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On shelf stability of freeze-dried poly(methylidene malonate 2.1.2) nanoparticles

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Abstract

Freeze-dried poly(methylidene malonate 2.1.2) (PMM 2.1.2)-made nanoparticles were evaluated for their 12-month stability under various storage conditions of temperature and illumination. At different period of time after their preparation, nanoparticles were resuspended and tested for their size, and the molecular weight and reversed-phase HPLC profiles of their components. The suspension pH, its turbidity at 400 nm as well as its in vitro cytotoxicity on CEM T cell line were also measured. Generally, nanoparticles maintained at 40°C underwent significant alterations revealed by the suspension pH decrease, the progressive modification of the HPLC chromatogram and the decreased cytotoxicity. Degradation of the polymer side chains and generation of carboxyl moieties could account for these observations. From the 1 year measurements here reported, lyophilized PMM 2.1.2 colloidal nanoparticles conserved at room temperature or below, either in darkness or in daylight, may be assumed to have a satisfactory shelf-life.

Keywords: Nanoparticles; Polymeric colloids; Methylidene malonate; Freeze-dried colloid stability; Colloid physico-chemical characterization

1. Introduction

The potential interest of polymeric nanoparticulate structures for the controlled delivery of therapeutic agents was first underlined in the late 1970s and early 1980s (Birrenbach and Speiser, 1976; Kreuter and Speiser, 1976; Couvreur et

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al., 1977, 1979; Gurny et al., 1981). Since that time, various polymers or co-polymers were developed to elaborate new biodegradable colloidal carriers capable of optimizing the bioavailability and the biodistribution of drugs throughout the body after parenteral or enteral administration (Allémann et al., 1993). From such efforts, especially emerged poly(alkylcyanoacrylate) (PACA), poly(alkylmethacrylate) (PAMA), poly(D,L-lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA) and poly(ϵ-caprolactone) (PECL)-made particles whose characteristics and potentialities were extensively described in the past 15 years literature (Rolland et al., 1986; Couvreur, 1988; Couvreur and Vauthier, 1991; Coffin and McGinity, 1992; Gref et al., 1994; Hanes et al., 1995).

Poly(methylidene malonate 2.1.2) nanoparticles have recently been developped with the aim of improving the intrinsic properties of polymeric colloids used in drug delivery and targeting strategies or in other pharmaceutical purposes (Breton et al., 1994a; Lescure et al., 1994). Some studies carried out until now highlighted their satisfactory degradability/erodibility (Breton et al., 1994a; Lescure et al., 1994) as well as their low in vitro (Breton et al., 1994a; Lescure et al., 1994) and in vivo (Breton et al., 1994b) toxicity. Furthermore, additional works focused on the preparation and characterization of monoclonal antibody-covered PMM 2.1.2 nanoparticles (i.e. anti-CD4 and anti-β_2 microglobulin immunonanoparticles) (Breton et al., 1996; Velge-Roussel et al., 1996) which were shown to have a potential importance in the treatment of AIDS (Bru-Magniez et al., 1994, 1996).

As for many other kinds of colloidal preparation, PMM 2.1.2 nanoparticles were conveniently stored under freeze-dried form. The scope of the present investigation was to determine the precise physicochemical stability of lyophilized PMM 2.1.2 nanoparticles kept at different temperatures, in broad daylight or in the dark.

2. Materials and methods

2.1. Chemicals

Methylidene malonate 2.1.2 (MM 2.1.2) was synthesized by UPSA organic chemistry department (Rueil Malmaison, France) as described by Bru-Magniez et al. (1988) and Lescure et al. (1991). Sulfur dioxyde-saturated MM 2.1.2 was maintained in 10 ml sealed ampoules at -20°C until used. Dextran ($M_r = 70\,000$) was purchased from Fluka Chemie AG (Buchs, Switzerland). The reagent 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chimie (L'Isle d'Abeau Chesnes, France). Polymerization buffer was prepared with phosphate salts from Prolabo (Paris, France) whereas RPMI 1640 cell culture medium, fetal bovine serum (FBS), L-glutamine and antibiotics were provided by Gibco (Cergy-Pontoise, France).

2.2. Cells

Nanoparticle in vitro cytotoxicity was evaluated on CEM T lymphoblastoid cell line kindly provided by Dr. J.C. Chermann (INSERM U322, Marseille). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 100 μg ml^{-1} neomycin. They were seeded at 5×10^4 cells ml^{-1} and split every 4 days.

2.3. Preparation of poly(methylidene malonate 2.1.2)-made nanoparticles

Freeze-dried PMM 2.1.2 nanoparticles were prepared according to a scaled-up adaptation of the process reported by Breton et al. (1994a) and Lescure et al. (1994). The methodology and the instrumentation employed are precisely given on Fig. 1. The yield of nanoparticle formation was calculated as described by Lescure et al. (1994). Nanoparticle characteristics gathered together in Table 1 provided the evidence that the semi-industrial methodology herein used lead to colloidal suspensions (250 ml) similar to those previously produced at low scale (10 ml) (Breton et al., 1994a; Lescure et al., 1994). Particles were kept either at 4, 25 or 40°C in the dark or at 25°C in daylight for different periods of time.

