Synthesis, Structural Characterization, and Water Solubility of a Novel Modified Poly(maleic anhydride-*co*-vinyl acetate)/ Acriflavine Conjugate

Yeni Modifiye Poli(maleik anhidrid-*ko*-vinil asetat)/Akriflavin Konjugatının Sentezi, Yapısal Karakterizasyonu ve Suda Çözünürlüğü

Research Article

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ABSTRACT

Novel functional poly(maleic anhydride-*co*-vinyl acetate)/acriflavine conjugate, MAVA/AF, was synthesized in two different ways that copolymer:drug ratio was designed as 1:1 and 1:2, respectively. Nontoxic polymeric drug carrier was prepared for conjugation reaction. The purified copolymer, MAVA, was modified with an antiexternal fungal and anticancer active agent acriflavine (AF) at 60°C for 72 hours in dimethylformamide (DMF), using triethylamine (TEA) as the catalyst. Structural characterizations were carried out by Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (1H-NMR). The spectroscopic results confirmed that conjugated/modification reaction was successfully carried out by amidification reaction.

Key Words
Poly(maleic anhydride-*co*-vinyl acetate) modification; acriflavine; FTIR, NMR.

ÖZ

Yeni fonksiyonel poli(maleik anhidrit-*ko*-vinil asetat)/Akriflavin konjugatı, MAVA/AF, iki farklı yolla kopolimer:ilaç oranı sırasıyla 1:1 ve 1:2 olacak şekilde sentezlendi. Konjugasyon tepkimesi için toksik olmayan polimerik ilaç taşıyıcı hazırlandı. Saflaştırılan kopolimer, MAVA, antikanser aktif akriflavin (AF) ajanı ile 60°C'da 72 saat dimetilformamid (DMF) içinde, trietilamin (TEA) katalizörlüğünde modifiye edildi. Yapısal karakterizasyon Fourier Transform Infrared (FTIR) ve Nükleer Magnetik Rezonans (1H-NMR) ile yapıldı. Spektroskopik analiz sonuçları konjugasyon/modifikasyon tepkimesinin başarılı bir şekilde amidleşme reaksiyonu ile olduğunu doğrulamaktadır.

Anahtar Kelimeler
Poli(maleik anhidrid-*ko*-vinil asetat) modifikasyonu; akriflavin; FTIR, NMR.

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INTRODUCTION

Drug discovery research is focus on to new formulations that ensure a greater pharmacological response, which in turn would lead to lower doses and therefore the minimization/disappearance of side effects. It is necessary to improve the bioavailability of drugs. Bioavailability is affected by several factors such as physical and chemical characteristics of the drug, the dose and concentration, the frequency of dosing, and the administration route. Thus, research into drug delivery systems (DDS) seeks to improve the pharmacological activity of drugs by enhancing pharmacokinetics (absorption, distribution, metabolism and excretion) and also by changing pharmacodynamics properties, including the mechanism of action, pharmacological response, and affinity to the site of action [1]. Polymeric systems have played an integral role in the advancement of drug delivery technology by providing controlled release of therapeutic agents in constant doses over long periods, cyclic dosage, and tunable release of both hydrophilic and hydrophobic drugs [2].

Maleic anhydride (MA) copolymers also known as polyanhydrides, consist of MA (not capable of self-homopolymerization) and vinyl-based monomers, is suitable for synthesis of functional macromolecules using radical initiators by free radical chain polymerization reactions. They contains reactive anhydride units which makes it susceptible to chemically modification reactions. MA based copolymers applied as drug carriers for biomedical applications. Modification of the MA polymers through derivatization or conjugation forms a highly reactive anhydride ring that can be attacked by amino (-NH_2) or hydroxyl groups (-OH) of nucleophilic reagents, [3-6] which results in either ester/carboxylic acid or amide/carboxylic acid (Scheme 1) by ring opening reaction. Recent methods have been commonly applied as solubilizing agents for nanoparticulate formation, surface modification, macromolecular drug carriers, diagnostic imaging agents, implants, etc. [7]. Generally water-soluble polymer conjugates have been designed as drug carriers. Many research have been focused on the rational design of polymeric-based prodrugs [8,9] which is based on a model for polymer-drug conjugate originally proposed by Helmut Ringsdorf (Figure 1) [10]. Poly(styrene-*co*-maleic acid/anhydride) (SMA) is well-known commercialized vinyl-based copolymer [3,11] that SMANCS, a conjugate of an SMA copolymer and the antitumor protein neocarcinostatin (NCS) [9], has also been marketed in Japan for the treatment of hepatocellular carcinoma, producing few side effects and yielding considerable therapeutic potential [12].

