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# Development of poly (dialkyl methylidenemalonate) nanoparticles as drug carriers

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## Summary

Two novel dialkylmethylidenemalonate (DAMM) analogs: diallylmethylidenemalonate (AAMM, **1b**) and cyclohexylethylmethylidenemalonate (CEMM, **1c**) were synthesized and evaluated for their capacity to polymerise to stable nanoparticles and to adsorb primaquine diphosphate (PQ). PQ loading onto poly-AAMM (PAAMM, **1b**) and poly-CEMM (PCEMM, **1c**) was up to 90% in the pH range from 6.0 to 8.5. A significant improvement of the physicochemical characteristics and the PQ desorption capacity of the nanoparticles was achieved in this series of PDAMM analogs.

## Introduction

Colloidal drug carriers are gaining success by achieving reduced toxicity, enhanced efficacy and site-directed action (Gabizon and Barenholz, 1988; Gregoriadis, 1988; Daoud et al., 1989).

The major limitations of current anticancer, antiparasitic and antiinfectious agents are their toxicity and their lack of specificity. In many instances, the inclusion of these drugs in particulate systems has led to significant advantageous improvement of their pharmacokinetics and metabolic patterns (Gabizon et al., 1982). Furthermore, intracellular infections in which the causal microorganisms are located within the cytoplasmic organelles are difficult to eradicate. Thus, the development of site-directed drug carriers to efficaciously treat such diseases is of major importance. Among the most studied endocytosable drug delivery systems, liposomes (Gregoriadis, 1988), nanocapsules and nanoparticles (Birrenbach and Speiser, 1976; Kreuter and Speiser, 1976) are gaining attention (Couvreur et al., 1982; Kreuter, 1983, 1988; Lenaerts et al., 1984). Along this line, polymethylidene malonate esters (polydialkylmethylidine malonates, PDAM-

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M) based nanoparticles are currently being investigated as potential drug carriers (De Keyser et al., 1991; Mbela et al., 1992).

This paper reports the preparation of polydiallylmethylidene malonate (PAAMM, **1b**) and polycyclohexylmethylidene malonate (PCEMM, **1c**) nanoparticles and the evaluation of their fundamental manufacturing parameters as new drug vehicles.

## Materials and Methods

*Synthesis of AAMM(1b) and CEMM (1c) monomers*

As described previously (Mbela et al., 1992), dialkyl malonic acid esters were submitted to reaction with paraformaldehyde and anthracene in the presence of $CuSO_4$ to yield the adducts **2a–c**. Subsequent retro-Diels-Alder thermolysis of **2a–c** (220°C, mineral oil) in the presence of maleic anhydride yielded the corresponding monomers **1a–c**. The monomer purity was determined by TLC, GLC, GC-MS and NMR (TLC system: silica gel F 254 TLC plates (Merck); ethyl acetate/ $CHCl_3$/ methanol (Merck, 98:2.5:0.5 v/v). Prior to TLC analysis, samples of the monomers were brominated (bromine in diethyl ether). The GLC analysis was performed on a Hewlett-Packard (HP) M 5710A using the following conditions: column, OV17 3%; detector, FID; carrier gas, nitrogen; temperature, 100°C (2 min) to 270°C (rate: 10°C). Mass spectrometry (GC-MS) was carried out on an HP using a capillary column (Ultra-2; 5% phenylmethyl silicone) and helium as carrier gas. The $^1$H-NMR spectra were recorded with a Varian 360 spectrometer; chemical shifts ($\delta$) were measured at room temperature using tetramethylsilane as internal standard; samples were dissolved in $DCCl_3$ to form 0.25 M solutions. The monomers were stored below 0°C under nitrogen.

