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Review article

Biocompatible poly(methylidene malonate)-made materials for pharmaceutical and biomedical applications

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Abstract

In the past 20 years, mainly with the sponsorship of Laboratoires UPSA (France) and, afterwards, its spin-off company Virsol (France), several authors have studied methylidene malonate-based polymers used in drug delivery approaches and in the development of novel biomaterials. The present paper aims at summing up the preparation of methylidene malonate monomers, and essentially a novel asymmetric diester structure: 1-ethoxycarbonyl-1-ethoxycarbonylmethylenoxycarbonyl ethene named methylidene malonate 2.1.2. Their polymeric and copolymeric derivatives and a few of their applications which were reported in the literature are also presented. It encompasses the manufacturing of particulate systems such as nano- and macroparticles designed for the delivery of hydrophilic or hydrophobic drugs and biomolecules. This review article also describes their use as biomaterials of interest in the fields of tissue repair, as drug reservoirs or ophthalmology, as implants. Copolymers based on these monomers offer a large range of properties and could be used as new surfactants, micellar vectors, or particulate systems for gene delivery. Therefore, this review, certainly the first dedicated exclusively to methylidene malonate-based materials, highlights the great biomedical and pharmaceutical technology potential of these new materials.

Keywords: Methylidene malonate; Polymers; Polymeric materials; Cationic polymers; Drug delivery; Biomaterials; Implants; Particles; Encapsulation

1. Introduction

In 2000, the biomaterial engineering served a huge global market evaluated approximately at US$ 39 billion. Expectedly, at an average annual growth rate of 12%, estimations forecasted a worldwide turnover of US$ 55 billion for 2003. Presently, orthopaedic, cardiovascular, drug delivery, dental, surgical and wound care sectors represent the largest sources of applications and revenues for biomaterial-based products while, according to available data, drug delivery, urology and ophthalmic fields showed the highest annual growth rate, at over 16%. Many significant companies, like Baxter Healthcare, Bausch & Lomb, Convatec, Smith & Nephew, Alkermes, ALZA, Biocompatibles, Boston Scientific, Cordis (J&J), Genzyme Biosurgery, IsoTis, etc. are involved in this very competitive market segment [1].

Besides ceramics and metals, synthetic polymers and polymer-based biomaterials were first extensively considered for biomedical applications in the fifties, sixties and seventies and are still the topic of many investigations throughout the world [2–6]. Especially, Merrill's group spent considerable efforts understanding what could cause biocompatibility of a material when it was in contact with blood or other physiological fluids [7,8]. For the past 40

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years both hydrophobic (*e.g.* poly(methyl methacrylate), polysiloxanes, polyethylene, polyurethanes, etc.) [9] and hydrophilic (*e.g.* poly(ethylene oxide)) [10] polymers were studied and tested in various biomedical or pharmaceutical applications. Synthetic polymeric materials have been used in a wide range of formulations such as gels, hydrogels, films, coatings or particle suspensions and became key elements of tissue engineering, constituants of implantable devices, drug reservoirs or drug vehicles. The design, preparation and characterization of such materials now represent a still growing part of worldwide biomaterials R&D efforts and investments [11]. Either bioresorbable or fully stable over time, these polymeric systems incorporate various types of homopolymers or copolymers made of poly(esters), poly(anhydrides), poly(acrylates) or many others. In the context of chemical engineering science and biopharmaceutical engineering, these polymeric materials have played a crucial role in the development of controlled drug delivery and targeting systems and were the topic of many reviews in this field [12–15].

As a matter of fact, during the mid-late seventies, synthetic polymers and especially acrylic polymers started to be carefully considered for the advanced formulation of drugs [16]. Poly(methylmethacrylate) [17], poly(acrylamide) [18], poly(*N*-(2-hydroxypropyl)methacrylamide) [19], poly(styrene) [20] and poly(alkylcyanoacrylate) [21] were first retained for such an application and drug-loaded particulate systems (*i.e.* nanospheres or microspheres) were produced and carefully studied.

