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# Development and characterization of cross-linked poly(malate) microspheres with dipyridamole

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**Abstract**

Biodegradable cross-linked microspheres containing up to 63 wt.% of the active substance were obtained in a polycondensation process between D,L-malic acid and the tetrahydroxy compound dipyridamole. The in vitro release mechanism from biodegradable cross-linked microspheres has been studied. It was found that dipyridamole was released due to two-step hydrolysis of the ester bonds of the network. Initially, the only product of the hydrolytic degradation was found to be an oligomeric ester fraction with $M_w = 1000$ Da. The release of the free drug started after 8 days due to a further hydrolysis of the oligomers in solution. It was found that blood plasma enzymes in rats did not affect the hydrolytic processes. Biodegradable poly(malate) microspheres containing an anti-aggregating agent dipyridamole can be considered as a novel drug delivery system for a prolonged period of time implying a future parenteral application. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Biodegradable microspheres; Sustained release; Malic acid polymer; Antiplatelet agent; Drug delivery system

## 1. Introduction

Biodegradable drug delivery systems have been of particular pharmaceutical interest providing possibilities to achieve long term sustained drug release (Heller, 1984). Two are the preparative methods for such systems: physical incorporation of drug into the polymer carrier or its chemical bonding to the polymer carrier during the preparation of the drug systems (Okada and Toguchi, 1995). While the first method has been the subject of a number of studies (Beck and Tice, 1983; Ogawa et al., 1988; Mehta et al., 1994), the chemical systems have been less investigated (Arshady, 1989). The most widely used carriers for these systems are various natural (proteins, polypeptides and polysaccharides) and synthetic polymers (polyesters of hydroxy acids, polyanhydrides, poly(ortho esters) etc). In this view, malic acid and its polyesters (polyhydroxyalkanoates) are suitable as a biodegradable polymeric carriers for

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drug delivery systems because of their advantages. They are biodegradable and biocompatible (Rossignol et al., 1999) as well. The availability of free functional groups allows preparation of drug delivery systems with chemical drug bonding (Belcheva et al., 1993; Cammas et al., 1999). Our earlier studies have shown the possibilities to achieve sustained drug release of the original bulgarian bronchodilator vephylline at its chemical crosslinking with malic acid (Lambov et al., 1997).

The present investigations aim at revealing the possibilities to prepare cross-linked microspheres with dipyridamole based on malic acid as a potential novel biodegradable drug delivery system for parenteral administration. Dipyridamole is an attractive active drug because of its anti-aggregating effect and low toxicity. Some authors demonstrated (Ricevuti et al., 1991) that dipyridamole-beta-cyclodextrin complex showed better bioavailability and no adverse side effects in comparison with conventional dipyridamole. Aldenhoff et al. reported that photochemical covalent coupling of dipyridamole to polyurethane surfaces leads to an improved thromboresistance (Aldenhoff et al., 1997). In our opinion, the introduction of dipyradamole into biodegradable systems spreads the possibility to use its anti-aggregation activity for treatment of patients with vascular diseases. Moreover, the availability of four free hydroxyl groups in its molecule is a prerequisite for cross-linking with malic acid under suitable conditions.

## 2. Materials and methods

### 2.1. Materials

Dipyridamole ( 2,2′,2″,2‴-[(4,8-Dipiperidinopyrimido[5,4-d] pyrimidine-2,6-diyl) dinitrilo]tetraethanol) was obtained from Sopharma Ltd. (Sofia, Bulgaria). D,L-malic acid, *N*,*N*′-dicyclohexylcarbodiimide (DCC), tetrahydrofuran (THF), diethyl ether and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade.

