic and bactericidal capacity of peritoneal macrophages. The experiments were carried out on mice divided into groups of 7 animals each and treated orally as follows: Group 1 (control – INN) indomethacin, Group 2: P(HEMA-co-U)$_{PVA}$ (98.25/1.75), Group 3: P(HEMA-co-U)$_{PVA}$ (98.25/1.75)/INN, Group 4: P(HEMA-co-U)$_{PVA}$ (96.5/3.5), Group 5: P(HEMA-co-U)$_{PVA}$ (96.5/3.5)/INN, Group 6: P(HEMA-co-U)$_{PVA}$ (90/10), and Group 7: P(HEMA-co-U)$_{PVA}$ (90/10)/INN. 24 h and 14 days after substances administration, blood samples (0.5 ml) were taken from retro-orbital plexus to assess blood count, GOT, GPT and LDH activity. In the 14th day, serum opsonic capacity (OC) (using *Staphylococcus aureus 94* cultures) was also determined. At the end of the experiment animals were sacrificed, after ethylic ether anaesthesia and the peritoneal macrophages were removed from the intact peritoneal cavity with 10 ml HANKS solution (37 °C thermostated). Samples were centrifuged (1000 rpm for 10 min), brought into contact with *S. aureus 94* cultures, incubated for 48 h at 37 °C and re-inseminated on culture media. The following immune parameters: phagocyte capacity (PC) and bactericidal capacity (BC) of peritoneal macrophages were evaluated. Data were expressed as mean ± SEM (standard error of mean) and statistically analysed using *t*-student of Windows EXCEL program test.

2.10.3. Somatic nociceptive experimental model (tail flick test)

For this test, male white Swiss mice (20–25 g) were used. Lighting was on a 12 h light/dark cycle (lights on at 6:00 a.m.), with standard laboratory food and tap water freely available, except during the time of the experiments. Before the experiment, mice were placed on a raised wire mesh, under a clear plastic box and allowed 2 h to acclimate to the testing room. The animals were divided into groups of 7 animals each and treated orally (using an esogastric device) as follows: Group 1 (control – INN) indomethacin, Group 2: P(HEMA-co-U)$_{PVA}$ (98.25/1.75), Group 3: P(HEMA-co-U)$_{PVA}$ (98.25/1.75)/INN, Group 4: P(HEMA-co-U)$_{PVA}$ (96.5/3.5), Group 5: P(HEMA-co-U)$_{PVA}$ (96.5/3.5)/INN, Group 6: P(HEMA-co-U)$_{PVA}$ (90/10), and Group 7: P(HEMA-co-U)$_{PVA}$ (90/10)/INN.

Antinociception was assessed using the tail-flick test (tail-flick Panlab Hardvard/Apparatus). Mice were positioned on a flat surface and held gently by the operator. Tail withdrawal latencies were recorded in response to heat from a light beam focused on the dorsal surface of the tail (approximately 2 cm from the tip) (Mogil, 2009). When the animal flicks its tail, the light beam activates the photocell, closing a switch which turns off the heat source. The amount of time taken for the animal to move its tail away from the heat, representing the response latency period, was recorded (Le Bars et al., 2001).

The baseline latency (before drug injection) in the tail flick test was 4.2 ± 0.2 s (mean ± standard error of mean – SEM). The recommended cut-off time of 12 s was used to prevent tissue damage. Differences between the experimental and baseline latencies are interpreted as an index of analgesia. Increases in the latency for the mouse to flick its tail are indicative of analgesia, while decreases in tail-flick latency are indicative of hyperalgesia. The tail-withdrawal latency (seconds) was measured before administration of any drug or vehicle. Latency time response was measured 15, 30, 60, 120 min, 4, 6, 8, 10, 12 h after substances administration. Results of tail flick response from each group were calculated as mean ± SEM of the latency time response. The data were presented as ±SEM and significance was tested by SPSS Statistics for Windows version 13.0 and ANOVA method. *p*-Values less than 0.05 are considered statistically significant comparing with those of control group.