After the modification of partially water-insoluble MA copolymers observed in increased water solubility depending on the increase of the carboxyl group. On the other hand conjugated drug excess of polar functional group and also hydrogen bonding capacity with water molecules increases the water solubility. The main important point here is that amides preferred than esters for conjugated products amide can hydrolyze with amidases in the organism, due to the electron delocalization on the nitrogen, the hydrolysis of the amides is more difficult and therefore stable than the esters. Primary and secondary amides have both hydrogen bonding acceptors and hydrogen bonding donors making them versatile, which increases water solubility [13].

Acriflavine generally known Acriflavinium chloride is a topical antiseptic dye which is also used for the treatment of gonorrhea, meningitis, intestinal infections, diphtheria, pneumonia, cholera and infected wounds. Recently, it has been shown to have anticancer activity and used in AIDS treatments. Acriflavine, actually known as a dye, is a kind of topical antiseptic agent derived from acridine, topical antibacterial against sleeping sickness. It is widely used for photosensitizer, analytical reagents for sensing, acid–base indicator, luminescence sensor, etc. Systematic IUPAC name, molecular formula and molecular mass of Acriflavine (AF) are as follows: 3,6-diamino-10-methylacridine10-ium chloride, $C_{14}H_{14}ClN_3$, and 259.73 g.mol^{-1} with two primary amine groups [14].

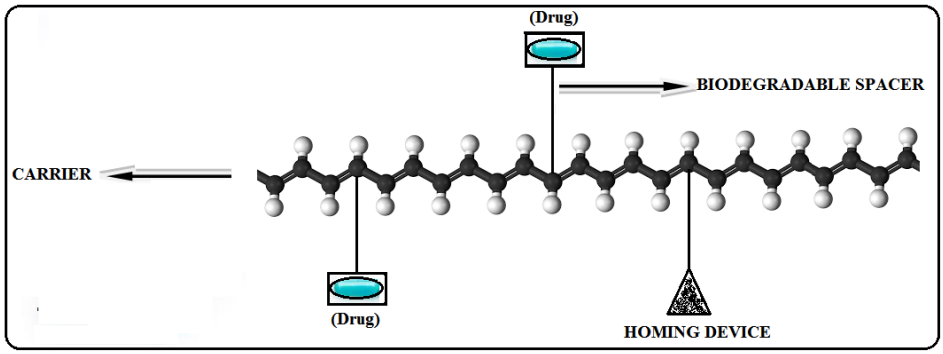
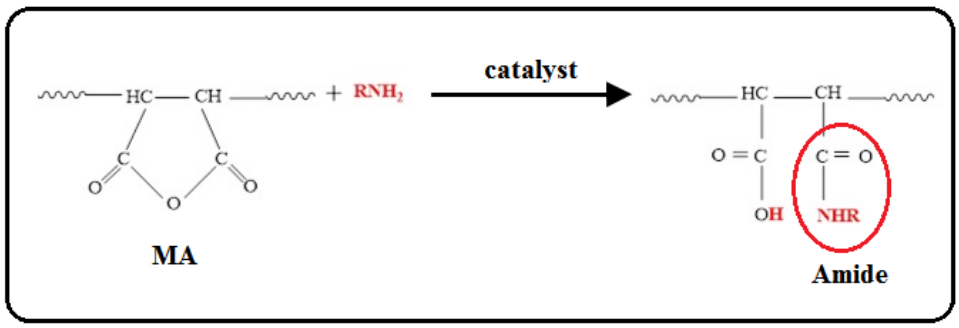


Figure 1. The Ringsdorf model.



Scheme 1. Representation of amide reaction for maleic anhydride.