In the mid-1980s, Laboratoires UPSA, a French analgesia world leader company of high repute for its unique know-how in production of effervescent drug formulations, wished to perpetuate and reinforce its leadership in pharmaceutical technology and decided to invest in the development of proprietary pharmaceutical technologies based on the use of poly(methylidene malonate)-made materials [22,23]. Initially involved in the preparation of novel nanoparticulate systems that can be drug loaded [24], poly(methylidene malonate) and, more specifically, poly(methylidene malonate 2.1.2) (PMM 2.1.2), were extensively studied by Laboratoires UPSA and then by VIRSOL, after Bristol-Myers and Squibb took over Laboratoires UPSA in 1994. In the past years, VIRSOL has explored different pharmaceutical and biomedical applications where methylidene malonate 2.1.2- (MM 2.1.2) and PMM 2.1.2-based materials could be of interest.

This paper aims at reviewing the main R&D advancements having been accomplished for the past 15 years on MM 2.1.2, PMM 2.1.2 and all of their derivatives.

2. Methylidene malonate monomers

2.1. General structure

The general formula of methylidene malonate species is depicted on Fig. 1a. The structural backbone is a malonic acid, for which the two carboxyl functions are esterified

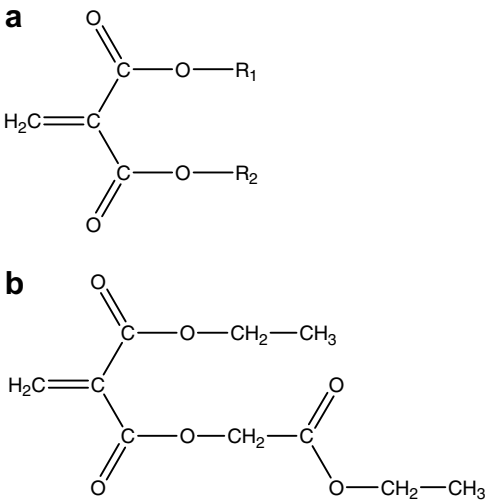

Fig. 1. General molecular structure of methylidene malonate (a) in which R_1 and R_2 are identical or different and can represent H, an alkali metal or alkaline earth metal atom, a linear or branched alkyl radical having from 1 to 6 carbon atoms, an alicyclic group having from 3 to 6 carbon atoms, an alkenyl radical having from 2 to 6 carbon atoms, defined in their *cis* or *trans* configuration, or an alkynyl radical having from 2 to 6 carbon atoms, the said groups optionally being substituted by one or more functional groups such as ether, epoxide, halogeno, cyano, ester, aldehyde, ketone, aryl or hydroxide. Molecular structure of methylidene malonate 2.1.2 (MM 2.1.2) or 1-ethoxycarbonyl-1-ethoxycarbonylmethylenoxycarbonyl ethene (b).

by various residues R_1 and R_2, identical or different, that can represent linear or branched alkyl, alicyclic, alkenyl or alkynyl groups optionally being substituted by one or more functional groups such as ether, epoxide, halogeno, cyano, ester, aldehyde, ketone, aryl, etc. Carbon 2 of malonate is substituted by a methylene group through a polarized double bond that is quite easily reduced in the course of a Michael's addition or a polymerization reaction [25,26].

2.2. Synthesis

Synthesis of simple methylidene malonate esters had been the topic of several reports in the literature since the late thirties up to the 1980s [27–34]. Among the chemical processes implemented, the most frequently described was the Knoevenagel condensation of paraformaldehyde with symmetric malonic acid esters in presence of various catalysts. However, except for the methylidene malonate di-*tert*-butyl ester [34], under the usual experimental conditions, yielded methylidene malonate esters were very unstable and polymerized very easily.

Noteworthy were the work and data patented by researcher groups at Eastman Kodak Co. [32] and also published by Ponticello [33]. To stabilize dialkyl methylidene malonates, these authors described similar chemical procedures in which acrylic derivatives were trapped by various dienes using a Diels–Alder mechanism to avoid polymerization in the reaction mixture. Such adducts were

easy to produce, could be subsequently modified or substituted, and the olefinic product was recovered through a retro Diels–Alder reaction by pyrolysis at high temperature. Hawkins et al. [32] only described the use of linear dienes like a substituted pentadiene or a hexadiene whereas Ponticello [33] demonstrated the interest of cyclic dienes, such as cyclopentadiene, to form substituted norbornenes which then could undergo various chemical reactions that led to different asymmetric diesters. After pyrolysis, asymmetric methylidene malonate diesters could be obtained. Unfortunately, the above chemical pathways showed some major and troublesome drawbacks that seriously compromised any industrial scaling-up.