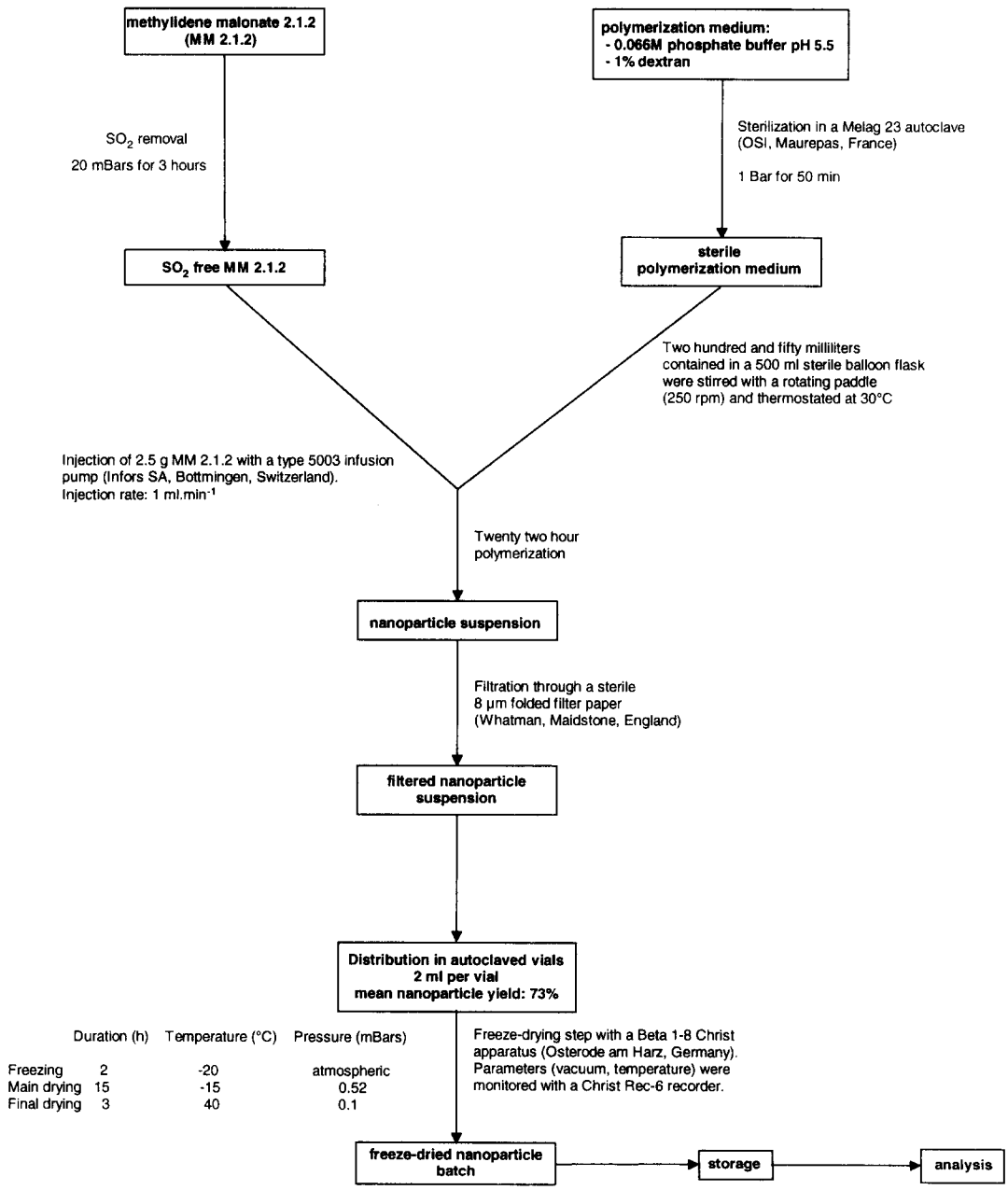

Fig. 1. Schematically summarized PMM 2.1.2 nanoparticle preparation process.

2.4. Nanoparticle physico-chemical assessment

At each time selected, freshly resuspended freeze-dried nanoparticles (2 ml water per vial) were analyzed for the following parameters.

2.4.1. pH

Determination of the colloidal suspension pH was carried out without any preliminary dilution with a well-calibrated M240 Corning pH meter (Corning, NY).

Table 1
Comparison of two different size PMM 2.1.2 nanoparticle batches prepared through distinct procedures

	Batch size (ml)		Significance
	10 ($n=3$)	250 ($n=9$)	
pH ($\pm$ S.D.)	5.32 $\pm$ 0.10	5.20 $\pm$ 0.09	NS
Optical density ($\pm$ S.D.)	0.88 $\pm$ 0.06	0.93 $\pm$ 0.05	NS
Size ($\pm$ S.D., nm)	470 $\pm$ 47	545 $\pm$ 56	NS
Molecular weight ($\pm$ S.D., polystyrene eq.)	631 $\pm$ 11	645 $\pm$ 10	NS
CC_{50} ($\pm$ S.D., μg ml^{-1})	11.1 $\pm$ 1.1	11.6 $\pm$ 1.0	NS

NS, not significant. Student's t-test was applied with $P<0.05$.
S.D., standard deviation.
CC_{50}, 50% cytotoxic concentration determined on 10^6 CEM cells ml^{-1}.

2.4.2. Turbidity

Seventy five μl of nanoparticle suspension were diluted in 2 ml water contained in a 1-cm path length plastic cuvette hold in the stage of a Model 25 spectrophotometer (Beckman, Gagny, France). Turbidity was read at 400 nm.

2.4.3. Size

A 37.5-μl nanoparticle suspension aliquot were added to 3 ml water. The resulting suspension was introduced in a 1-cm path length plastic cuvette placed in the stage of a Model N4 submicrometer particle sizer (Coulter Electronics, Hialeah, FL). Particle diameters were expressed as the mean of five consecutive measurements.