2.10.4. Visceral nociceptive experimental model (writhing test)

Male white Swiss mice (20–25 g) were used. Lighting was on a 12 h light/dark cycle (lights on at 6:00 a.m.), with standard laboratory food and tap water freely available, except during the time of the experiments. Before the experiment, mice were placed on a raised wire mesh, under a clear plastic box and allowed 2 h to

acclimate to the testing room. The experiments were carried out on 5 identical lots of 6 groups of 7 white Swiss mice (20–25 g) each: Group 1 (control – INN) indomethacin, Group 2: P(HEMA-co-U)$_{PVA}$ (98.25/1.75), Group 3: P(HEMA-co-U)$_{PVA}$ (98.25/1.75)/INN, Group 4: P(HEMA-co-U)$_{PVA}$ (96.5/3.5), Group 5: P(HEMA-co-U)$_{PVA}$ (96.5/3.5)/INN, Group 6: P(HEMA-co-U)$_{PVA}$ (90/10), and Group 7: P(HEMA-co-U)$_{PVA}$ (90/10)/INN that received the substances orally (using an esogastric device), at the same moment. The model of visceral pain consists of writhing test with acetic acid 0.6% (10 ml/kg). The writhing response is used to screen for both peripherally and centrally acting analgesic activity (Le Bars et al., 2001). The abdominal constriction response is thought to involve, in part local peritoneal receptors. Acetic acid causes algesia by stimulating the release of endogenous substances including H+, K+, 5HT, histamine, prostaglandins, bradykinin, substance P and many other active substances that excite pain nerve endings. Intraperitoneal injection of irritant agent acetic acid causes typical stretching responses named writhes (abdominal constrictions and full extension of hind limb-named writhes) as a pain reaction (Shin et al., 2006). Acetic acid (0.6%) was administered intraperitoneally at the dose of 10 ml/kbw to the lots of each group, according to the following schedule: Lot I – a half an hour, Lot II – 1 h, Lot III – 3 h, Lot IV – 6 h, and Lot V – 12 h after substances administration. Immediately after administration of acetic acid, the animals were placed in glass cages, and the number of writhing per animal was recorded during the following 10 min. Writhing movement was accepted as contraction of the abdominal muscles accompanied by stretching of hind limbs. Comparative with control animals, decreases in the writhes number of mice are indicative of analgesia, while increases in the number of behavioural manifestations are indicative of hyperalgesia (Ness, 1999). Results of writhing test response from each group were calculated as mean ± SEM of the number of writhes. The data were presented as ±SEM and significance was tested by SPSS Statistics for Windows version 13.0 and ANOVA method. *p*-Values less than 0.05 are considered statistically significant comparing with those of control group.

3. Results and discussion

3.1. The analysis of particles size and distribution

The analysis of particles size and distribution is presented in Fig. 3. The synthesized particles varied in size from the micron and submicron range with a relatively broad size distribution. This was not unexpected since it is well known that small changes to the synthesis procedure can result in significant changes to the resulting microspheres.

Thus, it is evidenced the benefit effect of the comonomer presence in reducing the particle dimensions attributed to the intervened intra- and intermolecular physical bonds. Also, it is confirmed the polymeric matrices capacity for coupling the INN: practically small increase of the particle size takes place after preparation of the biocomposites. On the other side, the size distribution of the bioactive microspheres was less broad than for the nonactive batch. This behaviour is attributed to the wrapping and compatibility between polymeric template and INN. These facts are owing to the functional groups of the polymeric template and PVA, as well as to PVA capacity as dispersant, and both polymer structures capacity for coupling INN.

Table 2
The zeta potential and conductivity values.

Sample code	Zeta potential, mV	Conductivity, mS/cm
P(HEMA)$_{PVA}$	−33.4	0.0532
P(HEMA-co-U)$_{PVA}$ (98.25/1.75)	−16.2	0.0266
P(HEMA-co-U)$_{PVA}$ (96.5/3.5)	−14.9	0.0395
P(HEMA-co-U)$_{PVA}$ (90/10)	−12	0.032
P(HEMA)$_{PVA}$/INN	0.98	5.69
P(HEMA-co-U)$_{PVA}$ (98.25/1.75)/INN	−1.58	4.12
P(HEMA-co-U)$_{PVA}$ (96.5/3.5)/INN	−3.5	1.3
P(HEMA-co-U)$_{PVA}$ (90/10)/INN	−8.7	1.14
INN	−3.5	0.5

3.2. Zeta potential

As it is well known zeta potential measurements give information about the overall surface charge of the particles, which is affected by changes in the environment: pH, presence of counter-ions, adsorption of drugs, *etc.* Zeta potential size gives also indications on systems stability, and the surface properties of the colloidal systems, which are critical in determining their drug carrier potential. Table 2 presents the zeta potential and conductivity values for the studied samples. Between the characteristic values of the zeta potential and the conductivity of witness (polymeric samples without INN) and the prepared bioactive compounds there are differences considered the result of the interactions among the active substance and the carrier, respectively the drug encapsulation within the body of the particles.