### 2.2. Methods

#### 2.2.1. Preparation of microspheres

Briefly, to a mixture of 0.505 g (1.0 mmol) dipyridamole, 0.17 ml (1.25 mmol) pyridine and 2.28 g (11.0 mmol) *N*,*N*′-dicyclohexylcarbodiimide (DCC) in tetrahydrofuran (THF) was added dropwise a solution of 0.67 g (5.0 mmol) D,L-malic acid and 0.08 g (0.4 mmol) *p*-toluenesulfonic acid in THF. The reaction was carried out at 40–45 °C temperature and continued for 24 h. After filtration of the reaction mixture the yellow precipitate of the cross-linked polyester (dipyridamole-malate) was purified by washing with hot ethanol, then with diethyl ether and dried.

#### 2.2.2. Drug loading

A weighed quantity of microspheres was hydrolyzed in 100 ml 1 N NaOH medium. After suitable dilution the absorbance of samples was measured spectrophotometrically at $\lambda = 284$ nm (Hewllet Packard 8452 a diode array spectrophotometer equipped with HP Vectra 386/25 computer). The amount of drug was determined from standard plot of dipyridamole. The amount of dipyridamole loaded in poly(malate) microspheres was determined after complete hydrolysis and was found to be 63 wt.%.

#### 2.2.3. Determination of surface morphology and microsphere size

The shape and surface characteristics of microspheres were examined by scanning electron microscopy (SEM) using a JSM 5300 scanning microscope (JEOL 5300, Japan). Samples were coated with gold to a thickness of 200–500 Å prior to SEM examination.

#### 2.2.4. In vitro drug release

In vitro drug release was conducted in a shaker bath (75 rpm) at 37 °C temperature, in a phosphate buffer (pH 7.4). The amount of dipyridamole was measured spectrophotometrically ($\lambda = 284$ nm) (Hewllet Packard 8452 A).

Thin layer chromatography (TLC) of samples during drug release was performed on Merck DC-Alufolien, Kieselgel, 60 $F_{254}$ (0.20 mm) plates

and butanol:water:acetic acid (68:22:10 v/v) was used as a mobile phase.

High-performance liquid chromatography (HLPC) was performed with a liquid chromatography apparatus equipped with a UV-detector Waters 991. The HPLC conditions included a flow rate of 0.4 ml/min, C-18 ($100 \times 8$ mm) column, UV-detection at 284 nm, temperature 22 °C and a mobile phase methanol:water (30:70 v/v) mixture.

Infrared spectra of D, L-malic acid, dipyridamole and cross-linked poly(malate) microspheres were recorded on a Shimadzu FTIR-8101 M spectrometer.

#### 2.2.5. Gel-permeation chromatography (GPC)

The molecular weight of oligomeric fractions during drug release from poly(malate) microspheres were determined using a Waters gel-permeation chromatograph equipped with a differential refractometer R-410 and a UV-detector Waters 490. The chromatographic separation was done by a Ultrahydrogel columns with porosities 250 and 500 Å. Methanol/water mixture was used as a mobile phase (15:85 v/v) at a flow rate of 0.8 ml/min and 36 °C temperature.

#### 2.2.6. Enzyme hydrolysis

Enzyme hydrolysis was carried out using a method described by Mork (Mork et al., 1990). Poly(malate) microspheres were stirred (75 rpm) at 37 °C temperature in a rat plasma, diluted to 80% with 0.05 M phosphate buffer (pH 7.4). The release of free dipyridamole from microspheres was studied by HPLC chromatography.

#### 2.2.7. Differential scanning calorimetry (DSC)

The thermal behavior of malic acid, dipyridamole and cross-linked microspheres was analyzed by a Perkin–Elmer DSC-7 differential scanning calorimeter (Perkin–Elmer, USA). The samples (5 mg) in sealed aluminium pans were scanned at a heating rate 10 °C/min in the 10–190 °C temperature range, under nitrogen atmosphere.