2.4.4. Molecular weight of nanoparticle-constituting molecules

The molecular weight distribution of PMM 2.1.2 polymers was investigated by Size Exclusion Chromatography (SEC). Equipment from Shimadzu (Kyoto, Japan) comprised a LC-6A solvent delivery module, a RID-6A differential refractometer and a CTO-6A column oven. Maintained at 35°C, serially installed Shodex KF.800P pre-column (1 $\times$ 0.46 cm), Styragel HR1 (30 $\times$ 0.78 cm) and Styragel HR4E (30 $\times$ 0.78 cm) columns (Waters, Saint-Quentin-en-Yvelines, France) were calibrated with a polystyrene standard. Eluant was tetrahydrofurane (THF) flowed at 1 ml min^{-1}. For sampling, nanoparticles were pelleted (120 000 $\times$ g for 5 min) and solubilized in THF containing 0.2% toluene as an internal reference. Residual dextran was eliminated by filtration through a Fluoropore (PTFE/polypropylene) filter (Millipore, Yonezawa, Japan). Thirty μl of this solution were injected via a 20-μl loop (injection valve from Rheodyne, Cotati, CA) through both columns. Relative refractive index was monitored versus time and data processing achieved by a GPC software (version 4.02) from Polymer Laboratories (Church Stretton, England) provided molecular weights (M_w and M_p) expressed in polystyrene equivalent (p.e.).

2.4.5. HPLC analysis of PMM 2.1.2 polymers

High performance liquid chromatography analyses were achieved with an 110B isocratic HPLC pump from Beckman equipped with a C-18 Hypersil ODS reversed-phase column (25 $\times$ 0.46 cm) (Touzart et Matignon, Vitry-sur-Seine, France) kept at 30°C in a Croco-Cil oven (Prolabo). The mobile phase was water/acetonitrile (70:30, v/v) plus 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany). Ultra-violet 166 programmable detector module (Beckman) operated at 210 nm. Sample preparation was as described for SEC, except that nanoparticle pellets were dissolved in the mobile phase prior to be filtered and injected (20 μl/injection). Data processing was performed with a DS 4000 chromatography workstation (version 5.00) (Polymer Laboratories).

Table 2
Comparison of nanoparticle characteristics before and after lyophilization

	Before lyophilization[a] ($n = 3$)	After lyophilization[a] ($n = 10$)	Significance
pH (± S.D.)	5.25 ± 0.10	5.20 ± 0.09	NS
Optical density (± S.D.)	0.99 ± 0.06	0.93 ± 0.05	NS
Size (± S.D., nm)	505 ± 45	545 ± 56	NS
Molecular weight (± S.D., polystyrene eq.)	643 ± 13	645 ± 10	NS
CC_{50} (± S.D., $\mu g\ ml^{-1}$)	10.9 ± 0.9	11.6 ± 1.0	NS

[a] 250-ml batches.

2.4.6. Nanoparticle in vitro cytotoxicity

Colorimetric MTT-based assay was performed according to Denizot and Lang (1986). Briefly, CEM cells suspended in phenol red-free medium (10^6 cells per ml) were distributed into 96-well plates (Costar, Cambridge, MA) (100 μl per well) and incubated at 37°C in a humidified and CO_2-enriched (5%) atmosphere with increasing nanoparticle concentrations for 48 h. Then, 10 μl of 5 mg/ml MTT in a complete phenol red-free medium were added for an additional incubation period at 37°C in the same conditions as described above. Only viable cells could metabolize MTT into water insoluble MTT formazan. Four h later, 100 μl per well of an isopropanol/HCl 1 N (90:10, v/v) mixture allowed purple MTT formazan crystals to get smoothly solubilized after gentle back and forth pipetting. Optical density of the resulting solution was read against control at 570 nm on a EL310 Bio-tek Instrument microplate reader (Winooski, VT). Accordingly, nanoparticle cytotoxicity was expressed in percentage of cell viability as it follows:

$$\text{Cell viability}(\%) = \frac{OD_{sample}}{OD_{control}} \times 100$$

$OD_{control}$ was determined from untreated CEM cells. From each viability curve, the 50% cytotoxic concentration (CC_{50}) was calculated with version 3.00 Grafit software (Erithacus Software, Staines, UK).

3. Results

A preliminary investigation showed that the lyophilization performed as described in the section above did not impair the colloidal suspension characteristics (Table 2).

Stability of freeze-dried PMM 2.1.2-made nanoparticles was studied over a year of storage under different conditions of temperature and illumination. Several physico-chemical parameters of freshly reconstituted colloidal suspensions were monitored. Variations of the mean nanoparticle size, their in vitro cytotoxicity on cell culture, the molecular weight (M_w and M_p) and the reversed-phase HPLC profile of their constituents were evaluated concomitantly with pH and turbidity at 400 nm.

On Fig. 2 is shown the pH evolution versus time. Nanoparticles maintained at 40°C and, in a lesser extend, at 25°C lead to more and more acidic suspensions. As temperature had a dramatic effect on proton concentration, illumination did not seem to have a crucial influence since

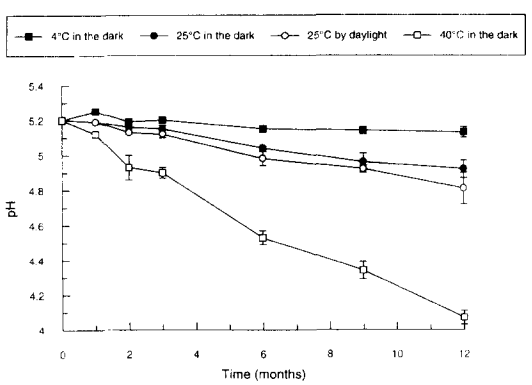

Fig. 2. Evolution of the pH of PMM 2.1.2 nanoparticle suspension. At each time considered, freeze-dried particles, until then kept in different storage conditions, were extemporaneously resuspended before measuring proton concentration ($n = 4$).