*Preparation of free nanoparticles and lyophilization*

50 $\mu$l of the monomer **1b** or **1c** were introduced under mechanical stirring into 5 ml of the polymerization medium containing 0.1 M phosphate buffer, 1% (w/v) dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden) and 5% (w/v) of glucose (Janssen Chimica, Beerse, Belgium). The pH of the medium was kept at 7.4 and stirring at ambient temperature (22–25°C) was maintained until polymerization was complete (24 h). The milky suspension obtained was filtered, dialysed twice against 2 l of 0.1 M phosphate buffer at pH 7.5 for 24 h at 25°C and freeze-dried in a Lyovac GT2, Leybold Heraus. Samples of the suspension were removed before and after freeze-drying for size determination.

*Nanoparticle size distribution measurements*

10 $\mu$l of the nanoparticle suspension were diluted in 2 ml of distilled water. The mean size and size distribution of nanoparticles were determined by laser beam light scattering measurement on a Coulter sub-micron particle analyzer (Nanosizer, N4MD, Coulter Electronics Ltd, Luton, U.K.).

*Preparation of PQ-loaded nanoparticles*

*In situ PQ-loaded nanoparticles* 50 $\mu$l of the monomer **1b** or **1c** were introduced under mechanical stirring in 5 ml of the polymerization medium containing 0.1 M phosphate buffer (pH 7.5), 1% w/v dextran T 70 and various primaquine diphosphate salt concentrations ranging from 0.5 to 5.0 mg. PQ diphosphate was purchased from Sigma, St Louis, MO U.S.A. The polymerization was carried out at room temperature (22–25°C). After 24 h, free PQ concentration was evaluated in the supernatant by withdrawing an aliquot of the yellow milky suspension (5 $\mu$l) which was centrifuged at $14\,000 \times g$, for 15 min at 15°C and assayed for PQ content by UV spectrometry. The filtration and lyophilization of the drug-loaded nanoparticles were carried out as described above. Samples of the nanoparticles were taken out before and after lyophilization for size determination on a Coulter sub-micron particle analyzer as described above.

*PQ-post-loaded PDAMM nanoparticles* Lyophilized free nanoparticles (450 mg) were, successively, resuspended with stirring in 5 ml of solutions of PQ diphosphate at concentrations ranging from 0.5 to 5.0 mg/ml in 0.1 M phosphate

buffer at pH varying from 6.0 to 8.2. The suspensions, protected from light (photooxidation; Brossi et al., 1987) were stirred for several days at various temperatures: 20, 25 and 37°C. Twice a day, 5 $\mu$l of the suspensions were taken up, centrifuged and assayed for free PQ content in the supernatant. When the adsorption of PQ on the polymer was complete, the yellow suspensions were filtered and lyophilized. Aliquots of the suspensions were taken out before and after freeze-drying for size determination.

*PQ content determination*

Determination of PQ content was performed on the supernatant of the centrifuged nanoparticles. The PQ concentration was calculated as described previously (Mbela et al., 1992). UV spectrometry conditions: Perkin Elmer 556, double beam, double wavelength, fully automated. Analytical conditions: scanning interval, from $\lambda = 350$ nm to $\lambda = 340$ nm; mode, second derivative; scanning rate, 240 nm per min.

Calibration and dilution curves: 2.5 ml of the supernatant of free nanoparticles were introduced into the blank and sample cells. 1 ml of serial dilutions of PQ diphosphate or of the supernatant of centrifuged nanoparticle suspension were introduced into the sample cell. The absorbances were measured as the height of the PQ absorbance plots and were computed on a Macintosh SE/30 for linear regression. The unknown PQ concentrations of the supernatant of nanoparticles suspensions were calculated (Cumps and Derese, 1990).

*Drug release from nanoparticles*

Freeze-dried PDAMM nanoparticles (450 mg) containing 5 mg of PQ were suspended in 20 ml of a normal phosphate buffer saline, PBS (Mishell and Shiigi, 1980) and were incubated at 37°C at various pH values from 6.0 to 8.5 under slight agitation (70 cycles per min) and light protection. At different time interval, aliquots (50 $\mu$l) were taken out, centrifuged ($14\,000 \times g$, 15 min) and assayed for the free PQ in the supernatant by UV spectrometry. The medium volume was maintained constant by adding PBS.