## 3. Results and discussion

### 3.1. Preparation and characterization of poly(malate) microspheres

The cross-linking of dipyridamole and malic acid was carried out by polycondensation in the presence of dicyclohexylcarbodiimide, as suggested by Belcheva for the preparation of polyester networks of malic acid with low molecular weight diols (Belcheva et al., 1993). Main condition for the reaction is the availability of at least two free hydroxyl groups in the drug molecule. Therefore, dipyridamole having four OH-groups in its molecule was used. The investigations carried out aim at establishing an optimum regime for the preparation of a poly(malate) system in the form of microspheres. First, dipyridamole, pyridine and DCC were completely dissolved in tetrahydrofuran. A solution of D,L-malic acid in the same solvent was added dropwise to this mixture under stirring. Tetrahydrofuran was used as a solvent because dipyridamole and malic acid, and all other reagents are soluble in it.

A series of experiments allowed us to determine that the stirring rate during polycondensation is the most important factor, which influences the shape of microspheres. Microspheres prepared at stirring rate lower than 900 rpm were non-spherical and formed agglomerates. The stirring rate at 900–1000 rpm was determined to be the optimum one because the microspheres obtained were with spherical shape and smooth surfaces having particle diameters in the range from 1 to 15 μm (Fig. 1).

Another factor affecting the formation of microspheres is the temperature at which the polycondensation is run. It has been found that the cross-linking and the yields (7%) are rather poor at reaction temperatures in the range 20–25 °C. A raise of the temperature (up to 40–45 °C) theoretically enhances the reaction and probably increases the number of groups reacted, hence cross-linking effectiveness improves considerably (46% yield). Further increase of the temperature did not affect significantly the yield.

The poly(malic acid) network comprising dipyridamole is in fact a novel compound, a

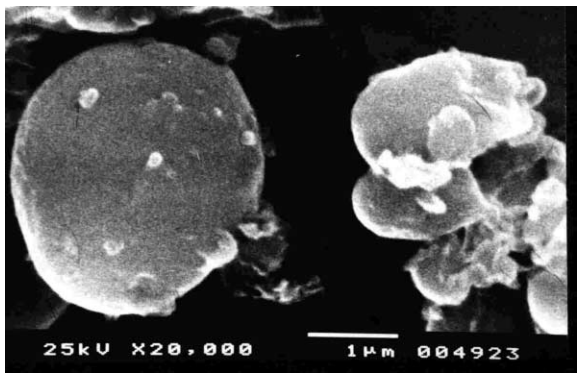


Fig. 1. Scanning electron microscopy of poly(malate) microspheres containing dipyridamole.

specific type of Prodrug. Therefore, it should be characterized so that the nature of the chemical bonds and hence the mechanism of drug incorporation can be revealed.

The infrared spectral studies (IR) of cross-linked microspheres show absorption peaks at 1165.0 per cm ($\nu$C–O–C ether) and at 1741.0 per cm ($\nu$C=O ester) characteristic for ester bonds which are missing in the IR spectrum of the free dipyridamole (Fig. 2). As seen from the comparison of the two spectra the absorption intensity characteristic for the hydroxyl groups of dipyridamole (3240–3250 per cm) in the IR spectrum of the microspheres is lowered. The fact gives enough grounds to assume that most of the hydroxyl groups of the drug have reacted with the carboxylic groups of malic acid. Unlike the spectra of the cross-linked microspheres there are no absorption signs for C–O–C bonds in the spectrum of non-crosslinked malic acid. An interesting fact is the disappearance of signs for the hydroxyl group of the malic acid (3445.3 per cm) in the IR spectra of the cross-linked poly(malate) microspheres. This reveals the potentiality for the formation of an ester bond between molecules of the malic acid itself. Consequently, the ester bonds evidenced by the IR spectra of the cross-linked poly(malate) microspheres can occur both between the drug and the malic acid and between the molecules of the malic acid itself. In order to find out which one of the discussed esterifications prevails autopolycondensation of malic acid (without a drug) was run under the same conditions. It has been established that the molecular weight of the products determined by GPC is not higher than 600 Da. Meanwhile, GPC chromatograms of the products from the degradation of the dipyridamole-malate microspheres at the initial minutes of alkaline and acidic hydrolyses were taken. These chromatograms indicated high molecular weight fractions of mass over 15000 Da (Fig. 3, peaks 1, 2) and fractions of average molecular weight approximately 1000 Da. Hence, the spectral and GPC studies allow the hypothesis that the high molecular weight fractions are frag-