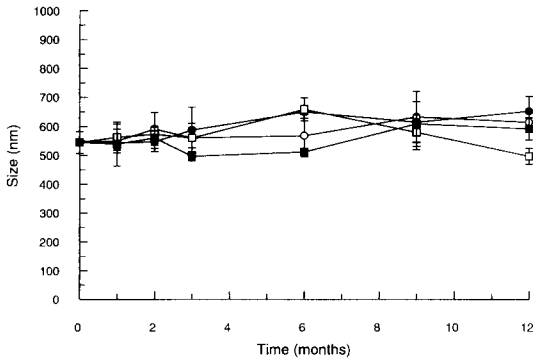

Fig. 3. Variations of the size of freeze-dried PMM 2.1.2 nanoparticles over 12 month storage in the same conditions as in Fig. 2 (see Fig. 2 for dot legend) ($n = 4$).

curves obtained from colloids stored at 25°C in the dark or in daylight are roughly superimposable.

Whatever the storage conditions considered, no significant change was noticed about nanoparticle size (Fig. 3) and turbidity which depends of the size and the concentration of suspended particles (Fig. 4). Similarly, size exclusion chromatograms did not evolve and revealed no noticeable modification in the molecular weight distribution of intrinsic polymers and oligomers (Fig. 5).

Going over Fig. 6 provided the evidence of a time-dependent impairment of the HPLC chromatogram observed when injecting constituents of nanoparticles kept at 40°C in the dark. Indeed, in these storage conditions, as the relative area of peak 1 increased from 5% at $t = 0$ to 20% at

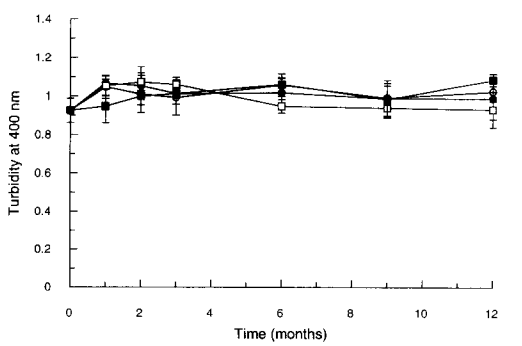

Fig. 4. Turbidity variations of freshly resuspended freeze-dried PMM 2.1.2 nanoparticles maintained for various periods of time in the conditions described in Section 2 (see Fig. 2 for dot legend) ($n = 4$).

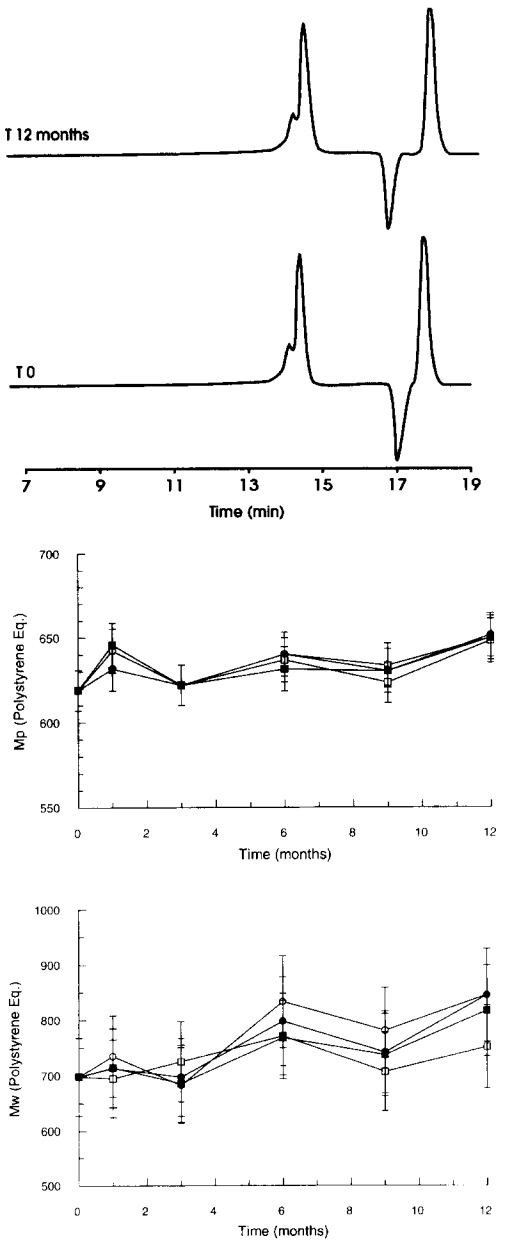

Fig. 5. Size exclusion chromatography analysis of constituents of PMM 2.1.2 nanoparticles stored in various conditions (see Fig. 2 for dot legend) for different times. Profiles recorded at $t = 0$ and $t = 12$ months (40°C in the dark) were superimposed for comparison (top panel). Data processing at 0, 1, 3, 6, 9 and 12 months allowed the monitoring of molecular weights M_p (middle panel) and M_w (bottom panel) over 1 year ($n = 5$).