*Influence of pH on the polymerization rate.*

50 $\mu$l of the monomer **1b** or **1c** were introduced into a series of test tubes containing the previous described polymerization medium at pH values ranging from 5.5 to 8.5. Stirring was performed at ambient temperatures (22–25°C). To assess the progress of polymerization, samples were taken at regular time intervals and evaluated spectrometrically for turbidity (Griffin and Griffin, 1985; Rollot et al., 1986) at 321 nm. The readings were expressed as the percent of the maximum absorbance of a standard suspension of PDEMM (**1a**) nanoparticles prepared as follows: 10 mg of dry **1a** nanoparticles were redispersed in 1 ml of the polymerization medium at 25°C (pH 7.5). 1 ml of the colloidal suspension was introduced into the sample cell and calibrated with distilled water in the blank cell.

*Influence of pH on the nanoparticle size*

Monomers **1b** and **1c** were submitted to polymerization at pH values ranging from 5.5 to 8.5 at 25°C as described above. After 48 h of polymerization, the resulting suspensions of the nanoparticles were filtered, dialysed twice against 2 l of 0.1 M phosphate buffer at pH 7.5 for 24 h at 25°C and analysed for size determination on a Coulter sub-micron particle analyzer.

*Influence of the stabilizer*

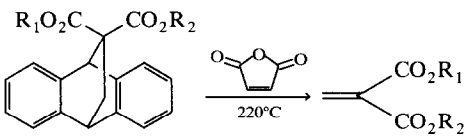
Nanoparticles were prepared as described previously at pH 7.5 in a series of polymerization media containing Pluronic F68® (non-ionic surfactant, from ICI France, Clamart) or various types of Dextran® (40, 70, 500 and 6000–30 000) used as stabilizers. After polymerization, filtration and dialysis, the mean size and populations of the resulting nanoparticles were assessed using the Coulter sub-micron particle analyzer.

*Influence of the temperature*

Empty and in situ PQ-loaded nanoparticles were prepared as described previously at various temperatures: 15, 20, 25 and 30°C. The mean size and populations of the resulting nanoparticles were assessed by using the Coulter sub-micron particle analyzer.

TABLE 1

*Yields and physicochemical characteristics of DAMM monomers*




**2a–c** **1a–c**

| Compound | $R_1$ | $R_2$ | Yield (%) | b.p. (°C) (Torr) | MS (*m/e*) |
|---|---|---|---|---|---|
| **1a** | ethyl | ethyl | 60 | 62–64 (0.2) | 172 |
| **1b** | allyl | allyl | 51 | 65–67 (0.3) | 196 |
| **1c** | ethyl | cyclo-hexyl | 52 | 80–82 (0.1) | 226 |

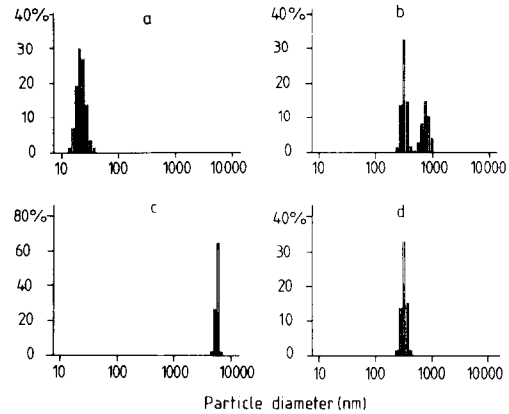



Fig. 1. Populations of nanoparticles formed in the presence of 5% of Dextran 500 (a) and 1% of Pluronic F68 (c), and with various concentrations of PQ diphosphate (b, d); pH 7.5.

## Results

### *Synthesis of AAMM (1b) and CEMM (1c)*

The yields of **1b** (51%) and **1c** (52%) were similar to those obtained previously with diethylmethylidene malonate (DEMM, **1a**; Mbela et al., 1992). The physicochemical and spectrometric characteristics (mass spectrometry) of the two analogs are shown in Table 1.

