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Evaluation of in vitro controlled release polymeric material loaded with poly(styrene–alt–maleic anhydride) as anticancer and antimicrobial

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Abstract Delivery of drugs by new technologies is a highly topical challenge of polymer chemistry. This report describes the preparation of antibacterial activated poly(styrene–alt–maleic anhydride) (PSMA) in which aminophenyl antibiotic is covalently bonded onto the polymeric framework. The prepared copolymers were purified and their structures characterized by FT-IR spectroscopy. In vitro drug releasing was performed under specific conditions to investigate the influence of pH on the releasing rate. Poly(Styrene co-maleic anhydride) (SMA) is a synthetic copolymer with interesting chemical and biological properties. The anhydride group can also easily react with hydroxyl and amine containing compounds viz aminobenzoic acid at 90 ^{0}C to obtained modified SMA polymer with high yield. The Tg of the polymer were higher than that of SMA due to H-bonding interaction between the polymer chains. The detailed structural study of polymer complexes was done using method of molecular spectroscopy.The antiproliferative activity of poly(styrene–alt–maleic anhydride) released from polymer towards MCF-7 (human breast cancer) and BALB/3T3 (murine fibroblast) cell lines was evaluated. Moreover, antimicrobial potency of the released drug was tested against (*Bacillus subtilis* and *Escherichia coli* as a Gram positive and Gram negative bacterial pathogen, while *Candida albicans* was used as a pathogenic yeast.

Keywords poly (styrene–alt–maleic anhydride), antiproliferative activity, MCF-7, BALB/3T3, antimicrobial potency

Introduction

Polymers have played a major role in the development of controlled release systems. Amphiphilic graft copolymers have been extensively studied due to their wide applications in pharmaceuticals [1]. Organic functional groups that decompose slowly in an aqueous environment have attracted wide interest as potential linkers for the covalent conjugation of drugs to polymers for a range of drug delivery applications [2]. Styrene and maleic anhydride are known to produce alternating copolymers Poly(styrene-co-maleic anhydride) or styrene maleic anhydride (SMA) copolymers have gained interest as a functional polymer due to the reactivity of the maleic anhydride (MA) groups in the backbone [3]. Poly(styrene-co-maleic anhydride) copolymers (SMA) is an inexpensive commercially available copolymer having reactive groups in the main structure that can be easily modified chemically to yield a

variety of very useful compounds which could be used in multiple of applications [4]. Poly(styrene-alt-maleic anhydride) (SMA) is one of the most appropriate intermediate polymers that can be converted into bioactive polymers as its succinic anhydride units can react with any bioactive agents with low molecular weights containing amino and hydroxyl groups [5]. Antimicrobial polymers, also known as polymeric biocides, is a class of polymers with antimicrobial activity, or the ability to inhibit the growth of microorganisms such as bacteria, fungi or protozoans [6]. This synthetic method involves first synthesizing the polymer, followed by modification with an active species. The following kinds of monomers are usually used to form the backbone of homopolymers or copolymers: vinylbenzyl chloride, methyl methacrylate, 2-chloroethyl vinyl ether, vinyl alcohol, maleic anhydride [7]. The polymers are then activated by anchoring antimicrobial species, such as phosphonium salts, ammonium salts, or phenol groups via quaternization, substitution of chloride, or hydrolysis of anhydride [8]. Poly(styrene-alt-maleic anhydride)s (PSMAs) have excellent properties such as high thermal stability, tuneable solubility and adjustable compatibility when blending with styrenic polymers and polar polymers is desired [9]. The large number of anhydride groups in the alternating PSMA copolymer offers the possibility for interaction with polar groups such as –OH, -NH_2, etc., present on all kinds of polar surfaces, which is expected to give good adhesion to the surface of interest. Clinical applications of PSMA have been well studied and reported [10]. Various polymeric prodrugs have been prepared using PSMA with enhanced physicochemical, pharmacokinetic and pharmacodynamics properties [11].