$t = 12$ months, the one of peak 2 went down from 71% to 47% (Fig. 6, top and bottom). At 4 or

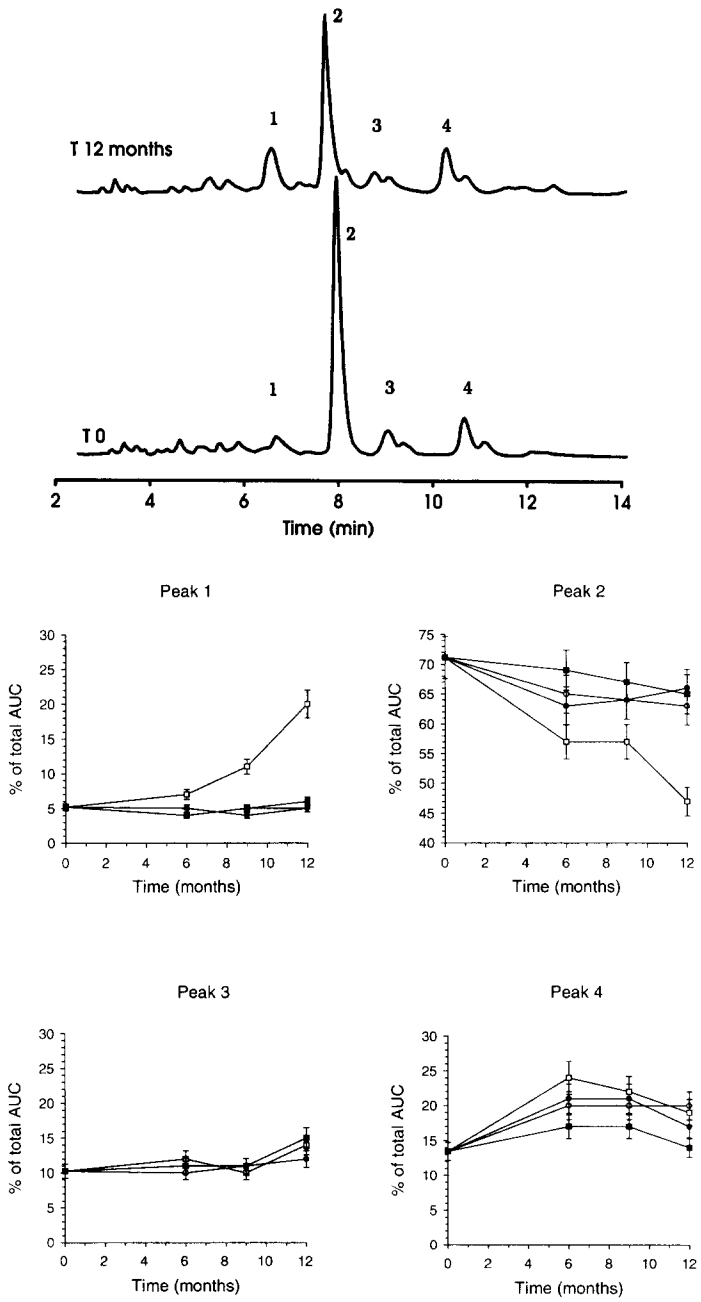

Fig. 6. Methylidene malonate 2.1.2 oligomerization products involved in nanoparticle formation were analyzed by HPLC after 0, 6, 9 and 12 months of colloid storage in the conditions described in Fig. 2. Compared profiles obtained at $t = 0$ and $t = 12$ months (40°C in the dark) showed four peaks numbered from 1 to 4 (top panel). Eluant flow rate was maintained at 0.5 ml min^{-1} under a constant pressure of 1000 psi. Time evolution of relative area-under-curve (AUC) for each of these peaks was observed for all the duration of the present study (bottom panel) ($n = 4$).

25°C, peak 1 remained unchanged throughout the study, while peak 2 slightly decreased to about 65% (Fig. 6, bottom). Meanwhile, irrespective of storage temperature or illumination, peak 3

integration was approximately constant with an area-under-curve covering $13 \pm 2\%$. Concerning peak 4, no clear tendency could be released before additional studies were done to clarify oligomerization/polymerization processes and to elucidate resulting molecular structures.

In vitro cytotoxicity of PMM 2.1.2 nanoparticles was conducted on CEM cells, 0, 1, 2, 3, 6, 9 and 12 months after nanoparticle preparation. The graph shown on Fig. 7 demonstrates that CC_{50} values were independent of storage conditions and remained approximately the same at about 9–13 $\mu g\ ml^{-1}\ 10^6\ cells^{-1}$ for the first 6 months. Beyond this time, nanoparticle cytotoxicity seemed to decrease (CC_{50} increased) and appeared to be temperature sensitive since at the conclusion of the study CC_{50}s were about 29 $\mu g\ ml^{-1}\ 10^6\ cells^{-1}$, 22 $\mu g\ ml^{-1}\ 10^6\ cells^{-1}$ and 16 $\mu g\ ml^{-1}\ 10^6\ cells^{-1}$ at 40, 25 and 4°C, respectively. Once again, no influence of illumination could be noticed.

4. Discussion

The pharmaceutical and biopharmaceutical value of polymeric colloidal drug carriers is now well-established by many potential applications reported in the literature. Obviously, the preservation of these particulate systems in conditions maintaining their integrity and their usefulness for a long period of time was one of the important features which should early be considered before any further development.

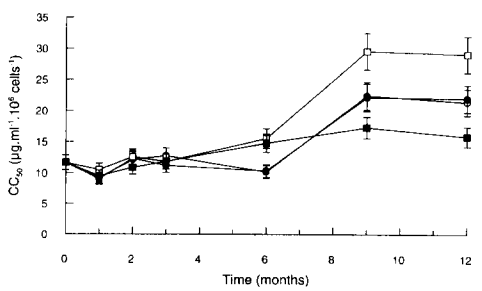

Fig. 7. Fifty percent cytotoxic concentration (CC_{50}) variations of PMM 2.1.2 nanoparticles kept up to 12 months in the conditions given in Fig. 2 and incubated with CEM cells as described in Section 2 (see Fig. 2 for dot legend) ($n = 4$).