### *Preparation of free nanoparticles and lyophilization*

Empty nanoparticles ranging between 100 and 562 nm were obtained with an unimodal distribution (Fig. 1d). The particle size and homogeneity were not affected by lyophilization (results not shown). These data are in agreement with those published previously (Couvreur et al., 1982; De Keyser et al., 1991; Gaspar et al., 1991; Mbela et al., 1992).

### *In situ PQ-loaded nanoparticles*

As reported previously for polydiethylmethylidene malonate (PDEMM, **1a**), the polymerization process seems to be faster in the presence of PQ in the incubation medium. When concentrations of 0.5–1.5 mg of PQ are present in the polymerization medium, particle sizing between 100 and 316 nm are obtained. These lower PQ concentrations do not affect significantly the nanoparticle

TABLE 2

*Mean size and in situ PQ adsorption of nanoparticles formed with various concentration of PQ diphosphate*

| [PQ] (mg/ml) | PAAMM (**1b**) [a] | | PCEMM (**1c**) [a] | |
|---|---|---|---|---|
| | Size ± SD (nm) | % PQ adsorbed ± SD | Size ± SD(nm) | % PQ adsorbed ± SD |
| 0.5 | 100 ± 10 | 92.7 ± 0.03 | 100 ± 32 | 92.6 ± 0.05 |
| 1.0 | 156 ± 40 | 95.8 ± 0.05 | 100 ± 5 | 95.4 ± 0.07 |
| 1.5 | 133 ± 16 | 96.4 ± 0.003 | 100 ± 10 | 96.0 ± 0.02 |
| 2.0 | 315 ± 63 | 97.6 ± 0.02 | 316 ± 10 | 96.8 ± 0.02 |
| | 1650 ± 210 | – | 1000 ± 185 | – |
| 4.0 | 333 ± 250 | 98.8 ± 0.02 | 562 ± 73 | 98.5 ± 0.02 |
| | 3000 ± 1800 | – | 1780 ± 220 | – |
| 5.0 | 1620 ± 320 | 99.6 ± 0.01 | 310 ± 250 | 99.3 ± 0.01 |
| | 3100 ± 110 | – | 1000 ± 110 | – |

[a] Polymerization medium: 1% Dextran T 70; 0.1 M phosphate buffer, pH 7.4; temperature, 22–25°C; $n = 3$.

size homogeneity. Particle distribution profiles are not different from that of the unloaded nanoparticles. Higher concentrations of the drug resulted in a bimodal size distribution of particles related to the PQ concentrations (Fig. 1b). The appearance of increasing amounts of microparticles for PQ concentrations up to 2 mg/ml was reported in our previous study on PDEMM and by Gaspar et al. (1991).

In contrast with our early findings on PDEMM nanoparticles, the PQ dosage in the PAAMM and PCEMM nanoparticles is not concentration dependent: a high level of PQ entrapment (up to 90%) is always achieved in this series of PDAMM (Table 2).

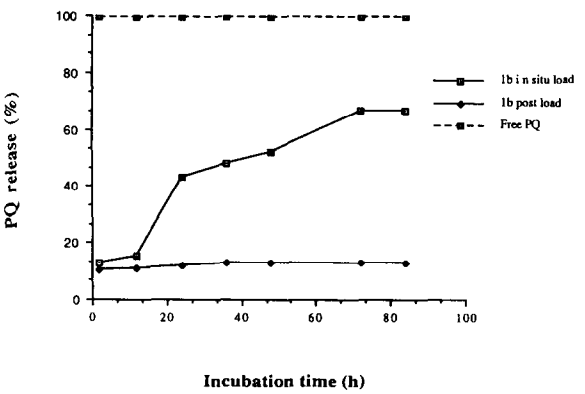



Fig. 2. Desorption of PQ from polyDAMM **1b** and **1c** as a function of incubation time; Medium, PBS, pH 7.5; temperature, and 37°C.