Antimicrobial agent-bound polymers usually exhibit their microbial activities by slowly releasing the active agents through hydrolysis, but some other polymers are antimicrobial by themselves [12]. Phenols release the intracellular constituents of the cell by targeting cell membrane leading to cell lysis, hence they are known as biocidal agents. Phenols are considered as environmental safe antimicrobialagents and also as algaecide, bactericide and fungicide [13].

The reason for choosing anhydride containing polymers is that anhydride groups can be used for different reactions such as imidization, ammonolysis and cross-linking with diamines or dihydrazides. Anhydride groups of PSMA can easily react with hydroxyl, sulfhydryl or amine groups. Modification of biologically active compounds with polymers is one of the methods for altering and controlling their biodistribution and, very often, toxicity. Moreover, anchoring the p-aminophenyl prepared SMA to obtain the SMA–AP conjugate. AP was chosen as an active agent since phenols are known to be antimicrobial [14]. Amino drugs containing –NH_2 were linked up with PSMA by amide bond formation, and then, hydrolysis reactions were carried out in buffer solution (pH 1.3) at 37 °C, as the human gastric simulated conditions [15]. In addition, the hydrophilic surface of SMA micelles is comprised of carboxyl terminated maleic acid groups, allowing easy surface modification or conjugation with targeting moieties [16].

In this work, the chemical loading of the aminophenyl antibiotic on poly(styrene-alt-maleic anhydride) (PSMA) was performed by direct grafting of the aminophenyl on PSMA backbone. Also, the physical loading of aminophenyl on amine modified PSMA was carried out. In vitro, drug releasing from the antibiotic loaded polymers was investigated and compared with each other.

Materials and Experimental Techniques

Materials

Styrene was purchased from Merck and was purified by distillation in reduced pressure. Maleic anhydride, benzoyle peroxide (BPO), tetrahydrofuran (THF), p-aminophenyl (AP) and triethylamine (TEA) were purchased from Merck and used without further purification.

Experimental Techniques

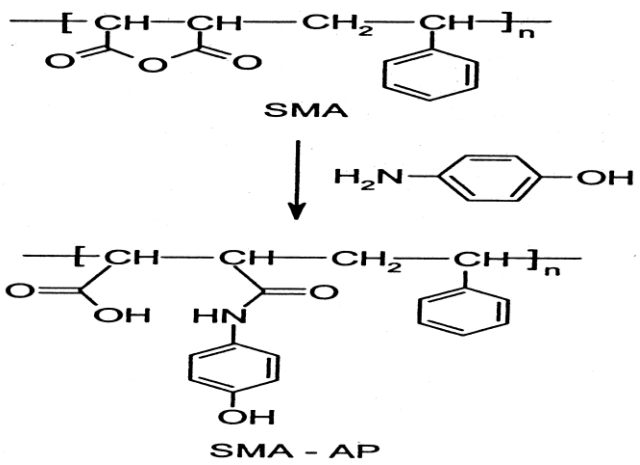
Synthesis of Poly(styrene-alt-maleic anhydride)

The poly(styrene-alt-maleic anhydride) was prepared through a thermally initiated free-radical polymerization of styrene and maleic anhydride according to the literature method [9]. Briefly, equimolar amounts of styrene and maleic anhydride were combined in a 100 mL round bottomed flask with benzoyl peroxide (BPO) in dried

tetrahydrofouran (THF). The mixture was degassed by nitrogen to remove oxygen from the reaction vessel prior to polymerization. Polymerizations were carried out for 6 h in 80°C by stirring under nitrogen atmosphere. The polymerization product was diluted in THF followed by dropwise addition into a 100-fold excess (v/v) of cold diethyl ether to precipitate pure PSMA polymer, which was then filtered and dried under vacuum at room temperature as shown in Scheme 1.