3.3. SEM studies

SEM morphological features of the studied copolymer networks are illustrated in Fig. 4a–c. Differences between the suprastructure characteristics of the synthesized copolymers surfaces as a function of U amount are proof. Astonishing the size of the network meshes decreases with the increasing of U, suggesting well packed structure sustained by the physical bonds. The shape of the pores is also different when the U amount is increasing. Thus, in case of P(HEMA-co-U)$_{PVA}$ (90/10) copolymer the holes get longer and distinct channels are practically formed (Fig. 4c).

3.4. Swelling studies

Polymers with temperature and pH-sensitivity present increased applicability in their use as template for the controlled

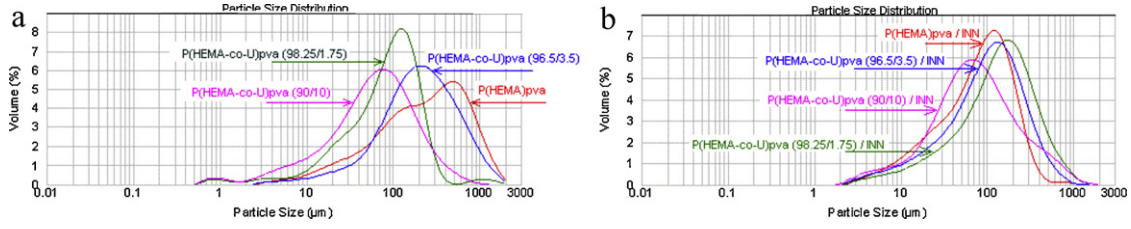

Fig. 3. Particle size distribution of the polymeric matrices (a) and the polymeric template loaded with INN (b).

