Poly(HEMA-co-MOEP) MICROPARTICLES: OPTIMISATION OF THE PREPARATION METHOD AND *IN VITRO* TESTS

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În ultimii ani 2-hidroxietil metacrilatul (HEMA) a fost unul dintre cei mai studiaţi monomeri sintetici pentru utilizare în domeniul biopolimerilor. Alături de acesta, metacriloiloxietil fosfatul (MOEP) prezintă proprietăţi care îl fac interesant în ceea ce priveşte testele de mineralizare.

În această lucrare se prezintă o aplicaţie a copolimerului acestora, p(HEMA-co-MOEP), în sisteme pentru eliberare controlată. În acest scop, au fost sintetizate şi s-a optimizat metoda de obţinere a microparticulelor, care, mai departe, au fost caracterizate prin SEM, FTIR, analiză elementală, grad de gonflare şi au fost supuse testelor de citotoxicitate.

In the last few years 2-hydroxyethyl methacrylate (HEMA) has been considered one of the most interesting synthetic monomers for general biopolymer purposes. In addition, methacryloyloxyethyl phosphate (MOEP) has also different useful properties, which made it interesting for mineralization tests.

In this paperwork it is presented a possible application of their copolymer, p(HEMA-co-MOEP), as drug delivery system. In this respect, we have synthesised and we have optimised the method for the obtaining of the microparticles which we further characterised by SEM, FTIR, elemental analysis, swelling rate, and we submitted to cytotoxicity assays.

Keywords: copolymer of 2-hydroxyethyl methacrylate and methacryloyloxyethyl phosphate, microparticle, elemental analysis, cytotoxicity tests.

1. Introduction

The range of bioactive compounds emerging as potential drug candidates, together with those currently used in research and development, continue to provide major challenge for efficient drug delivery and targeting. Various

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strategies for formulations design using different chemicals as formulation excipients are available, and numerous materials are being considered and developed to provide specific functionalities in the design of medicines [1]. A number of approaches to formulation and drug delivery highlight their need and value [2].

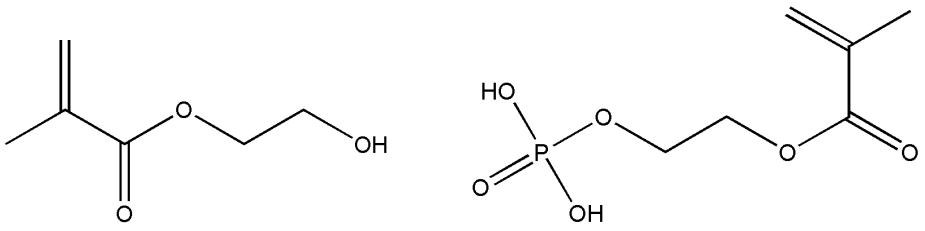
It has been a constant ambition of scientists to optimise drug delivery systems in order to provide a defined dose, at a chosen rate and selected time to a targeted biological site. While improvements in drug delivery over recent years are impressive, there is still some way to go in fully achieving these objectives. Key issues requiring continuing research and study range from fundamental understanding of the biosystems and targets and basic characterisation of novel classes of bioactive agents to the development of intelligent materials which provide required excipient or carrier properties to achieve modulated and targeted drug delivery [1,3].

The types of carrier materials used, the drug substance and the biological environment for drug delivery influence the mechanisms of drug release. The complex matrix of variables and interactions which influence and ultimately control drug release will clearly continue to provide major challenges for scientists working in drug delivery and targeting [2].

Functionalisation confers a wide array of interesting properties such as new and improved characteristics, bioadhesive properties, and it prevents aggregation of the microparticles, imparts biostability and solubility, reduces toxicity, and provides site-specific delivery. This makes the system an intelligent tool for diagnostics, prognostics, and controlled and sustained delivery of protein, peptide, pDNA, and other therapeutic agents to specific targets (tissue, cell, and intracellular). Various types of functional systems, such as carbon nanotubes, quantum dots, polymeric micelles, dendrimers, metallic nanoparticles, and liposomes, are being extensively explored. However, high tissue accumulation of nonbiodegradable nanoparticles has caused toxicity problems and rendered them as not-so-popular therapeutic and diagnostic systems. The toxicity and safety of nonbiodegradable nanoparticles are subject to present research [3-5].