Preparation of SMA copolymer with AP

SMA was reacted with AP in the presence of triethylamine to yield SMA–AP with an isolation yield of 85%. The SMA copolymer and THF (50 mL) were poured in a three neck flask equipped with condenser, magnetic stirring bar and ultrasonic irradiation probe. TEA was charged in the flask too. Reaction mixture was refluxed for 5 h. and dried by vacuum oven at 60° C for 24 h. (yield 75 %).These amphiphilic graft copolymers readily formed core shell type nanoparticles of size < 100 nmas shown in Scheme 1.



Scheme 1

Cells

Cell lines: MCF-7 (human breast cancer) and BALB/3T3 (murine fibroblast) are being maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. All cancer cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA) and are being maintained in the Institute of Immunology and Experimental Therapy (Wrocław, Poland). MCF-7 cells were cultured in Eagle medium (IIET, Wroclaw, Poland) supplemented with 2 mML-glutamine, 10% fetal bovine serum, 8 ug/mL of insulin and 1% mem non-essential amino acid solution 100x (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). BALB/3T3 cell line was cultured in DMEM (Gibco, UK) supplemented with 2 mML-glutamine, 10% fetal bovine serum (GE Healthcare, Logan, UT, USA). All culture media were also supplemented with antibiotics: 100 μg/ml streptomycin (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and 100 units/ml penicillin (PolfaTarchomin SA, Warsaw, Poland). All cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere.

Instruments

Fourier transforms infrared (FTIR) spectroscopy

The FTIR spectra of samples were recorded using a Jascow FTIR 430 (Japan) instrument.

Transmission electron microscope

Transmission electron microscopy (TEM) of the prepared samples was examined with a JEOL JX 1230 technique with a microanalyzer electron probe. This technique was used to determine the particle size of the investigated polymer.

Particle size distribution and zeta potential measurements

The particle size distribution and zeta potential of the prepared samples were measured, using Nicomp TM 380 ZLS size analyzer, USA. Leaser light scattering was used at 170 °C in case of particle size detection where zeta potential was measured at 18 °C. The micelles had a zeta potential of -35 ± 3 mV.

Differential scanning calorimetry DSC

Differential scanning calorimetry DSC131 evo (SETARAM Inc., France) was used to perform the differential scanning calorimeter analysis, Nanomaterial Investigation laboratory, Central Laboratories Network, National Research Centre (NRC) of Egypt. The instrument was calibrated using the standards (Mercury, Indium, Tin, Lead, Zinc and Aluminum). Nitrogen and Helium were used as the purging gases. The test was programed including the heating zone from 25°C to 250°C with a heating rate 10 °C / min. The samples were weighted in Aluminum crucible 120 ul and introduced to the DSC. The thermogram results were processed using (CALISTO Dataprocessing software v.149).

An anti-proliferative assay in vitro

24 hours before addition of the released drugs, the cells were plated in 96-well plates (Sarstedt, Germany) at density of $1x10^4$ cells per well. The assay was performed after 72 hours exposure to varying concentrations of the released drugs. The *in vitro* cytotoxic effect of all released drugs was examined using the SRB assay.

Cytotoxic test SRB

The details of this technique were described by Skehan *et al* [17]. The cells were attached to the bottom of plastic wells by fixing them with cold 50% TCA (trichloroacetic acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 hour and then washed five times with tap water. The cellular material fixed with TCA was stained with 0.4% sulphorhodamine B (SRB, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 minutes. Unbound dye was removed by rinsing (fife times) in 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (POCH, Gliwice, Poland) for determination of the optical density (λ = 540 nm) in Synergy H4 multi-mode microplate reader (BioTek Instruments USA).

Results

The FT-IR spectra clearly indicated that the absorption peaks of anhydride carbonyl groups (1850 and 1780 cm^{-1} in Fig. 1a) disappeared completely, as shown in Fig. new peaks emerged in the range of 1665– 1709 cm^{-1}, which probably correspond to carboxyl and amide groups. This result indicates that the succinic anhydride rings in the SMA– AP backbone were almost completely opened by the reaction with AP. TEM image as in figure 2 show that the particle size range in the nanoparticles from 20 to 50 nm. Fig 2.