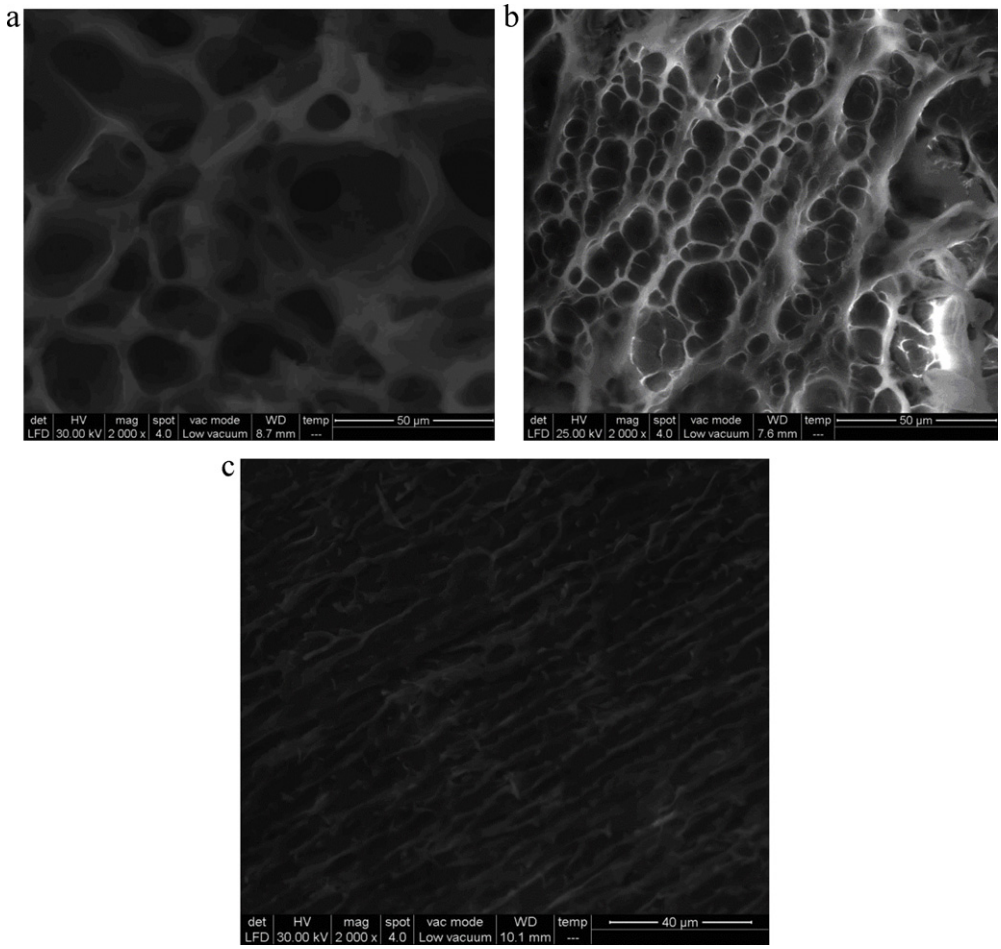

Fig. 4. SEM micrographs of copolymers P(HEMA-co-U)$_{PVA}$ (98.25/1.75) (a), P(HEMA-co-U)$_{PVA}$ (96.5/3.5) (b), P(HEMA-co-U)$_{PVA}$ (90/10) (c) at 2000× magnification.

drug delivery field. This kind of polymeric networks for the controlled delivery presents also different behaviour during swelling in various physiological media.

The swelling studies confirm the influence of the polymer composition and external conditions (change of pH and temperature) on the dynamic and equilibrium swelling properties of the copolymer networks. The pH and temperature sensitivities of P(HEMA-co-U)$_{PVA}$ copolymers are presented in Fig. 5a–f. At 24 °C the P(HEMA-co-U)$_{PVA}$ (98.25/1.75) and P(HEMA-co-U)$_{PVA}$ (96.5/3.5) samples are almost non-pH sensitive (Fig. 5a). On the other hand, with the increase of the temperature these samples becomes pH sensitive, the swelling degree equilibrium (q_e %) decreases with the pH increase (Fig. 6). Such temperature dependence is not so pronounced in the case of P(HEMA-co-U)$_{PVA}$ (90/10) copolymer. The best manifestation of the dual sensitivity at pH and temperature corresponds to the samples with 3.5% U amount in compositions. Also, the maximum swelling degree was registered in case of P(HEMA-co-U)$_{PVA}$ (90/10) copolymer samples, behaviour which is in good agreement with the polymer network formation owing to the comonomer presence.

At lower pH, when the U comonomer is more sensitive and the spiroacetal moieties decompose, the polymer samples uptake more acidic buffer (necessarily also for the decomposition process) and swell more. At higher pH, there are more favourable conditions for the intramolecular hydrogen bonds formation between hydroxyl and spiroacetal groups and, as a consequence, the polymeric networks swell less with a lower swelling degree. The exception is constituted by the P(HEMA-co-U)$_{PVA}$(90/10) sample which, at 37 °C and pH = 7.4, has the maximum swelling degree, behaviour which is also sustained by the morphology of this polymer network variant (SEM morphology – Fig. 4c).

3.5. The evaluation of the homogeneity of drug in polymeric matrices

NIR chemical imaging method (NIR-CI) provides information about the spatial distribution of the components comprising the sample and is used as fast and non-destructive alternative method for the quantification of excipients and active compounds within the polymeric drug delivery systems (Rayn et al., 2008; Reich, 2005; Nita et al., 2010). The opportunity to visualize the spatial distribution of the bioactive substances (drugs) throughout the sample enables the degree of the chemical and/or physical heterogeneity within a given sample to be determined. Fig. 7 show the score images derived from the indomethacin component class. In the score images, the pixels with higher and lower score values are indicated by light gray and dark code colours, respectively. For the polymeric network loaded with indomethacin, the code colour in the most regions of the score images is gray, the intermediate colour between light gray (the code colour of indomethacin) and dark colours (the cod colour of the copolymers). The predominantly gray score images evidences the homogeneity distribution of drug in the polymeric matrix no matter the composition of the polymeric template. At the same time, the code colour evidences the qualitative differences between the polymeric particles loaded with drug comparative with the witness network.