MATERIALS AND METHODS

Materials

For copolymer synthesis: Maleic anhydride, (MA), was purified before use by recrystallization from anhydrous benzene and by sublimation in vacuum, (BPO) radical initiator, was purified by recrystallizing twice from chloroform solution by methanol dried under vacuum, were obtained from Merck (Schuchardt, Germany). Ethyl alcohol (95%) was obtained from Carlo-Erba (Rodano, Italy). Vinyl acetate (VA), ethyl acetate and petroleum ether were obtained from Sigma-Aldrich (St. Louis, USA). Other reagents including organic solvents were purified by ordinary methods.

For copolymer/drug conjugation: Methyl ethyl ketone (MEK), dimethylformamide (DMF) and triethylamine (abbreviated as TEA or Et3N) were obtained from Merck (Germany). AF was purchased from Sigma-Aldrich (St. Louis, MO, USA).

For purification: Ethyl alcohol Merck (Schuchardt, Germany) was used for purification step for the conjugated product.

Synthesis of MAVA copolymer

Maleic anhydride (MA) was purified before use by recrystallization from anhydrous benzene. Poly(maleic anhydride-*co*-vinyl acetate), MAVA copolymer, was synthesized by free radical polymerization of MA and VA monomers, at a 1:1 molar ratio in MEK using BPO preferred as the free radical initiator instead of azobisisobutyronitrile (AIBN) for 24 h at 80°C (Table 1) and conducted in the fume hood [15]. Un-reacted vinyl acetate or homopolymerization products were removed by incubating the precipitate in ethyl acetate for 24 h, then precipitated with petroleum ether, filtered under vacuum, and dried in a vacuum incubator at 55°C for 24 h.

Synthesis of MAVA/AF copolymer/drug conjugate

Poly[MA-*co*-VA]/AF polymer/drug conjugate was synthesized in two different ways that pharmaceutically active ingredient (AF) covalently conjugated to copolymer backbone. The purified copolymer was modified with an anti-external fungal and anti-cancer active agent, acriflavine

Table 1. Reaction conditions of MAVA copolymer and MAVA/AF conjugate.

Sample	Molar Proportions	Initiator and Catalyst	Solvents	Time (h)	Temperature (°C)
MAVA	MA:VA ~ (1:1)	BPO	MEK	24	80
MAVA/AF	MAVA:AF ~ (1:1)	TEA	DMF	72	60
	MAVA:AF ~ (1:2)	TEA	DMF	72	70

BPO: Benzoyl peroxide, Triethylamine (TEA or Et3N), DMF: Dimethylformamide.

(AF) at 70°C for 72 h in dimethylformamide organic media, using triethylamine (TEA or Et3N) as the catalyst [16]. Copolymer:drug ratio was designed as 1:1 (60°C for 72 hours) and 1:2 (70°C for 72 hours), respectively (Table 1). Briefly the conjugated product, MAVA/AF, was precipitated with excess of cold ethyl alcohol (-20°C), kept at -80°C for 24h and dried in a vacuum incubator for 24h at 45°C [16,17]. The MAVA copolymer powder (0.25 mmol, 42.53 mg) was dissolved in DMF (1.25 mL) to obtain transparent homogeneous solution, TEA (10 µL) was added while under stirring to MAVA-DMF solution. Bioactive agent, AF, (0.25 mmol, 64.925 mg) was dissolved in DMF (2.2 mL) (before solved in 1.5 mL ultra-pure water) and was then added drop-wise into the copolymer solution at room temperature [16]. The final mixtures was stirred separately by shaking for 2 h at 50°C, and the reaction was continued for further 70 h at 60°C for 1:1 ratio and also 70°C for 1:2 ratio until the obtained, dark brown color, viscous solution and cooled to room temperature again for 2 h. Cold viscous solution then purified repeatedly with excess of cold ethyl alcohol (-20°C) and incubated for 24 h at -80°C to obtain dark brown precipitate. Precipitate was collected by centrifugation for 10 min at 3,000 rpm and liquid phase was removed. Precipitate was dried in a vacuum incubator for 48h at 45°C.