If particles are stored as a suspension in an aqueous medium, degradation and/or solubilization of the polymer, drug leakage, drug desorption and/or drug degradation may occur (Kreuter, 1985). Thus, lyophilization probably represents one of the most useful methodology to ensure the long-term conservation of unloaded or drug-bound polymeric nanoparticles. Kreuter and Hartmann (1983) were among the first in reporting the freeze-drying of poly(butylcyanoacrylate) nanoparticles. Soon, this technique became widely employed (Douglas et al., 1985; Krause et al., 1985; Manil et al., 1986; Kubiak et al., 1988; Magenheim and Benita, 1991) and later on extended to other kinds of colloidal drug carriers such as solid lipid nanoparticles (SLN) (Müller et al., 1995) or hydrosol (Gust et al., 1995).

Results of experiments solely based on pre- and post-freeze-drying size measurements of colloids made of different materials demonstrated the relevance of this dessication process applied to these brittle particles, although it requires the use of cryoprotectors such as trehalose, maltose, lactose, mannitol, sorbitol or glucose (Auvillain et al., 1989; Seijo et al., 1990). Moreover, there were very few published studies which really scrutinized the pertinence of lyophilization with regard to the stability of freeze-dried particles over several month storage. Only papers by Fouarge et al. (1989) and Gaspar et al. (1991) underlined that the size of PIHCA nanoparticles and the level of drug binding were not altered after 12 or 24 month storage in a deep-freezer.

Freshly prepared PMM 2.1.2 nanoparticles were freeze-dried in presence of 1% dextran (w/v), acting alone at one and the same time as a colloidal stabilizer and as a cryoprotector. The stability in different storage conditions of these preparations was assessed by monitoring the evolution of some physico-chemical parameters whereas, simultaneously, their in vitro cytotoxicity was controlled. Among the measured nanoparticle characteristics, suspension pH, toxicity on cell culture and HPLC profile of constitutive oligomers were shown to be significantly sensitive to storage temperature conditions. It may be assumed that these three concomitant

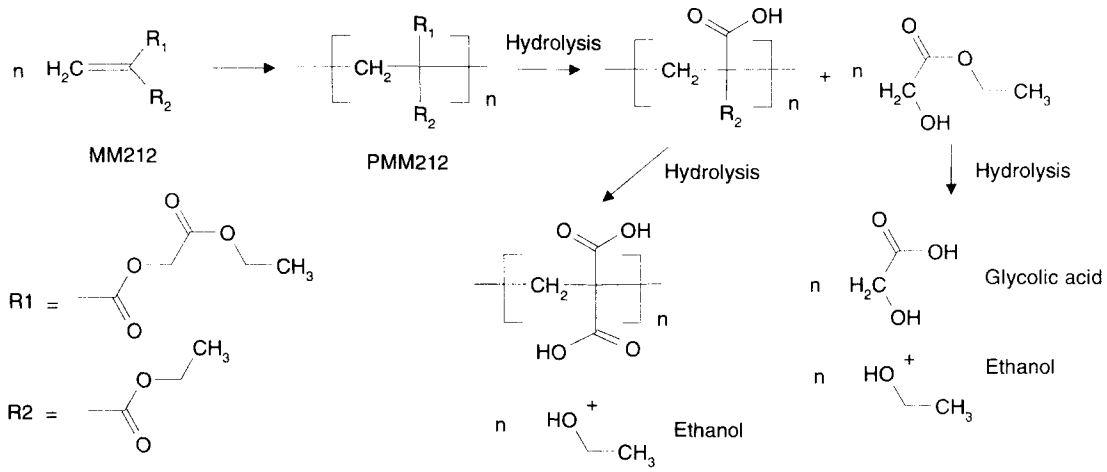

Fig. 8. The putative degradation pathway of PMM 2.1.2 side chains.

observations could probably reveal the same nanoparticle impairing phenomenon. Indeed, Breton et al. (1994a) and Lescure et al. (1994) previously demonstrated that nanoparticle-constituting PMM 2.1.2 polymers could undergo an erosion of their side chains via an ester hydrolysis which yields ethanol and carboxyl-bearing polymers. Even if it happened through a slower kinetic, this degradation process first reported for nanoparticles suspended in aqueous media, could occur with non-anhydrous freeze-dried nanoparticles and generate water soluble acidic polymers responsible for lowering the pH of the aqueous reconstituted colloidal suspensions.

High performance liquid chromatography profiles of solubilized nanoparticles showed four major peaks numbered from 1 to 4 (Fig. 6). Whatever the storage conditions considered, peaks 3 and 4 did not significantly evolve to allow clear and plausible interpretations. However, only for nanoparticles maintained at 40°C, the integration of chromatograms brought the evidence that peak 1 area increased as that of peak 2 concurrently decreased. Recent and not yet published data showed that, in the present experimental HPLC conditions, only oligomers up to pentameric structures were identified while higher molecular weight polymers were not eluted from the column. Hypothetically, it may be considered that compound(s) corresponding to peak 2 would progressively be altered through ester hydrolysis (Fig. 8). Thus, peak 1 could then identify these new molecular species with dramatically different physico-chemical characteristics. Especially, these more hydrophilic products would interact in a lesser extend with the column stationery phase and, of course, would be eluted sooner than unmodified oligomers.

Does storage time and temperature dependence of PMM 2.1.2 nanoparticle in vitro cytotoxicity correlate with pH and HPLC profile changes? The cytotoxicity of PMM 2.1.2 nanoparticles incubated for 48 h with cultured cells was initially examined on L929 fibroblast cell line. Data then published by Lescure et al. (1994) determined a CC_{50} of about 50 μg ml^{-1} 10^5 cells^{-1}. In the present investigation, similar experiments provided side information whereby CEM cell line was much more sensitive to this polymeric material than L929 cells.