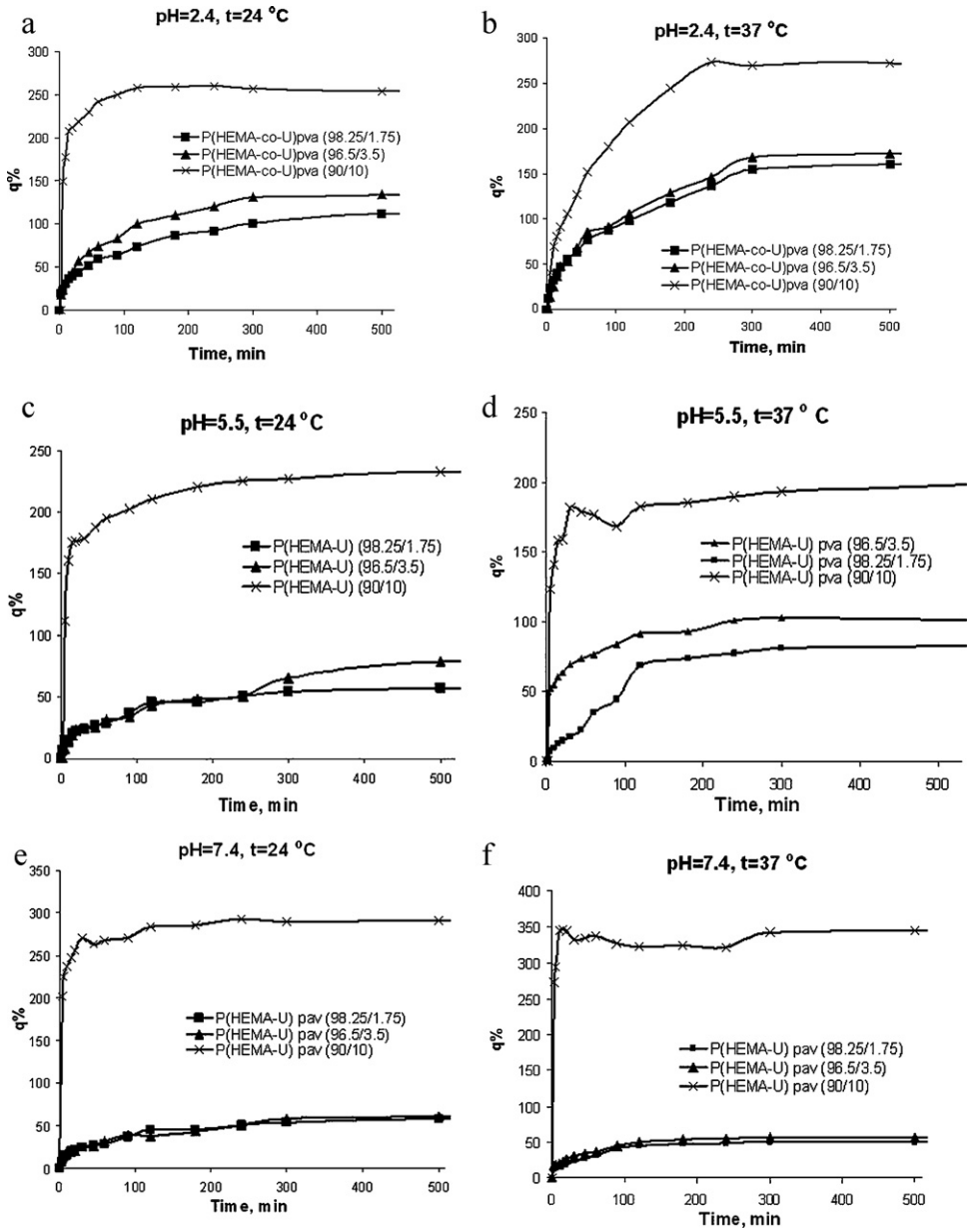

Fig. 5. pH and temperature sensitive swelling behavior of P(HEMA-U) networks.