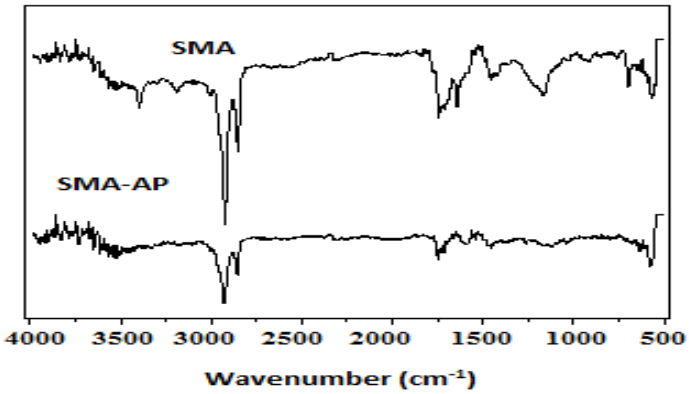


Figure 1: FT-IR spectra of SMA and SMA-AP

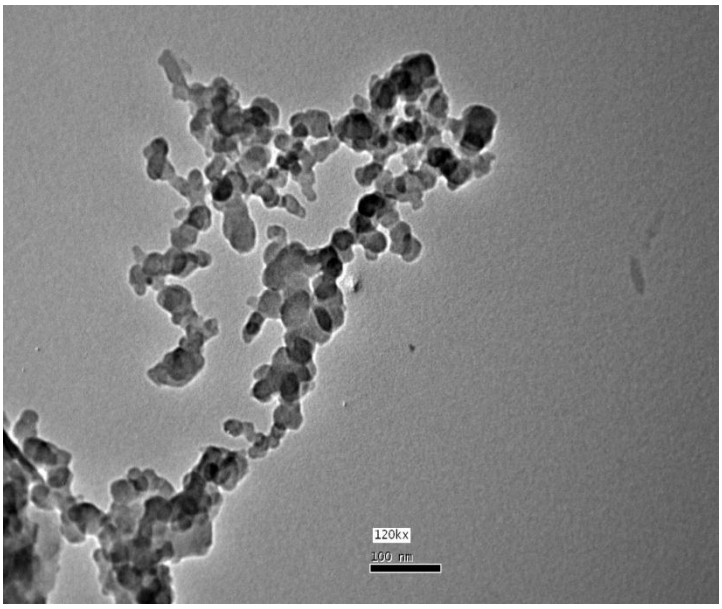


Figure 2: TEM image of SMA

DSC analysis

The DSC thermogram showed that the glass transition temperatures of polymers were higher than that of intermediate polymers due to hydrogen-bonding interactions. The Tg of the polymer were higher than that of SMA due to H-bonding interaction between the polymer chains. The glass transition temperature (Tg) of SMA was measured to be about 202 ^{o}C, which is very close to a previously reported value (Tg 201 ^{o}C). In contrast, the Tg of SMA-AP was 251 and 253 ^{o}C, respectively. The increased Tg of the new polymers probably resulted from enhanced molecular interactions due to H-bonding, as mentioned above Fig. 3

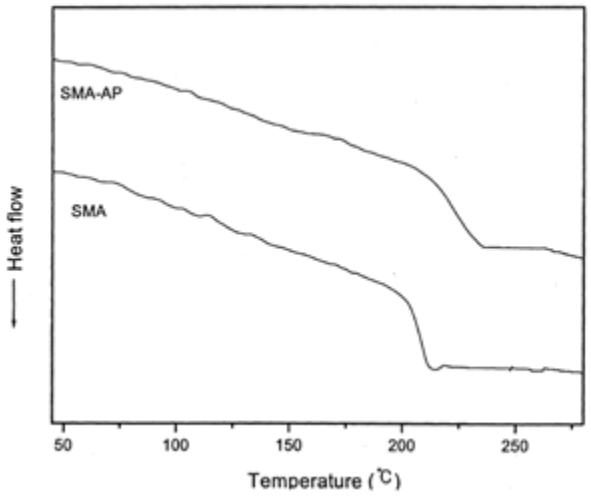


Figure 3: DSC Thermograph for SMA-AP polymer

Zeta Potential Study

Zeta potential is a significant parameter representing the stability of drug particles measured, i.e. not change in chemical structure or physical modification of drugs. The results represent as the zeta potential for the copolymer which modified with aminophenol was -16.58 mv.