Structural Characterization

The FTIR spectra of samples, MAVA copolymer and MAVA/AF conjugate, (KBr pellets) were recorded on an FTIR spectrophotometer (MATTSON 1000 Unicam, USA) at 400-4000 cm^{-1} with 4 cm^{-1} increments. Nuclear Magnetic Resonance, 1H-NMR, analysis were performed at 400 MHz (Bruker Avance III, Karlsruhe, Germany) using 6 mg of the copolymer and modified product, dissolved in 0.8 mL pyridine-d5.

Water Solubility Property and Stability in Phosphate Buffered Solution

MAVA/AF in dried powder form was incubated in ultra-pure water (1 g/1 ml). After dissolving in a fresh, daily prepared phosphate buffered saline solution (PBS; biotechnology grade, 137 mM NaCl, 2 mM KCl and 10 mM phosphate buffer, pH 7.4 0.1) it was incubated at 37°C for 2 h.

RESULTS

FTIR Analysis

Samples (copolymer, copolymer/drug conjugate, and drug) were represented by various colors that are abbreviated on each spectrum as follows:

MAVA copolymer (Figure 2a and 3a) had the expected anhydride units at 1855.48 cm^{-1}, and 1780.59 cm^{-1}, belongs to symmetric and asymmetric carbonyl (C=O) stretching vibrations of maleic anhydride (MA), respectively [18,19]. C-O-C stretching vibrations located on MA ring, were observed at 1025.21 cm^{-1} and 934.96 cm^{-1} [20]. C=O stretching vibrations of VA were appeared at 1715.7 and 1713.17 cm^{-1} [21]. Methyl and methylene groups of VA were detected at 1373.80 cm^{-1} and 1430.38 cm^{-1}, respectively [22,23]. $COCH_3$ stretching vibrations of VA observed at 1095.30 cm^{-1} [18]. Furthermore C-O-C and C-H stretching also located at 1214 cm^{-1}, and at 2943 cm^{-1}, respectively [24,25].

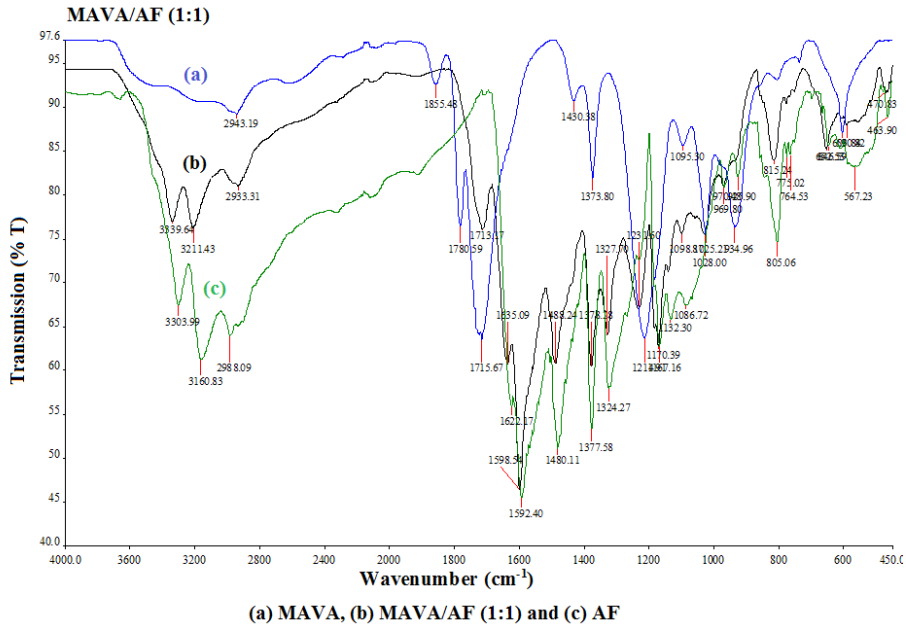


Figure 2. Overlapped FTIR spectra of the copolymer, conjugate and AF. (a) MAVA, (b) MAVA/AF (1:1), and (c) AF.