About 10 years later, with the aim of identifying a convenient and efficient access to large quantities of symmetric and asymmetric methylidene malonate esters, De Keyser et al. [35] and Bru-Magniez et al. [25] proposed an improved synthesis pathway. Earlier work by Bachman and Tanner [22], Buck [36], Ponticello [33] and Giral et al. [37] described the use of anthracene for the synthesis of an addition product with an unsaturated compound such as a diethyl methylidenemalonate or a cyanoacrylic acid ester. But anthracene could be unexpectedly and advantageously used as a diene to trap very efficiently the unsaturated methylidene malonate and to form an adduct which then allowed, after regeneration by thermolysis at rather low temperature, the easy preparation of an extensive panel of symmetric and asymmetric dialkyl methylidene malonate esters obtained in batches of several hundreds of grams. Interestingly, this patented process gave an access to novel asymmetric diester structures such as 1-methoxycarbonyl-1-methoxycarbonylmethylenoxycarbonyl ethene (*i.e.* methylidene malonate 1.1.1 or MM 1.1.1), 1-ethoxycarbonyl-1-ethoxycarbonylpropylenoxycarbonyl ethene (*i.e.* methylidene malonate 2.3.2 or MM 2.3.2) or 1-ethoxycarbonyl-1-ethoxycarbonylmethylenoxycarbonyl ethene (*i.e.* methylidene malonate 2.1.2 or MM 2.1.2) (Fig. 1b) [25,26], the latter and its polymerization products having been extensively studied, initially by Laboratoires UPSA and, afterwards, by VIRSOL, a start-up company, spin-off of Laboratoires UPSA.

3. Poly(methylidene malonate 2.1.2) (PMM 2.1.2)- and oligo(methylidene malonate 2.1.2) (OMM 2.1.2)-based particulate systems

3.1. Poly(methylidene malonate) and PMM 2.1.2 nanoparticulate systems

3.1.1. Preparation and characterization

In the late 1980s and early 1990s, the Department of Medicinal Chemistry of Catholic University of Louvain (Belgium) and Laboratoires UPSA (France) first developed and characterized poly(methylidene malonate) nanoparticles [24,38–42]. The group of Prof. Dumont at Catholic University of Louvain got focused on poly(dialkyl methylidene malonate) and, more specifically, on poly(diethyl methylidene malonate) (PDEMM)-made nanoparticles. In 1991, De Keyser et al. [38] published the aqueous polymerization of diethyl methylidene malonate in presence of 1% dextran (M_w (weight average molecular weight) = 70,000) to form nanoparticles (diameter: 140–250 nm). A very high proportion of these nanoparticles (*i.e.* 95%) produced at neutral pH, was found in the liver and in the spleen short after i.v. administration in mice, and only 10% were eliminated from these organs over a 3 months period. After oral administration to mice there was no evidence of nanoparticle uptake in the intestinal tract and no trace of PDEMM remained 24 h after gavage. Thus, PDEMM nanoparticles could not reasonably be considered for systemic drug administration, but, eventually might be envisaged as a drug vehicle for enteral administration.

Soon interested in nanoparticulate systems, researchers in the pharmaceutical technology group at Laboratoires UPSA, in collaboration with Prof. Couvreur's group at the School of Pharmacy of University of Paris XI, first focused on the evaluation of polyalkylcyanoacrylate nanoparticles as new potent drug carriers [43,44]. The PMM 2.1.2 nanoparticles were first easily prepared as described before for PDEMM nanoparticles [38] just by adding dropwise sulfur dioxide-free MM 2.1.2 into a phosphate buffer containing 1% dextran ($M_w = 70{,}000$) under vigorous stirring. In such experimental conditions, MM 2.1.2 readily polymerizes and spontaneously forms nanoparticles in the diameter range of 150–500 nm [24,41,42]. Nanoparticle suspensions could be easily and conveniently freeze-dried, stored and resuspended for further use [45].

The robustness of this manufacturing process and the stability of resulting nanoparticles stored in various conditions were carefully studied by Roy et al. [45] who, in addition, were able to successfully scale up (25×) the production of colloidal suspension batches up to a semi-industrial level. Kept over 1 year at various temperatures in the dark or in daylight, lyophilized PMM 2.1.2 nanoparticles remained stable, with an exception for the storage at 40 °C for which a polymer side chain degradation seemed to occur and might account for progressive and significant changes of some measurable endpoints such as a colloidal suspension pH decrease probably due to the generation of free carboxyl moieties [45].