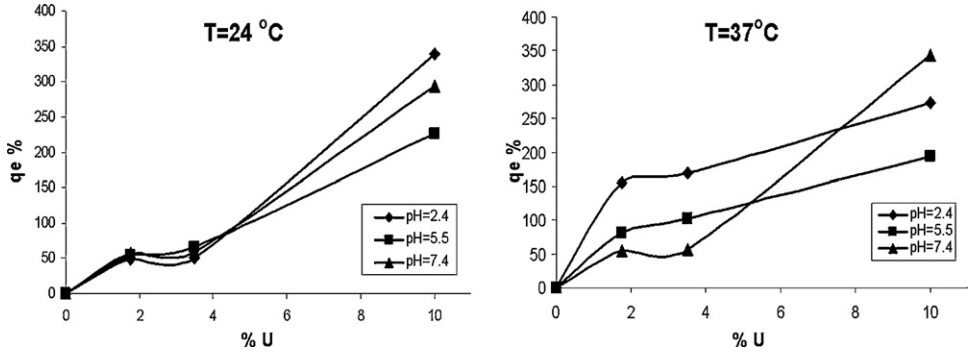

Fig. 6. pH and temperature sensitive swelling behavior of P(HEMA-U) networks as function of U% amount.

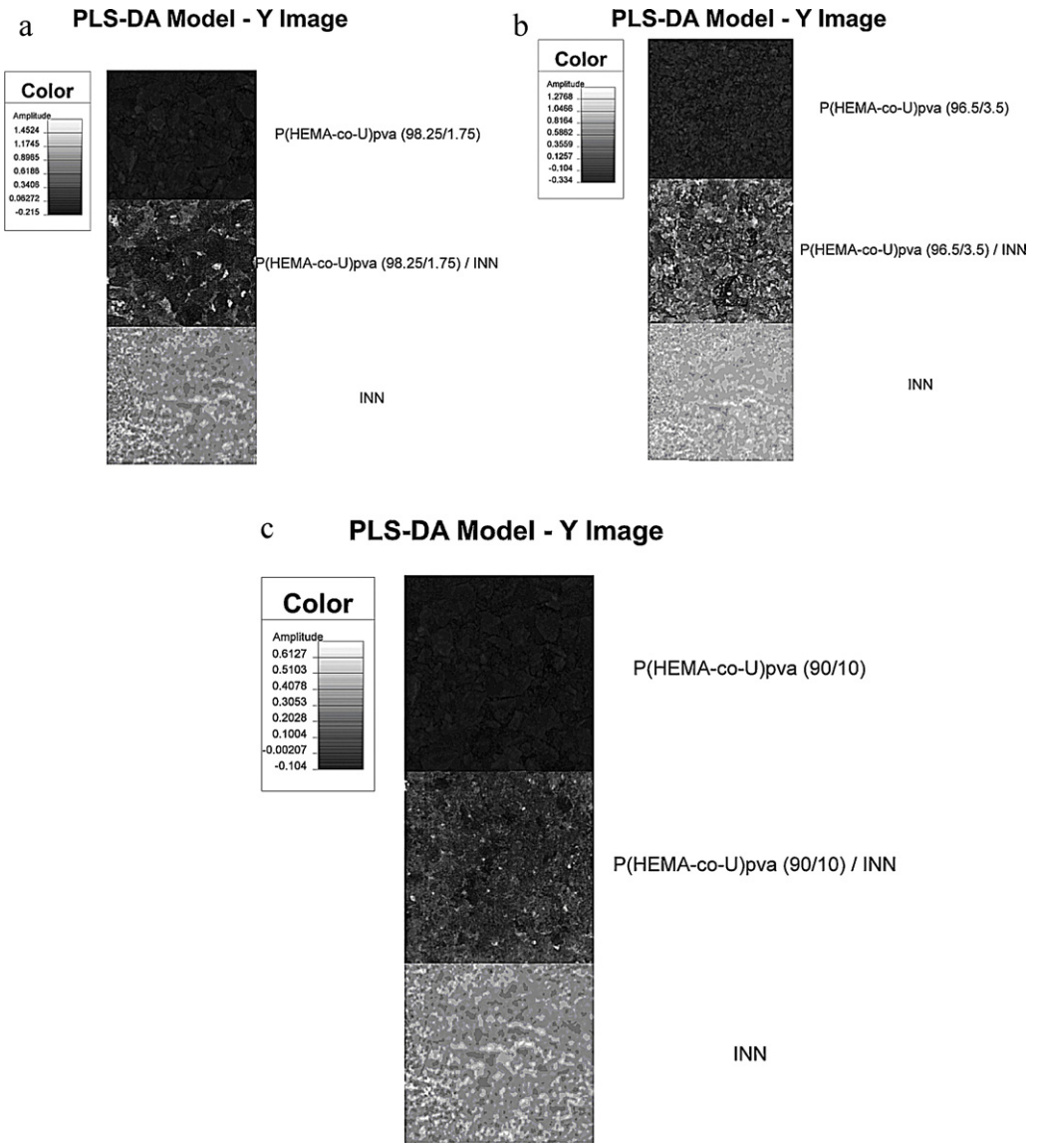

Fig. 7. Score images of the loaded indomethacin onto the polymeric templates.

3.6. In vitro controlled release of indomethacin from polymeric matrices

As it is well known the physicochemical properties of the polymeric template and the loading method determine the mechanism(s) by which the up-taken bioactive compound is released from the networks, as for example if the absorption of drugs is achieved after the inert network formation (which does not form chemical bonds with drug) the diffusion is the major driving force for biocompounds uptake and their release (Peppas & Merill, 1977; Tasdelen et al., 2004; Bajpai et al., 2008; Lin and Metters, 2006).

The INN release profile from P(HEMA-co-U) carriers into a physiological buffer solution (pH = 7.40, at 37 °C) is illustrated in Fig. 8.

All copolymer formulations present sustained release behaviour. The fastest release of INN with a marked "burst" effect corresponds to the $P(HEMA\text{-}co\text{-}U)_{PVA}$ (98.25/1.75) template, behaviour characterized by the rapidly release at the beginning followed by the gradually reached equilibrium value owing to the network structure with reduced crosslinking degree.

The increase of the comonomer amount in the copolymer composition induces a more tardily release rate of the bioactive product, aspect justified by the copolymer structure with the new possibilities for coupling and retain of the drug through physical bonds into the network meshes.