Polymeric nanoparticles have offered attractive alternative modules due to biocompatibility, nonimmunogenicity, nontoxicity, biodegradability, simple preparation methods, high physical stability, possibility of sustained drug release, and higher probability for surface functionalisation [1, 3].

In the last few years there have been presented opposite opinions concerning the copolymer system p(HEMA-co-MOEP) obtained from 2-hydroxyethyl methacrylate (HEMA) and methacryloyloxyethyl phosphate (MOEP), shown in Fig. 1.



HEMA MOEP

Fig. 1. Chemical structures of the 2 comonomers used, HEMA and MOEP

The incorporation of negatively charged groups, such as phosphate group, into the structure of biopolymers has been widely proved to be a method for inducing mineralization [6-8] or, on the contrary, in a more recent paper, that this system presents an inhibitory effect of the phosphate ions on the deposition of calcium and phosphate phases on methacrylic-based copolymers [9].

The reason for choosing p(HEMA-co-MOEP) as a possible drug delivery matrix is based exactly on this idea. In this respect, we have synthesised copolymeric microparticles that we further analysed for potential biomedical application.

2. Experimental

2.1 Materials

The general method used for the synthesis of HEMA-containing microparticles was described previously [10], and improved by our group [11].

We employed 2-hydroxyethyl methacrylate (HEMA), methacryloyl-oxyethyl phosphate (MOEP), benzoyl peroxide (BPO), 2-butanol, and ethylenglycol dimethacrylate (EGDMA), all these reagents being purchased from Sigma-Aldrich. The solvents, toluene and diethyl ether, were provided from Chimopar, Bucharest, and the star copolymer styrene-butadiene (pBuSt) and polybutadiene (pBu), as stabilising agents, from ICECHIM Bucharest.

The initiator BPO was purified by recrystallisation from ethanol and the comonomers were distilled under reduced pressure before use.

MTT, 3-(4,5-dimethyl 2-thiazolyl)-2,5-diphenyltetrazolium bromide, the dye used for cellular viability test, was provided from Fluka.

2.2 Synthesis of the polymers

We introduced into a three-neck reactor a solution of stabilising agent (w/v) in toluene (95:5 (w/w) vs. monomer). At 40°C and under stirring we slowly introduced the 2-butanol (firstly, we chose a 40:60 (v/v) ratio of solvent vs. non-solvent solution). Separately, we prepared a solution containing the comonomers (HEMA:MOEP = 95:5 and 90:10 (mol/mol)), initiator (BPO) (5 x 10^{-3} mol/L in the solution), and cross-linking agent (EGDMA) (2% vs. comonomers). We added dropwise this second solution to the first one, under mechanical stirring, and then we increased the temperature (to 75°C) and the stirring rate (to 800 rpm). Polymerisations were performed in a water-bath and under nitrogen atmosphere. An optimal result and a high conversion was noticed after 5 hours of reaction.

The copolymers obtained were washed repeatedly with toluene and with diethyl ether, in order to remove any traces of unreacted monomer and other organic residue with low molecular weight. The microparticles were then dried in the oven at 37°C for 24 hours. For the homogeneous dispersion of the microparticles in water, we first dispersed them into a saline solution. Afterwards we transferred them in an aqueous solution.

2.3 Characterisation of the microparticles

In order to characterise the microparticles obtained by heterogeneous polymerisation, we used Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Elemental Analysis. We also determined the swelling rate and the cytotoxic effect.

SEM was performed on a Philips XL30 - ESEM Turbo Molecular Pump (TMP), at 20 keV. The samples were first carbon-coated.

The FTIR analysis was performed with a Shimadzu spectrophotometer. All samples were mixed and ground with spectroscopic grade potassium bromide prior to being placed in the sample cell, and the diffuse reflectance spectra were scanned over the range of 400-4000 cm^{-1}.

Elemental analysis was performed in order to establish the chemical structure of the copolymer, using a LECO CHN2000, MI, USA instrument.

Swelling rate was carried out on samples of copolymers obtained by bulk polymerisation, in triplicate, using a solution of 9 g/L NaCl in distilled water, for three days at 37°C.

2.4 Biocompatibility tests

Polymers biocompatibility was verified by testing the *in vitro* cytotoxicity. For the *in vitro* tests, the microparticles were first exposed to UV light (long wave UV, 360 nm, 12W) for 8 hours.