The results were calculated as the IC_{50} (inhibitory concentration 50%), the concentration of released drug which inhibits 50% of the cells population. IC_{50} values were calculated for each experiment separately and mean values ±

SD are presented in the table. Each released drug at each concentration was tested in triplicate in a single experiment, which was repeated 3–5 times. The results of the studies on antiproliferative activity of released drugs are summarized in Table 1 and 2.

Table 1: Value of IC_{50} [µg/ml]

Cancer cell line MCF-7	
	Proliferation inhibition [%] ± SD (n=2)
Release time (days)	**4-aminobenzoic from polymer**
1	85.63 ± 1.5
14	93.19 ± 2.4
21	90.73 ± 3.1
28	88.96 ± 2.5

Table 2: Value of IC_{50} [µg/ml]

Normal cell line BALB/3T3	
	Proliferation inhibition [%] ± SD (n=2)
Release time (days)	**4-aminobenzoic from polymer**
1	78.97 ± 1.3
14	84.29 ± 2.0
21	86.54 ± 2.7
28	84.13 ± 3.4

Antimicrobial Activity

Chemical compounds were individually tested against (Bacillus *subtilis* ATCC 6633 *and E. coli* ATCC2592) as a Gram positive and Gram negative bacterial pathogen respectively. *Candida albicans* NRRL Y-477 was tested as a pathogenic yeast. 1mg of SMA polymer was added to test tubes containing 5 ml of nutrient broth (NB) for bacteria and potato dextrose broth (PDB) for yeast and received 100 µL of suspension containing 1 $x10^8$ CFU/mL of pathological tested bacteria and 1 $x10^6$ CFU/ml of yeast. The inculcated tubes were then incubated for at 37 °C a sample was taken at interval of time every 1 week to study the release of the (antimicrobial compound). After incubation time, a microbial growth was measured against un-inoculated sterilized medium. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

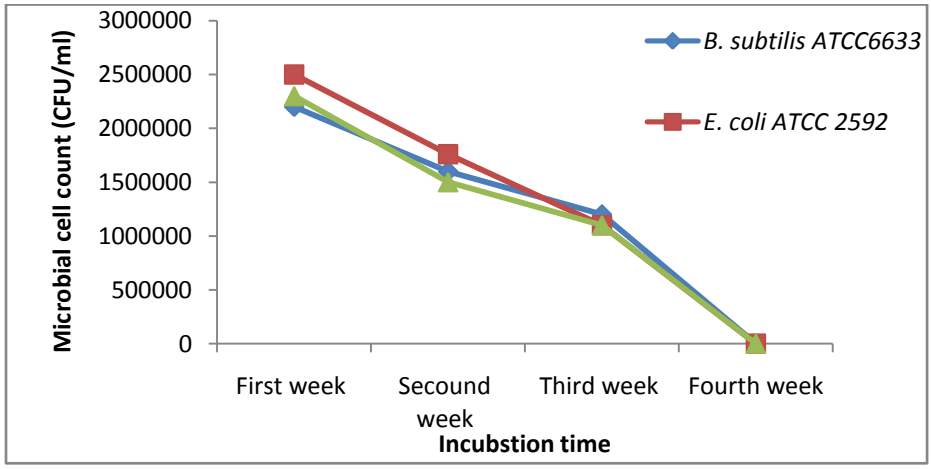


Figure 4: Growth inhibition of pathogenic bacteria along the incubation period with the drug

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