3.7. In vivo study

3.7.1. Biocompatibility tests

Laboratory analyses did not show significant differences of leucocyte formula elements, GOT, GPT and LDH levels, nor immune parameters (OC, PC, and BC) between control mice group (INN) and groups treated with copolymer samples with or without drug (in supplementary material for the article).

3.7.2. Somatic nociceptive experimental model (tail flick test)

Statistical analysis of the results obtained in tail flick test shows that: indomethacin determined a rapidly and statistically significant ($p < 0.01$) increasing of the latency time period of the response effect prolonged 4 h after oral administration of the substance. After this period, the effect of indomethacin was gradually decreased ($p < 0.05$) to 8 h after its administration (Fig. 9). As it was expected, the copolymer samples without INN do not influence the latency time response to thermal noxious stimulus, in tail flick test in

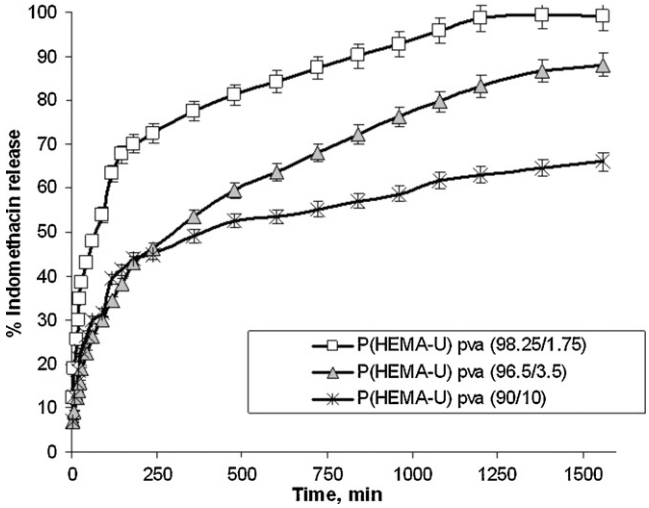

Fig. 8. INN release profile from copolymers template.

mice. Using different systems of indomethacin incorporation we obtained an increasing of the latency time pain reaction in tail flick test, statistically significant compared with indomethacin – the positive control substance administration. Thus, the *in vivo tests* prove the retard release of the drug from the copolymer networks as well as the better results obtained from the template which includes 10% of crosslinker comonomer.