The degradation pathway of PMM 2.1.2 side chains proposed by Lescure et al. (1994) and depicted on Fig. 8, postulated that ethanol and glycolic acid were produced. Up to about 100 μg

ml^{-1}, none of both compounds was shown cytotoxic or cytostatic after a 48 h incubation period with cultured L929 fibroblasts and, in the same way, no marked cell growth stimulation was noticed. So, although present investigations were conducted on a different cell line, these previous data suggest that neither ethanol nor glycolic acid could account for the decreased nanoparticle cytotoxicity observed between the time points 6 and 12 months (for conservation at 25°C and, especially, at 40°C). Degradation of PMM 2.1.2 into polycarboxylic acid could easily be achieved by treating nanoparticles in an alkaline medium which catalyzed ester hydrolysis of intact polymers. Thus, when incubated with L929 cells, these degraded and solubilized nanoparticles were approximately tenfold less cytotoxic than the untreated colloids (i.e. $CC_{50} \cong 400$ μg ml^{-1} versus 40 μg ml^{-1}) (Lescure et al., 1994). Based on this previous observation and because of their particular physico-chemical characteristics, it might be proposed that such water soluble carboxyl-bearing oligomers/polymers generated during the nanoparticle storage behaved as polyanions having a reduced cytotoxicity compared to the parental unmodified PMM 2.1.2. Moreover, according to the model proposed by Lherm et al. (1992), the in vitro cytotoxicity of PACA nanoparticles would essentially be due to high concentrations of polymer degradation products released in the close environment of plasma membrane after colloidal particles got adsorbed at the cell surface. This interpretation could also be evoked to explain the graph depicted on Fig. 7. Effectively, this phenomenon could occur with intact PMM 2.1.2 nanoparticles as well. It is obvious that more the colloidal system will be altered during its storage, less this phenomenon will have chance to apply since degraded PMM 2.1.2 being more hydrosoluble, less particles will go and interact with the cell surface and, consequently, lower potentially toxic concentrations of degradation erosion products will be generated in the immediate cell surrounding.

The present paper provides the most complete investigation ever published about the long-term stability of freeze-dried polymeric nanoparticles. Subject to storage temperatures set under 25°C (preferably 4°C), this study clearly highlights that PMM 2.1.2 nanoparticles prepared as herein described can be lyophilized and kept for at least 1 year without specific lighting requirement.

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References

Allémann, E., Gurny, R. and Doelker, E., Drug-loaded nanoparticles - Preparation methods and drug targeting issues. *Eur. J. Pharm. Biopharm.*, 39 (1993) 173–191.

Auvillain, M., Cavé, G., Fessi, H. and Devissaguet, J.P., Lyophilisation de vecteurs colloïdaux submicroniques. *STP Pharma*, 5 (1989) 738–744.

Birrenbach, G. and Speiser, P.P., Polymerized micelles and their use as adjuvants in immunology. *J. Pharm. Sci.*, 65 (1976) 1763–1766.

Breton, P., Roy, D., Marchal-Heussler, L., Seguin, C., Couvreur, P. and Lescure, F., New poly(methylidene malonate 2.1.2) nanoparticles: recent developments. In Gregoriadis, G., McCormack, B. and Poste, G. (Eds), *Targeting of Drugs, 4. Advances in System Constructs.* NATO ASI series, Series A: Life Sciences, Vol. 273, Plenum Press, New York, 1994a, pp. 161–172.

Breton, P., Seguin, C., Roy, D., Couvreur, P. and Lescure, F., In vivo evaluation of poly(methylidene malonate 2.1.2) nanoparticle toxicity. *Proc. Int. Symp. Control. Release Bioact. Mater.*, 21 (1994b) 608–609.

Breton, P., Guillon, X., Roy, D., Tamas, S., Marchal-Heussler, L., Bru, N. and Lescure, F., Evaluation of the interaction between poly(methylidene malonate 2.1.2) nanoparticles and an anti-CD4 by surface plasmon resonance (SPR). *Eur. J. Pharm. Biopharm.*, 42 (1996) 95–103.

Bru-Magniez, N., De Cock, C., Poupaert, J., De Keyser, J.L. and Dumont, P., Procédé de préparation de monoesters ou diesters de l'acide endoéthano-9,10dihydro-9,10anthracène dicarboxylique-11,11, nouveaux monoesters ou diesters ainsi préparés et utilisation de ceux-ci pour la préparation de methylidène-malonates symétriques ou asymétriques. *Eur. Patent EP 0 283 364 A2*, 21 September, 1988.

Bru-Magniez, N., Chermann, J.C., Lescure, F., Teulon, J.M. and Breton, P., Immunoparticles bearing monoclonal anti-

CD4 antibodies and utilisation thereof. *PCT World Patent WO 94/24168*, 27 October, 1994.

Bru-Magniez, N., Chermann, J.C., Lescure, F., Teulon, J.M., Breton, P. and Guillon, X., Immunonanoparticles coated with anti-beta-2 microglobulin monoclonal antibodies. *PCT World Patent WO 96/02278*, 1 February, 1996.

Coffin, M.D. and McGinity, J.W., Biodegradable pseudolatexes: the chemical stability of poly(D,L-lactide) and poly(ϵ-caprolactone) nanoparticles in aqueous media. *Pharm. Res.*, 9 (1992) 200–205.

Couvreur, P., Polyalkylcyanoacrylates as colloidal drug carriers. *CRC Crit. Rev. Ther. Drug Carrier Syst.*, 5 (1988) 1–20.