*PQ-post-loaded nanoparticles*

The adsorption of PQ diphosphate on preformed **1b** and **1c** nanoparticles was complete within 24 h for pH values ranging from 6.5 to 8.5 (**1b**) and from 7.5 to 8.5 (**1c**) (Table 3). In contrast with PDEMM (**1a**) nanoparticles, the drug adsorption onto free **1b** and **1c** nanoparticles was more rapid. The influence of pH, concentration and temperature was not significant. PQ concentrations up to 2 mg/ml in the incubation medium shifted the particles size to the micrometric range and gave a bimodal distribution (results not shown). Interestingly, in this polymer series, high pH values (up to 7.5) did not affect the stability of PQ as assessed by UV spectrometry.

*PQ release from nanoparticles*

The release of PQ in the incubation medium was very low. As reported in our previous study on PDEMM (**1a**), PQ seems to be strongly bound to the polymer. The effect of pH on the release of PQ was slight, although at pH 7.5 PAAMM released up to 60% of the entrapped drug within 72 h (Fig. 2).

*Influence of pH on the polymerization rate*

The effect of pH on the polymerization is significant at low pH values. The minimum pH values for the initiation of the polymerization reaction can be derived from Fig. 3 and are closely related to the monomer structure. Minima are respectively pH 5.5 for AAMM (**1b**) and pH 7.0 for CEMM (**1c**).

*Influence of pH on the nanoparticle size*

The particles size but not the size homogeneity is largely affected by the pH. A second-order

TABLE 3

*PQ adsorption onto empty poly(DAMM) nanoparticles at various pH values (PQ diphosphate concentration: 1 mg / ml [a])*

| Time (h) | pH 6.5 | pH 7.5 | pH 7.5 | pH 8.5 | pH 8.5 |
|---|---|---|---|---|---|
| | % adsorbed ± SD **1b** | % adsorbed ± SD **1b** | % adsorbed ± SD **1c** | % adsorbed ± SD **1b** | % adsorbed ± SD **1c** |
| 1 | 15.4 ± 0.03 | 30.6 ± 0.05 | 37.6 ± 0.05 | 30.0 ± 0.05 | 30.6 ± 0.02 |
| 3 | 42.9 ± 0.05 | 43.4 ± 0.07 | 58.4 ± 0.07 | 43.4 ± 0.07 | 40.4 ± 0.07 |
| 9 | 71.9 ± 0.02 | 58.8 ± 0.02 | 71.8 ± 0.02 | 58.08 ± 0.02 | 46.8 ± 0.02 |
| 12 | 82.5 ± 0.02 | 64.5 ± 0.02 | 73.5 ± 0.02 | 64.5 ± 0.02 | 58.5 ± 0.02 |
| 24 | 95.6 ± 0.02 | 73.3 ± 0.01 | 98.3 ± 0.01 | 75.0 ± 0.01 | 73.3 ± 0.01 |
| 72 | 99.5 ± 0.03 | 85.0 ± 0.02 | 98.5 ± 0.01 | 85.3 ± 0.03 | 87.3 ± 0.01 |

[a] Medium: 1% Dextran T 70; 0.1 M phosphate buffer, pH 7.4; temperature, 22–25°C; $n = 3$.

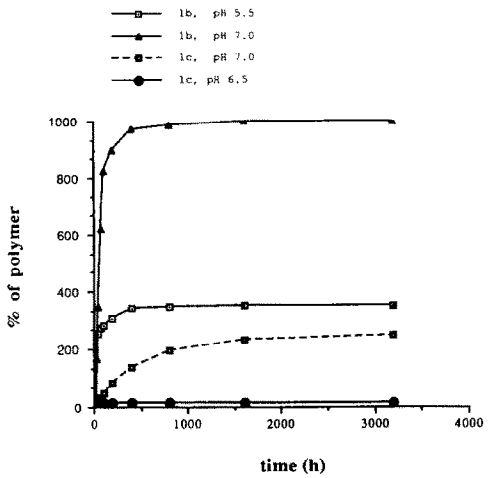



Fig. 3. Polymerization profile of polyDAMM as a function of the pH; medium, 0.1 M phosphate buffer, 1% dextran T 70; temperature, 22 and 37°C.

polynomial relationship (Fig. 4) clearly describes the pH influence. The optimal pH values for preparing submicron PAAMM and PCEMM nanoparticles of size less than 300 nm are 7.0 and 7.5 (PAAMM) and 7.5 (PCEMM).