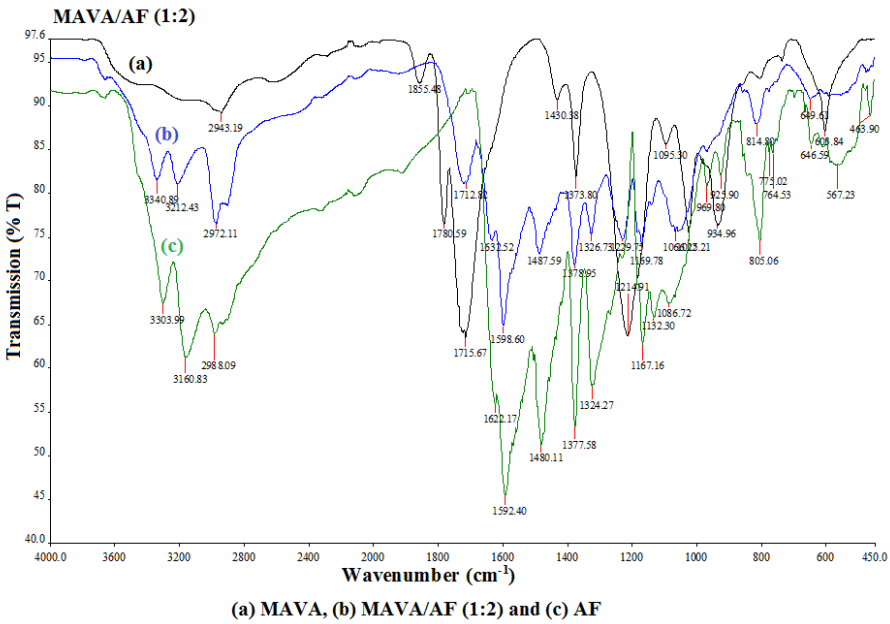


Figure 3. Overlapped FTIR spectra of the copolymer, conjugate and AF. (a) MAVA, (b) MAVA/AF (1:2), and (c) AF.

Anhydride ring peaks on MAVA/AF, for (1:1) and (1:2) conjugation, disappeared completely by ring-opening reactions (Figure 2b and Figure 3b). The disappearance of the characteristic anhydride peaks could suggest that almost all of the anhydride rings on polymeric backbone were opened by acriflavine (AF), via its two nucleophilic amino groups resulted in the formation of a carboxylic group and amide structure [26]. The -NH stretching of –CONHR mono-substituted amide group was observed in the MAAA/AF (1:1) and (1:2) spectra (Figure 2b): the intensity of the absorption bands of C=O on the anhydride groups, completely disappeared and following peaks was also appeared after the modification:

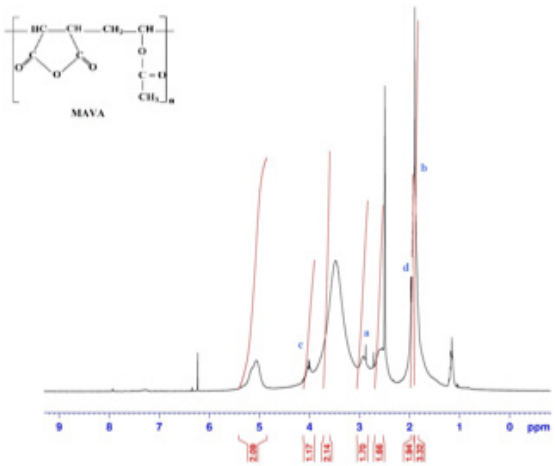


Figure 4a. ^{1}H-NMR spectra of MAVA copolymer.

a C-N stretching at 1028.00 cm^{-1} [27,28], and –NH wagging band at 815.24 cm^{-1} [29], due to the amide and free acid formation [3,5].