Each point is the mean ± SEM of latency time (seconds) for seven mice.

3.7.3. Visceral nociceptive experimental model (writhing test)

Statistical analysis of the results obtained in writhing test showed that the oral administration of indomethacin resulted in the gradual decrease of the number of writhes, immediately after acetic acid injection (0.5 h – $^{*}p < 0.05$), statistically significant ($^{**}p < 0.01$) comparing with control group, between 1 and 3 h and prolonged 6 h ($^{*}p < 0.05$) after peritoneal noxious stimulation (Fig. 10). In our experimental conditions the witness copolymers (without indomethacin) do not influence the behavioural manifestations due to chemical peritoneal irritation in writhing test in mice. Also, in this visceral pain model the samples loaded with drug do not influence the number of behavioural manifestations produced by chemical peritoneal stimulation.

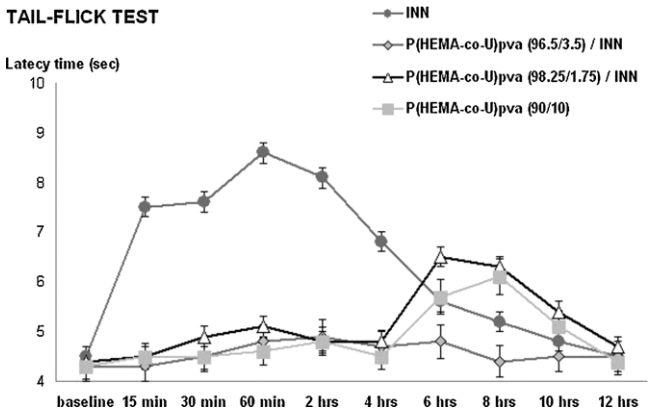

Fig. 9. The latency time period of the response to thermal noxious stimulus in tail flick test.

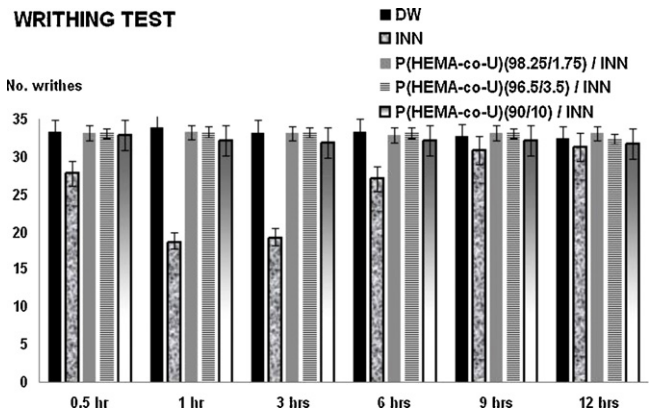

Fig. 10. Effects on acetic acid induced writhing in mice. Data represent mean ± SEM of the number of writhes of 7 animals.

4. Conclusions

Copolymer networks based on 2-hydroxyethyl methacrylate (HEMA) with different amounts of 3,9-divinyl-2,4,8,10-tetraoxaspiro [5.5]-undecane (U) (P(HEMA-co-U) prepared through radical dispersion polymerization and using poly(vinyl alcohol) as protective colloid, were used as template for indomethacin incorporation (INN) as model drug. The copolymers composition was confirmed by Fourier-transform infrared spectroscopy. Also, the porous microstructure of samples was evidenced by scanning electron microscopy. The swelling kinetic studies indicate that polymer network properties are significantly dependent on the comonomer amount as for example inducing pH and/or temperature sensitivity. The highest swelling degree was registered in case of P(HEMA-co-U) (90/10) copolymer. The release behaviour depends too by the copolymer composition the increasing of the comonomer amount attenuating the drug release. At the same time, the *in vivo studies* did not show significant differences of leucocyte formula elements, GOT, GPT and LDH levels, nor immune parameters (OC, PC, and BC) between control mice group (INN) and groups treated with copolymer samples with or without drug attesting the biocompatibility of polymer samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2012.01.028.

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