*Influence of temperature*

The polymerization rate did not depend upon the temperature used under our experimental conditions (20, 25 and 37°C). Neither the size, nor the polydispersity index was affected at temperatures ranging from 20 to 30°C (results not shown).

*Influence of the stabilizer*

For the dextran series used in this study, the molecular weight affected the particle size and

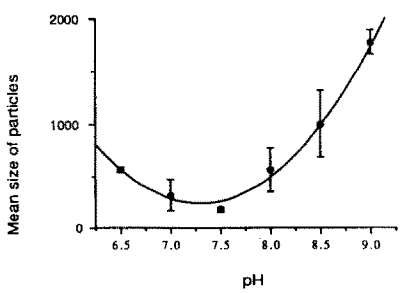



Fig. 4. Size and size distribution profile of PAAMM (**1b**) as a function of the pH; medium, 0.1 M phosphate buffer; 1% dextran T 70; temperature, 22 and 37°C.

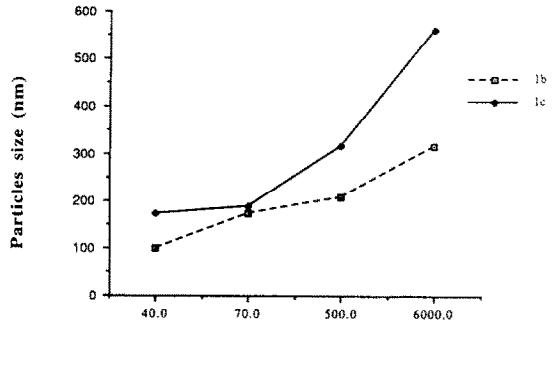



Fig. 5. Mean size of PAAMM and PCEMM nanoparticles as a function of the molecular weight of the stabilizer; medium, 0.1 M phosphate buffer, 1% dextran T 70, pH 7.5; temperature, 22 and 37°C.

homogeneity. Fig. 5 displays a quasi-linear relationship between the molecular weight of dextran and the particle size. To a much lower extent, high concentrations of the sugar (3–5% w/v) were shown to reduce the particle diameter to 10 nm in the particular case of PAAMM (Fig. 1a). No nanoparticles could be obtained when Pluronic F68 was used as surfactant (Fig. 1c).

## Discussion

In this study, the preparation of PAAMM and PCEMM nanoparticles was achieved by the polymerization reaction. In the polymerization approach, the influence of physicochemical factors, i.e., temperature, pH of the incubation medium, nature and concentration of the stabilizers or surfactants, etc., is one of the most important parameters to be considered for the resulting particle size and polydispersity index.

Our results show that the pH in the reaction medium and the concentration of PQ must be taken into account as the main factors which govern particle size.

The optimum pH range where particles size is minimal is 7.0–7.5 for both of the PDAMM congeners. This is advantageous since the nanoparticle preparation will be well tolerated by patients when given parenterally. From a pharmacotechnical point of view, this pH is also favourable to PQ

loading onto the polymer and to its subsequent desorption without any chemical decomposition.

Particle size alteration at lower and higher pH values has been reported and can partly be attributed to a change in the particle surface charges. Indeed, the pH value may alter the net charge of nanoparticles and, indirectly, their stability in the suspensions. With respect to this, the effects of the zeta potential on the stability of suspension were shown to be, in part, responsible for modulation of particle size (Lenaert et al., 1989; Lucks et al., 1990).

Concerning the drug adsorption characteristics, our results showed that PAAMM and PCEMM are superior to PDEMM in adsorbing PQ both in situ and in post-polymerization approaches. The adsorption is neither temperature- nor concentration-dependent and seems to be faster (post-polymerization process): 70–90% of the drug is adsorbed in less than 24 h.