It was used a murine fibroblasts L929 cell line, which was cultivated in culture medium (DMEM), supplemented with calf fetal serum and antibiotics. The cells culture was incubated in thermostat at 37°C, for a few days in the presence of 5% CO_2 and was examined daily during three days.

The cells were microscopically examined for detecting cytotoxicity visible signs, cellular lysis or cellular components dimensions and conformation.

For cytotoxicity evaluation, besides microscopically examination, it was also used the cellular viability.

3. Results and discussion

Efficient drug delivery remains an important challenge in medicine: continuous release of therapeutic agents over extended time periods in accordance with a predetermined temporal profile; local delivery at a constant rate to the tumour microenvironment to overcome much of the systemic toxicity and to improve antitumour efficacy; improved ease of administration, and increasing patient compliance required are some of the unmet needs of the present drug delivery technology. Microfabrication technology has enabled the development of novel controlled-release microchips with capabilities not present in the current treatment modalities [12].

The goal of every drug delivery system is to deliver the precise amount of a drug at a pre-programmed rate to the desired location in order to achieve the drug level necessary for treatment.

We synthesised p(HEMA-co-MOEP) microparticles with 5 and 10 mole % MOEP in the mixture by heterogeneous polymerisation and we characterised the products obtained by several specific methods.

We have verified the behaviour of several ratios solvent/non-solvent using pBu and we have compared pBu with another stabilizing agent, pBuSt, in several ratios, in order to obtain neat and homogenous polymeric particles.

Table 1 and Table 2 present the results concerning the formation of individual particles with specific diameter in case of using 5% and 10% MOEP, respectively.

Table 1

Experimental data for the synthesis of p(HEMA-co-MOEP 5%) microparticles

No. sample	Toluene/2-butanol (mL/mL)	Stabilising agent (g/100 mL toluene)	Results
1.	40/60	0.25 pBu	Good, particles of 1.5-2.5μm
2.	40/60	0.25 pBuSt	Good, particles of 1.5-2.5μm
3.	40/60	0.5 pBu	Good, particles of 1.5-2.5μm
4.	40/60	0.5 pBuSt	Good, particles of 1.5-2.5μm
5.	35/65	0.25 pBu	Agglomerated particles
6.	35/65	0.25 pBuSt	Agglomerated particles
7.	35/65	0.5 pBu	Agglomerated particles
8.	35/65	0.5 pBuSt	Agglomerated particles
9.	45/55	0.25 pBu	Good
10.	45/55	0.25 pBuSt	Good
11.	45/55	0.5 pBu	Good
12.	45/55	0.5 pBuSt	Very good, narrow distribution and dimension of the particles

Table 2

Experimental data for the synthesis of p(HEMA-co-MOEP 10%) microparticles

No. sample	Toluene/2-butanol (mL/mL)	Stabilising agent (g/100 mL toluene)	Results
1.	45/55	0.25 pBu	Particles not well-defined
2.	45/55	0.25 pBuSt	Particles not well-defined
3.	45/55	0.5 pBu	Particles not well-defined
4.	45/55	0.5 pBuSt	Particles not well-defined
5.	40/60	0.25 pBu	Agglomerated particles
6.	40/60	0.25 pBuSt	Agglomerated particles
7.	40/60	0.5 pBu	Agglomerated particles
8.	40/60	0.5 pBuSt	Agglomerated particles
9.	42/58	0.25 pBu	Good
10.	42/58	0.25 pBuSt	Good
11.	42/58	0.5 pBu	Good
12.	42/58	0.5 pBuSt	Very good, narrow distribution and dimension of the particles

The FTIR p(HEMA-co-MOEP) spectra, in comparison to a standard of the pHEMA, contained the peaks at 1240 (O=P–O^-), 1081 (P–O–C), specifically for phosphate-containing organic compounds.

The elemental analysis gave results which led us to the conclusion that the molar fractions in the copolymer obtained are close to the expected results, meaning that the MOEP reacted almost completely with HEMA (Table 3).

Table 3

The chemical structure of the copolymers obtained given by elemental analysis

MOEP in the comonomer mixture (%)	Elemental analysis (theoretical values)		Elemental analysis (obtained values)		MOEP in the copolymer (%) (calculated*)	EGDMA in the copolymer (%) (calculated*)
	C (%)	H (%)	C (%)	H (%)		
5	55.53	7.77	55.45	7.75	4.8	1.8
10	54.46	7.63	54.48	7.64	9.7	1.9

*from elemental analysis

The swelling rate of the obtained copolymers is very useful in the field of the controlled release, being noticed the fact that the microparticles swell to a certain extent (Fig. 2). Also, in comparison with pHEMA microparticles, these swell far less, which gives us information about the links created in the copolymer.