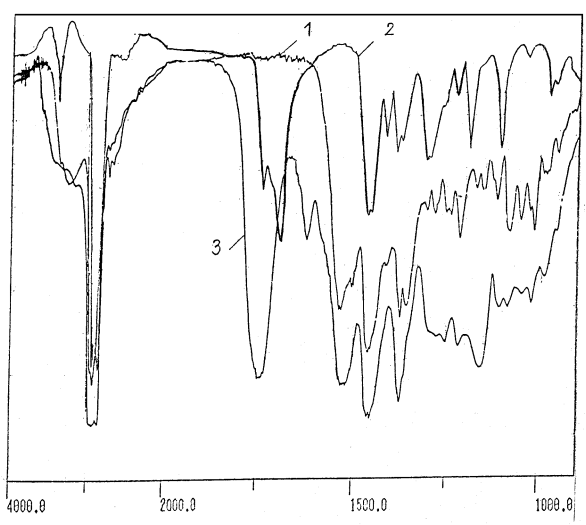


Fig. 2. Infrared spectra of dipiridamole (1), D, L-malic acid (2) and cross-linked poly(malte) microspheres (3).

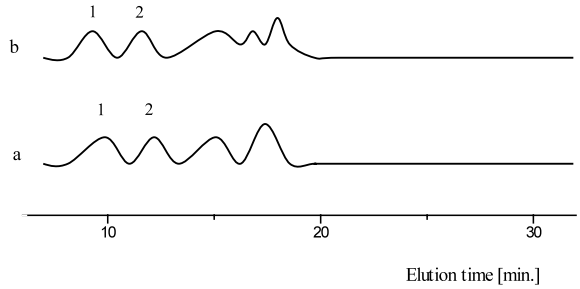


Fig. 3. GPC chromatograms of destructive products of poly(malate) microspheres obtained after the fifth minute hydrolysis: a) in alkaline medium (pH = 10.0); b) in acidic medium (pH = 1.2).

ments from the cross-linked poly(malate) chain, which has been formed by ester bonds between the drug and D,L-malic acid.

Taking into account the mechanism of esterification in the presence of carbodiimides as well as the effect of the catalyst and the acidity on the polycondensation, it can be assumed that D, L-malic acid predominantly participates with α-carboxylic group ($pK_a = 3.40$). However, an esterification of the β-carboxylic group ($pK_a = 5.11$) can not be excluded.

The theoretical structure of the cross-linked fragments from the polymalate system is based on the results achieved (Fig. 4). However, it is impossible to react all four hydroxyl groups of dipyridamole during polycondensation probably due to the steric hindrance. This fact is supported by the availability of free hydroxyl groups in the IR-spectra of cross-linked poly(malate) microspheres (Fig. 2).

### 3.2. In vitro drug release

The in vitro release of dipyridamole from the poly(malate) microspheres was studied under the conditions of a shaking bath in a phosphate buffer at pH 7.4. The release of dipyridamole