The 1327.70 cm^{-1} was assigned to asymmetric C-N stretching [30]. The symmetric –CNH deformation vibration was assigned to 1488.24 cm^{-1} [30]. Characteristic vibration for cyclic C-N-C observed at 1378.28 cm^{-1}. A symmetric stretching vibration of C=O imide was also observed at 1713.17 cm^{-1} [31]. C=O stretching vibration of tertiary and secondary amide was observed at 1635.09 cm^{-1} [32]. Because of 1170.39 cm^{-1} indicated of the CH_3 in AF molecule (Figure 2c and 3c) both of the peaks 1171.41 cm^{-1} and 970.48 cm^{-1} associated with various vibrations of CH-CH_3 group conformation [29]. Peak assignment for 3339.64 cm^{-1} could be attributed for both symmetric –NH_2 stretching [33] vibration and –OH band [29]. Most of the characteristic peaks of AF molecule such as 934.96 cm^{-1}, 969 cm^{-1}, 1132.30 cm^{-1}, 1170.39 cm^{-1}, 1324.27 cm^{-1}, 1377.58 cm^{-1}, 1480.11 cm^{-1}, and 1622.17 cm^{-1} were assigned to the FTIR spectrum of the pure acriflavine molecule [5,34]. These peaks were formed by an -NH bending indicating the -CONHR mono-substituted amide group (Figure 2b and 3b). These findings could be attributed to the formation of amide linkages. On the aromatic ring, the C=C frequencies were also represented at 1598.54 cm^{-1} [35]. It was also attributed to the C-N-C stretching related to NH_3^+ bending [31]. A C-O stretching vibration was also observed at 1231.60 cm^{-1} [36]. It can be clearly seen from the overlapped spectra (Figure2 and Figure 3) that most of the functional group peaks of AF observed in MAVA/AF for (1:1) and (1:2) spectrum. These findings strongly indicated that MAVA copolymer was modified with acriflavine (AF) by an amidization reaction. Furthermore it can be said that both FTIR spectra of MAVA/AF for (1:1) and (1:2) are almost identical.

NMR Analysis

Nuclear Magnetic Resonance, 1H-NMR, analysis were performed for both AF, MAVA, and MAVA/AF (1:1) overlapped with MAVA/AF (1:1) and the results are as follows:

^{1}H-NMR Results

The characteristic peaks appeared in the ^{1}H-NMR spectrum of MAVA (Figure 4a) are as following: a chemical shift of two protons on MA anhydride groups at 3-4 ppm, -CH_2 protons on VA approximately at 2.3-2.7 ppm, a multiplet peak for –CH, (bound to oxygen) approximately at 4.9-5.3 ppm, and three methyl protons, adjacent to carbonyl group, at 1.7-2.0 ppm [37].

Characteristic features of the MAVA/AF (1:1) spectrum (Figure 4b) confirmed conjugation by both MAVA and AF characteristic functional moieties, also amide linkages. Characteristic DMSO-d6 solvent peak was also shown at 2.50 and 3.3 ppm. The nitrogens of amide were observed at 3.87 ppm. Amide, -NH (-CONH-) protons were also observed at 7.74 and 7.93 ppm, confirming that AF bound to MAAA copolymer back bone by an amidation reaction [38].

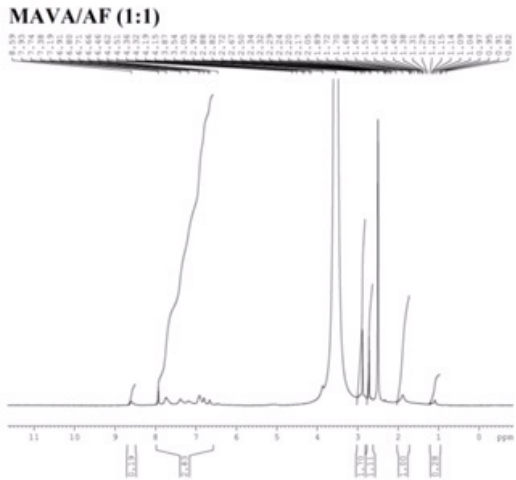


Figure 4b. ^{1}H-NMR spectra of MAVA/AF (1:1) 0-12 ppm range.