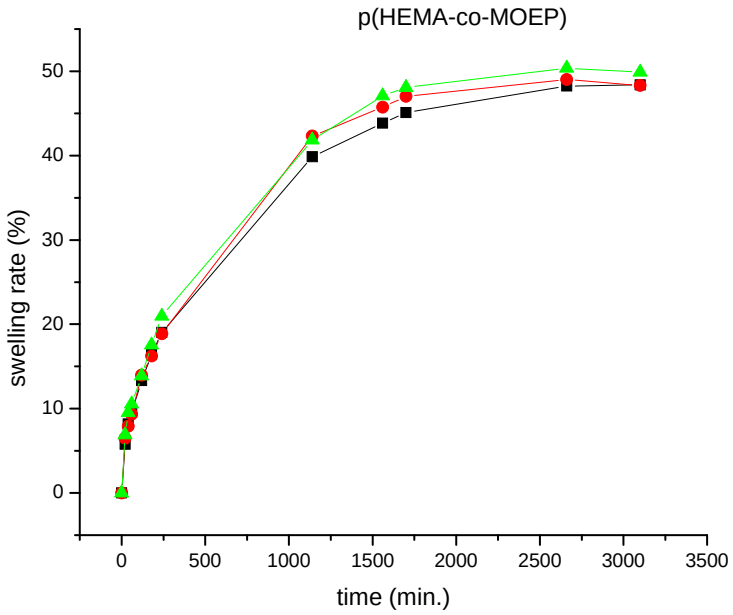


Fig. 2. Swelling rate of p(HEMA-co-MOEP).

SEM microphotographs confirmed the homogenous distribution of the microparticles obtained (Fig. 3).

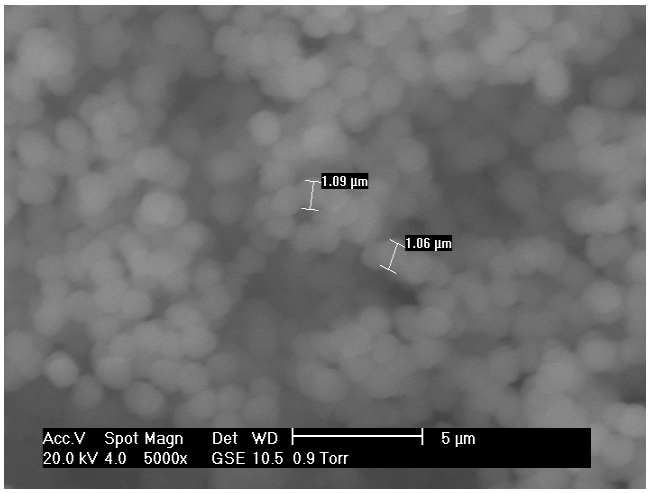


Fig. 3. Microparticles of p(HEMA-co-MOEP) obtained through heterogeneous polymerisation.

The cells were examined by inversion microscopy, before and after the incubation with the samples. There were not observed cytotoxic effects, the morphologic characteristics and the adherence being similar for the cells incubated in the presence or in the absence of the samples. In the same time, after the addition of the dye, MTT, both the cells incubated with the samples and those untreated reduced the MTT and formed formasan crystals (Fig. 4-6).