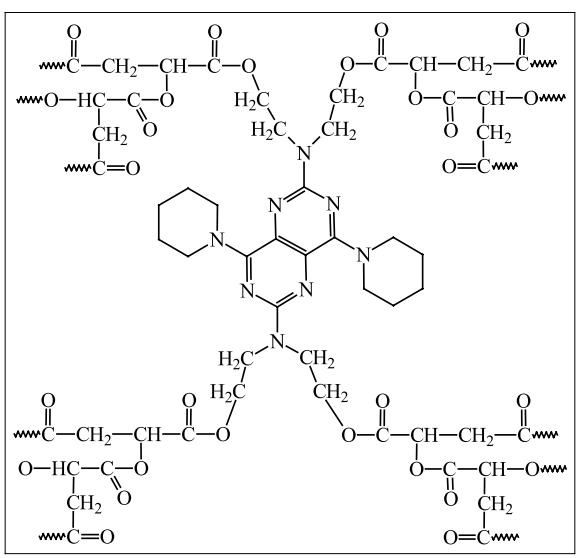

Fig. 4. Theoretical model of ester bonds between dipyridamole and malic acid.

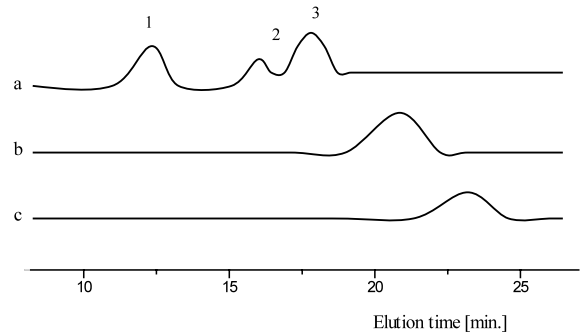


Fig. 5. GPC chromatograms: a) sample taken from the dissolution medium on the second day during the release process from cross-linked poly(malate) microspheres; b) free dipyridamole; c) non-crosslinked D,L-malic acid.

from poly(malate) microspheres has been followed both by UV-spectrophotometry and thin layer chromatography (TLC). The TLC-chromatograms of samples during drug release showed that the initial degradation of the system results not into a free dipyridamole but in an oligomeric products, which remain at the start of chromatograms. The free drug is found chromatographically only after the eighth day ($R_f = 0.54$).

The samples of dissolution medium were investigated by gel-permeation chromatography (GPC) for better understanding of the nature of the products released. Two fractions are available in the GPC-chromatograms of a sample taken on the second day of the release process: a high molecular weight one with $M_w$ up to 13250 Da (Fig. 5a, peak 1) and an oligomeric one of average $M_w$ 1000 Da (Fig. 5a, peaks 2 and 3). With release time the amount of high molecular weight fractions decreases while that of the oligomeric ones increases. However, after the eighth day is found fraction with average molecular weight 500 Da, which corresponds to the free dipyridamole ($M_w = 504.64$). This fact allows the statement that drug release from poly(malate) microspheres proceeds at two steps—initially the water soluble oligomers are released and subsequently they are hydrolyzed to free dipyridamole.

The amount of free dipyridamole at different time intervals was determined by high-performance liquid chromatographic measurements (HPLC). The data allow to plot the release profile

of the free drug. The total degradation of the microspheres is completed for 30 days, while the complete release of free dipyridamole takes 38 days (Fig. 6b). The data are proved by IR spectra of the medium sample taken on the thirtieth day during drug release. In the IR spectrum of the sample the following peaks were registered: 3250.0 per cm ($\nu$OH); 1740.0 per cm ($\nu$C=O); 1650.0 per cm ($\nu$C=O); 1165.1 per cm ($\nu$C–O–C). The results reveal that a mixture of free dipyridamole and dipyridamole-malate oligomers is still available in the sample. Only after this period, the above mentioned absorption peaks of ester bonds disappear in the IR spectra of medium samples.