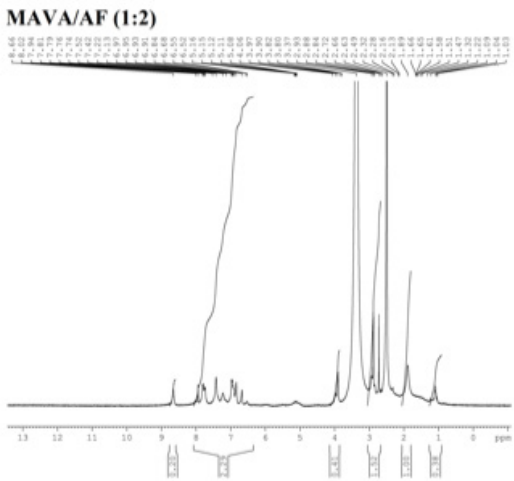


Figure 4c. ^{1}H-NMR spectra of MAVAAF (1:2) 0-13 ppm range.

Characteristic features of the MAVA/AF (1:2) spectrum (Figure 4c) almost identical to MAVA/AF (1:1) spectrum (Figure 4b). Characteristic DMSO-d6 solvent peak was located at 2.49 and 3.3 ppm. The nitrogens of amide were observed at 3.82-3.90 ppm. Amide, -NH (-CONH-) protons were also observed at 7.74 and 7.94 ppm, confirming that AF bound to MAAA copolymer back bone by an amidation reaction [38].

Observed peaks related to AF molecule (Figure 4d), as follows: 6.62-7.58 ppm (aromatic phenyl protons of benzene ring), 2.50 ppm (-CH of aromatic ring on AF), 2.07 ppm (-CH_2 of aromatic ring on AF). -N-H (attached to quaternary ammonium chloride proton peak located at 13.987 ppm and –NH_2 groups peak, attached to aromatic ring, observed at 7.28 ppm and 7.49 ppm. It can be seen clearly from the Scheme 3 that all of the aromatic protons come from the acriflavine molecule because MAVA copolymer has no any aromatic rings (Figure 4a). Furthermore it can be said that both ^{1}H-NMR spectra of MAVA/AF for (1:1) and (1:2) are almost identical.

Furthermore, for detail information, an enlarged image of MAVA/AF for (1:1) and (1:2) conjugation products spectra, showing the peaks between 0 and 12 ppm, was supplemented (Figure 5a and Figure 5b). Overlapped spectra were taken in different colors: Overlapped Figure 5a spectra [MAVA/AF (1:1), and MAVA/AF (1:2) represented as blue and red colors, respectively] and overlapped Figure 5b spectra [MAVA/AF (1:1), MAVA/AF (1:2), and AF represented as blue, red, and black colors, respectively].

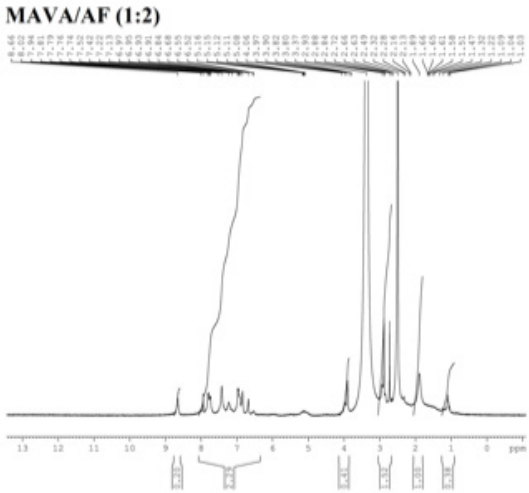


Figure 4d. 1H-NMR spectra of Acriflavine.

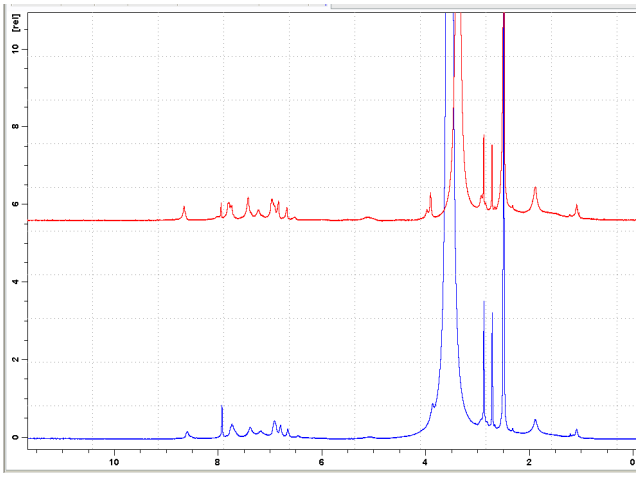


Figure 5a. Overlapped spectra of MAVA/AF (1:1) and MAVA/AF (1:2).