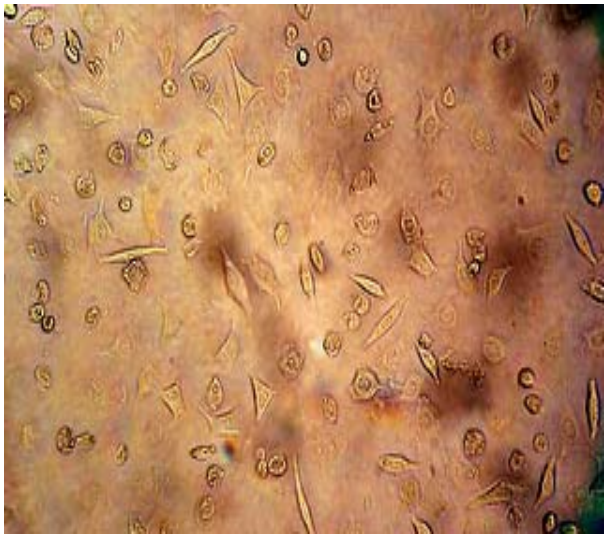
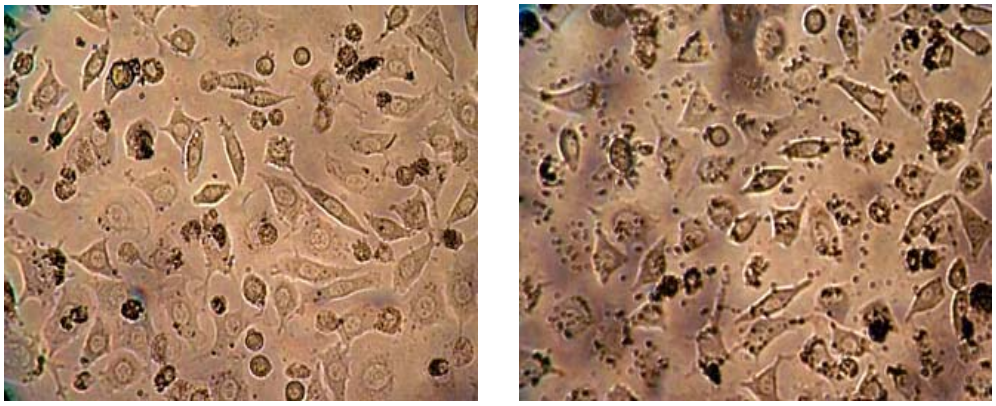


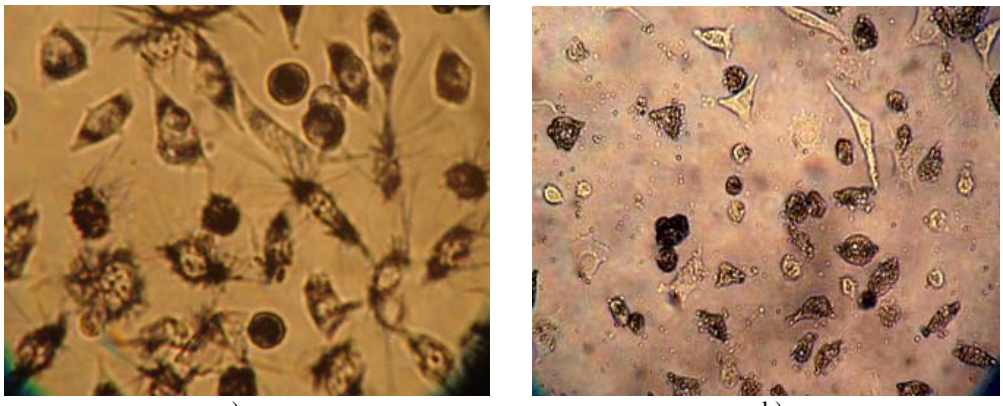
Fig. 4. Microscopic image of L929 cells in culture, in the absence of samples and before the addition of MTT



a) b)

Fig. 5. Microscopic image of L929 cell line in culture, incubated for 24h with:
a) copolymer with 5% MOEP, b) copolymer with 10% MOEP.

After the solubilisation of formasan crystals, we measured the optic density at 570 nm and we calculated the cellular viability in percentage versus the witness sample (cells incubated in the same conditions and volume).



a) b)

Fig. 6. Microscopic image of L929 cells in culture after incubation for 24h and addition of MTT in the presence of: a) copolymer with 5% MOEP, b) copolymer with 10% MOEP.

The *in vitro* tests of cytotoxicity and viability made on fibroblasts murine L929 line cell gave adequately results for all compositions obtained.

4. Conclusions

The need and the growing interest in polymers as biomaterials have led to the synthesis of new polymers with a variety of physico-chemical properties. Biomedical application of such materials not only depends on their physical properties but also on biocompatibility and biodegradability.

We obtained 1-μm microparticles, with a narrow distribution as dimension and surface. The elemental analysis proved a statistical binding of the comonomers, within the limits needed for functionalising the polymer. The swelling rate and the *in vitro* tests confirmed our belief that the present copolymer can be used as drug carrier, with satisfactory results. Both compositions used are biocompatible and non-toxic for the cells.

R E F E R E N C E S

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