The slow release rate is mainly due to the high cross-linked density of the poly(malate) system. In our case, it is a result from the large number of functional groups participating in the polycondensation. The high cross-linked density hinders diffusion of dissolution medium into the microspheres, leading to the slow hydrolysis. Similar dependence has been described by Larionova et al. (Larionova et al., 1999). On the other hand, it is well known that the appearance of crystalline phase in the polymeric structure hinders the performance of hydrolytical processes in these regions. DSC analysis of the dipyridamole and

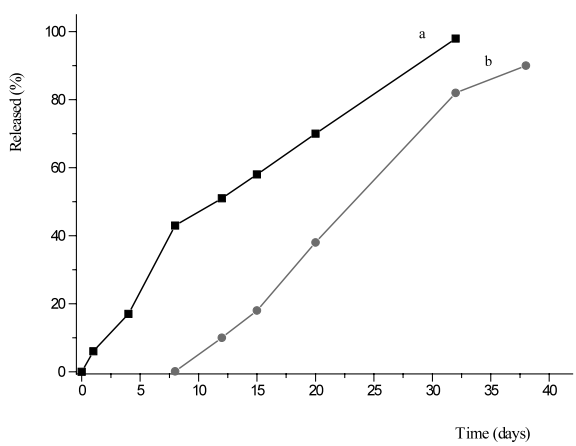


Fig. 6. In vitro drug release of dipyridamole from poly(malate) microspheres in a phosphate buffer (pH 7.4): (a) dipyridamole-malate oligomers; (b) free dipyridamole.

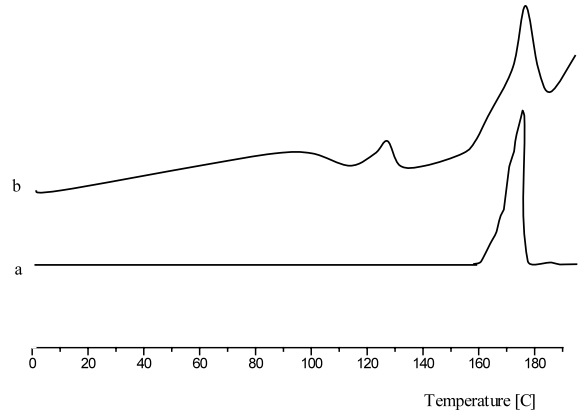


Fig. 7. DSC thermograms of pure dipyridamole (a) and dipyridamole-loaded poly(malate) microspheres (b).

cross-linked drug loaded microspheres was performed in order to characterize the state of model drug in the microspheres. The melting peaks of pure dipyridamole (169.6 °C) and D,L-malic acid (130.3 °C) are very close to the reported values, which are 162–168 and 128–130 °C, respectively. Both melting peaks are present in the DSC thermograms of cross-linked poly(malate) microspheres (Fig. 7b). First, the peak at 130.0 °C could be explained by an appearance of crystalline regions due to the ester bonded molecules of malic acid itself. On the other hand, the melting peak at 168.2 °C indicates that a part of dypiridamole is present in the polyester poly(malate) network in a crystalline state. Hence, dipyridamole release was prolonged additionally because of the appearance of a crystalline phase in the cross-linked poly(malate) chain.

For better understanding of the hydrolysis of microspheres a release study in blood plasma media was carried out. No difference in the release profiles of dipyridamole was found followed by HPLC chromatography. In both media, phosphate buffer (pH 7.4) and blood plasma, free dipyridamole appeared to release after the eighth day. No enzymatic degradation was registered in the blood plasma of a rat. Hence, drug release processes are controlled by the rate of chemical hydrolysis, which is a prerequisite for a good in vitro/in vivo correlation.

## 4. Conclusion

The investigations carried out show the possibility to prepare chemically cross-linked biodegradable poly(malate) microspheres containing dipyridamole via polycondensation. The optimal technological parameters providing the preparation of microparticles with regular shape and narrow distribution ranges (1–15 μm) have been established. The poly(malate) system prepared is a polyester resulting from the predominant esterification of hydroxyl groups of dipyridamole with carboxylic groups of malic acid. The system ensures sustained release of a drug for a long period of 38 days. Drug release proceeds in two steps: at first the water soluble oligomers are released which then hydrolyze to free dipyridamole. It could be concluded that biodegradable poly(malate) microspheres can be used for developing a novel drug delivery system with anti-aggregating activity.

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