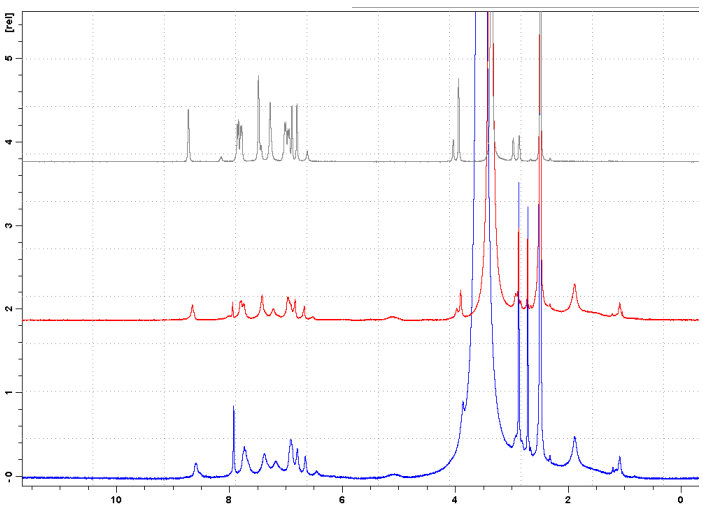


Figure 5b. Overlapped spectra of AF, MAVAAF (1:1) and MAVA/AF (1:2).

Water Solubility Property and Stability in Phosphate Buffered Solution

MAVA/AF in dried powder form was incubated in ultra-pure water (1 g/1 mL) at room temperature. It was partially soluble in water according to aromatic rings and also hydrophobic copolymer backbone. On the other hand it was shown that MAVA/AF conjugate kept its molecular integrity in phosphate buffered saline solution (PBS) for 2 h. PBS is a buffer solution commonly used in biological research because of its reference properties such as water-based salt solution containing disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate. PBS used for the stability, especially as a first approach for biocompatibility test, because the osmolarity and ion concentrations of the solutions match those of the human body (isotonic). It can be said that MAVA/AF displayed good properties for drug like compounds.

DISCUSSIONS

Structural characterizations, FTIR and 1H-NMR, strongly indicated that nontoxic MAVA copolymer [15] was modified with acriflavine (AF) by an amidization reaction. Because both FTIR and NMR spectra of MAVA/AF for (1:1) and (1:2) are almost identical in spite of copolymer:drug ratio was designed as 1:1 (60°C for 72 hours) and 1:2 (70°C for 72 hours), respectively. It can be said that suitable conjugation ratio, temperature and time for designed copolymer:drug was enough and also very successful as follows: Ratio (1:1), 60°C, and 72 hours.

Furthermore to the extent this study for especially stability and controlled drug delivery tests we have made some preliminary tests such as investigation of pH effect on zetasizer measurements, time effect on Particle size, Mobility and Zeta potential and also time effect of activity for poly(maleic anhydrite-*co*-vinyl acetate)/acriflavine (MAVA/AF) conjugate in simulated body fluids (5% Dextrose and PBS: biotechnology grade, 137 mM NaCl, 2 mM KCl, and 10 mM phosphate buffer, pH 7.4±0.1) by comparing with pure copolymer (MAVA) and also bioactive agent (AF). Initially, all simulated body fluid results indicate that this conjugate quite stable and is also suitable for the controlled drug release system.

MAVA/AF partially soluble in water according to aromatic rings and also hydrophobic copolymer backbone. In addition MAVA/AF displayed good properties as a first approach for biocompatibility test that kept its molecular integrity in PBS (simulated solution both osmolarity and ion concentrations match those of the isotonic human body for drug like compounds) for 2 h.

Disclosure Statement

The author of the manuscript solemnly declare that no scientific and/or financial conflicts of interest, exists with other people or institutions.

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