Couvreur, P. and Vauthier, C., Polyalkylcyanoacrylate nanoparticles as drug carrier: present state and perspectives. *J. Control. Release*, 17 (1991) 187–198.

Couvreur, P., Tulkens, P., Roland, M., Trouet, A. and Speiser, P., Nanocapsules: a new type of lysosomotropic carrier. *FEBS Lett.*, 84 (1977) 323–326.

Couvreur, P., Kante, B., Roland, M., Guiot, P., Bauduin, P. and Speiser, P., Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J. Pharm. Pharmacol.*, 31 (1979) 331–332.

Denizot, F. and Lang, R., Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods*, 89 (1986) 271–277.

Douglas, S.J., Davis, S.S. and Holding, S.R., Molecular weights of poly(butyl2-cyanoacrylate) produced during nanoparticle formation. *Br. Polym. J.*, 17 (1985) 339–342.

Fouarge, M., Dewulf, M., Couvreur, P., Roland, M. and Vranckx, H., Development of dehydroemetine nanoparticles for the treatment of visceral leishmaniasis. *J. Microencapsul.*, 6 (1989) 29–34.

Gaspar, R., Préat, V. and Roland, M., Nanoparticles of polyisohexylcyanoacrylate (PIHCA) as carriers of primaquine: formulation, physico-chemical characterization and acute toxicity. *Int. J. Pharm.*, 68 (1991) 111–119.

Gref, R., Minamitake, Y., Peracchia, M.T., Trubetskoy, V., Torchilin, V. and Langer, R., Biodegradable long-circulating polymeric nanospheres. *Science*, 263 (1994) 1600–1603.

Gurny, R., Peppas, N.A., Harrington, D.D. and Banker, G.S., Development of biodegradable and injectable latices for controlled release of potent drugs. *Drug Dev. Ind. Pharm.*, 7 (1981) 1–25.

Gust, R., Bernhardt, G., Spruβ, T., Krauser, R., Koch, M., Schönenberger, H., Bauer, K.H., Schertl, S. and Lu, Z., Development of a parenterally administrable hydrosol preparation of the 'third generation platinum complex' [(±)-1,2-bis(4-fluorophenyl)ethylenediamine]dichloroplatinum(II). Part 1. Preparation and studies on the stability and antitumor activity. *Arch. Pharm.*, 328 (1995) 645–653.

Hanes, J., Chiba, M. and Langer, R., Polymer microspheres for vaccine delivery (Chpt. 16). In Powell, M.F. and Newman, M.J. (Eds.), *Vaccine Design: the Subunit and Adjuvant Approach*, Vol. 6. Plenum Press, New York, 1995, pp. 389–412.

Krause, H.J., Schwarz, A. and Rohdewald, P., Polylactic acid nanoparticles, a colloidal drug delivery system for lipophilic drugs. *Int. J. Pharm.*, 27 (1985) 145–155.

Kreuter, J., Poly(alkyl acrylate) nanoparticles. *Methods Enzymol.*, 112 (1985) 129–138.

Kreuter, J. and Hartmann, H.R., Comparative study of the cytostatic effects and the tissue distribution of 5-fluorouracil in a free form and bound to polybutylcyanoacrylate nanoparticles in sarcoma 180-bearing mice. *Oncology*, 40 (1983) 363–366.

Kreuter, J. and Speiser, P.P., In vitro studies of poly(methyl methacrylate) adjuvants. *J. Pharm. Sci.*, 65 (1976) 1624–1627.

Kubiak, C., Manil, L. and Couvreur, P., Sorptive properties of antibodies onto cyanoacrylic nanoparticles. *Int. J. Pharm.*, 41 (1988) 181–187.

Lescure, F., Zimmer, C., Roy, D., Teulon, J.M. and Couvreur, P., Synthesis and evaluation of a new biodegradable monomer. *Proc. Int. Symp. Control. Release Bioact. Mater.*, 18 (1991) 325–326.

Lescure, F., Seguin, C., Breton, P., Bourrinet, P., Roy, D. and Couvreur, P., Preparation and characterization of novel poly(methylidene malonate 2.1.2.)-made nanoparticles. *Pharm. Res.*, 11 (1994) 1270–1277.

Lherm, C., Müller, R.H., Puisieux, F. and Couvreur, P., Alkylcyanoacrylate drug carriers. II. Cytotoxicity of cyanoacrylate nanoparticles with different alkyl chain length. *Int. J. Pharm.*, 84 (1992) 13–22.

Magenheim, B. and Benita, S., Nanoparticle characterization: a comprehensive physicochemical approach. *STP Pharm. Sci.*, 1 (1991) 221–241.

Manil, L., Roblot-Treupel, L. and Couvreur, P., Isobutyl cyanoacrylate nanoparticles as a solid phase for an efficient immunoradiometric assay. *Biomaterials*, 7 (1986) 212–216.

Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Mühlen, A., Weyhers, H., Freitas, C. and Rühl, D., Solid lipid nanoparticles (SLN) - An alternative colloidal carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm.*, 41 (1995) 62–69.

Rolland, A., Gibassier, D., Sado, P. and Le Verge, R., Méthodologie de préparation de vecteurs nanoparticulaires à base de polymères acryliques. *J. Pharm. Belg.*, 41 (1986) 83–93.

Seijo, B., Fattal, E., Roblot-Treupel, L. and Couvreur, P., Design of nanoparticles of less than 50 nm diameter: preparation, characterization and drug loading. *Int. J. Pharm.*, 62 (1990) 1–7.

Velge-Roussel, F., Breton, P., Guillon, X., Lescure, F., Bru, N., Bout, D. and Hoebeke, J., Immunochemical characterization of antibody-coated nanoparticles. *Experientia*, 52 (1996) 803–806.