The cytotoxicity of PMM 2.1.2 nanoparticles was evaluated *in vitro* on adherent L929 fibroblasts and was shown to be lower than the one of polyisohexylcyanoacrylate (PIHCA) or polyisobutylcyanoacrylate (PIBCA) nanoparticles [41,42]. Cell viability was also evaluated on non-adherent CEM T lymphoblastoid cell line after incubation with PMM 2.1.2 nanoparticles for 2 days. Under the conditions described, the 50% cytotoxic concentration (CC_{50}) was determined approximately at 11 $\mu g\,ml^{-1}$ 10^6 $cells^{-1}$ [45]. Administered intravenously to rats or mice, one single dose of PMM 2.1.2 nanoparticles of up to 500 mg/kg did not show any behavioral alteration or lethality of animals. Hepatic functions, metabolism, biochemical and hematological parameters remained mostly unchanged in both

rodent species which received i.v. injections for 5 consecutive days [46]. Their biodegradability was first evaluated *in vitro* in aqueous buffer of various pH, or in rat plasma by monitoring ethanol release due to polymer side chain erosion [41,42]. As expected, the more basic the pH, the more efficient the ester hydrolysis and the higher the ethanol concentration released. Similarly, in biological fluids, esterases were responsible for ethanol release. However, even after a period of incubation of 6 days, no more than 50% of releasable ethanol was detected in the supernatants, suggesting that the ethoxycarbonyl ester of ethoxycarbonylmethylenoxycarbonyl moiety is probably the only ester function that underwent hydrolysis. In the same experimental conditions, there was no evidence that the polymer backbone was altered so that the degree of polymerization certainly remained unchanged (Fig. 2).

^{14}C-radiolabeled backbone [47] PMM 2.1.2 nanoparticles were used to study the biodistribution and kinetics of excretion of PMM 2.1.2 [41,48]. Though to a much lesser extent than polyalkylcyanoacrylate (PACA) [49], polymethylmethacrylate [50] or polystyrene nanoparticulate systems [51], after one single intravenous injection in rats, most of radioactivity rapidly homed in the liver and in the spleen. Therefore, PMM 2.1.2 nanoparticles were cleared relatively slowly from the blood stream by reticuloendothelial defence system, especially by liver Kupffer cells. This observation indicated that specific physico-chemical properties of PMM 2.1.2 nanoparticles might lead to a sort of system with longer circulating properties in blood. Six days after injection, more than 85% of radioactivity were eliminated through faecal and urinary tracks [41,48]. However, results published in 1996 and 1998 by Breton et al. [52,53] somewhat invalidated these pharmaco-kinetic and pharmaco-dynamic data. Indeed, these authors showed that most of PMM 2.1.2 nanoparticles were very sensitive to dilution and got solubilized in aqueous medium. Thus, the biodistribution and kinetics of excretion data initially reported became difficult to interpret since they were in fact the reflect of the fate of PMM2.1.2 nanoparticles made of both insoluble PMM 2.1.2 long polymeric chains and soluble MM 2.1.2 oligomers (OMM 2.1.2).

Taken into account this observation, Breton et al. [53] conducted investigations to improve PMM 2.1.2 nanoparticle physico-chemical characteristics, and especially to increase the molecular weight of their main components to make the colloidal system much less sensitive to dilution. Two parameters of the initial process described by Lescure et al. [41] and Breton et al. [42] were the target of modulations: (i) pH or (ii) stirring speed of the polymerization medium. As suggested by previous data [41,42], a more basic pH, every other parameters being unchanged, strongly favored the occurrence of high molecular weights (*i.e.* 68% oligomers – trimers mostly – at pH 5.5 and 28% oligomers – trimers mostly – at pH 8.0). Similarly, decreasing the stirring speed from 700 rpm to 150 rpm, every other parameters being unchanged, also enhanced the mean molecular weight of nanoparticle components (*i.e.* 80% oligomers – trimers mostly – at 700 rpm and 30% oligomers – trimers mostly – at 150 rpm). In agreement with these observations and unlike PMM 2.1.2 nanoparticles prepared according to the process parameters initially set up [41,42] which showed a glass-transition temperature (T_g) of about −17 °C, these second generation of PMM 2.1.2 nanoparticles described by Breton et al. [53] have T_g values contained between 0 and 20 °C. Moreover, by using radioactive MM 2.1.2, it was shown [53] that these polymer-enriched nanoparticles were much less sensitive to aqueous dilution since just a few percent of radioactivity was associated to supernatants of colloidal suspensions. Whether Breton et al. [53] were able to dramatically improve the physico-chemical properties of PMM 2.1.2 nanoparticles, it is worthwhile to note that the yield of nanoparticle synthesis plummeted from about 90% to no more than 30%, most of PMM 2.1.2 formed being aggregated and stuck as a mass at the bottom of nanoparticle manufacturing