The concentration of 1 mg/ml of PQ diphosphate in the polymerization medium does not significantly change the mean particle size and even may induce a slight decrease (178–100 nm). This advantageous effect of PQ on the particle size could be attributed to a catalytic action of PQ (which is an amine) on the polymerization process as proposed previously for doxorubicin, vidarabin and insulin towards the anionic polymerization of cyanoacrylic monomers (Donnely et al., 1977; Couvreur et al., 1986; Damge et al. 1990; Guise et al. 1990). Furthermore, the polymerization reaction has been assumed to occur via a two-step mechanism: a nucleation step and a growth step (Vauthier-Holtzscherer et al., 1991). In the hypothesis that PQ could catalyze the generation of radicals in the continuous phase, the nucleation step should be instantaneous all over the dispersion system and consequently limit the growth step in the whole external aqueous phase of the emulsion for want of available monomer reservoirs.

Unfortunately, owing to its concentration in the medium, PQ may play another contradictory role during the polymerization process by increasing the particle size at high concentration. Similarly, Couvreur et al. (1986), Guise et al. (1990) and Damge et al. (1990) observed modifications of nanoparticle molecular weight profiles after drug loading onto polymers. Such an increase was attributed to colloidal instability resulting from the binding of drugs to nanoparticles at high concentrations (Douglas et al., 1984; Henry-Michelland et al., 1987; Brasseur et al., 1991). Furthermore, the occurence of a zeta potential alteration under PQ coating might not be excluded as a possible mechanism of nanoparticle agregation.

The influence of stabilizers on the nanoparticle size and size distribution was investigated and interpreted by reference to steric stabilization (Douglas et al., 1985). PAAMM and PCEMM stable nanoparticles can be prepared with a wide range of dextrans (Dextran® 40, 70, 500) in the concentration range of 0.1–1%. Interestingly, nanoparticles of size 10 nm have been obtained using high dextran concentrations (Dextran® 500, 5% w/v) in the polymerization medium. This result could be ascribed to the increased viscosity of the continuous aqueous phase of the emulsion due to the high concentration of the stabilizer. The viscosity could hinder the diffusion of sprayed fine monomer droplets and thus meeting through the continuous phase and growing into larger particles. Nevertheless, the use of 5% w/v of dextran is not suitable for the isotonicity of nanoparticle parenteral preparations since an equal amount of glucose must be added before freeze-drying.

Dextrans of molecular weight up to 500 are not able to stabilize the particle size which shifts to micrometrical size. We are not yet able explain this abnormal behaviour. The dramatic effect of Pluronic F68 on the polymerization of PDAMM also remains unexplained, since very small polycyanoacrylic nanoparticles have been prepared by using this surfactant (Seijo et al., 1990).

The release of PQ by PAAMM nanoparticles (up to 60% at pH 7.5) may be related to the polymorphism of the nanoparticles as a function of pH. Indeed, polymers display local crystalline and amorphous properties in a single molecule and the pH is a key parameter in the polymerization process leading to the formation of solid nanoparticles. In particular, the release of entrapped drugs appears to be modulated by the

degree of crystallinity of the solvated drug-loaded nanoparticles.

## Conclusion

Poly(dialkylmethylidenemalonate) esters constitute a new class of valuable biomaterials displaying much potential as alternative drug delivery systems. PAAMM and PCEMM have demonstrated many advantages compared to the corresponding polycyanopolymethyl-acrylic carriers: ease of multigram synthesis and polymerization in 100–300 nm particles, wide range of usable pH values, temperature and stabilizers, significant improvement of their drug loading and drug desorption capacity in comparison with PDEMM.

In view of the above results, PAAMM and PCEMM nanoparticles should receive more attention and could be proposed as effective drug carriers. More specifically, PAAMM which displays improved physicochemical and drug adsorption/release characteristics is an attractive candidate for further studies.

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