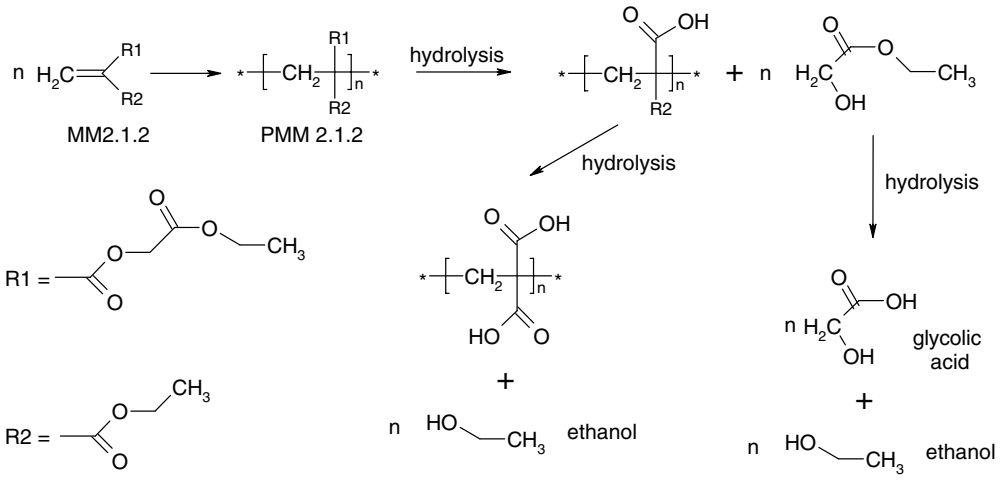

Fig. 2. Chemical structure of methylidene malonate 2.1.2 monomer, polymer and degradation pathway of PMM 2.1.2 side chains via hydrolysis, leading to a water soluble species. The potential backbone degradation process could occur through a reverse Knoevenagel's reaction.

vials. This major drawback led UPSA Laboratoires' preformulation group to further investigations.

Finally, Bru-Magniez et al. [54] were able to overcome this ultimate drawback and to establish what is currently the best and optimal methodology to produce PMM 2.1.2 nanoparticles: it has consisted, first, in solubilizing MM 2.1.2 monomer into an aprotic organic solvent miscible to water, such as acetone, and, second, to mix, under magnetic stirring, this organic solution to the aqueous polymerization medium. Alternatively, it was shown that the aqueous polymerization medium might also be added to the MM 2.1.2-containing organic solution with the same outcomes in term of nanoparticle physico-chemical characteristics and yields.

3.1.2. Identification of nanoparticle components

Since dealing with an anionic polymerization process initiated by hydroxyl anions, not surprisingly, pH of phosphate buffer was the key parameter to be controlled and very early Lescure et al. [41] et Breton et al. [42] showed that only a narrow and slightly acidic pH range (*i.e.* between 5.0 and 6.0) allowed the preparation of nanoparticles with acceptable physico-chemical characteristics. Interestingly, size exclusion chromatography analysis performed at various times after initiating the polymerization reaction highlighted the emergence of a main oligomeric species having a weight average molecular weight (M_w) of about 600–700 in polystyrene equivalent (pe). Though molecular weights were expressed in pe *i.e.* using a calibration curve for the determination of M_w done with polystyrene polymer standards of known molecular weight, and M_w measurement correlation between PMM 2.1.2 and polystyrene was unknown at that time, Breton et al. [42] who also illustrated the phenomenon via a high performance liquid chromatography (HPLC) methodology, first made the hypothesis that this main colloid component that accumulated over 22 h of nanoparticle manufacturing process might be the result of the cyclization of a trimer according to a Michael's reaction yielding a stable 1,3,5-trisubstituted cyclohexane ring. Further HPLC with various stationary and mobile phases, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and other mass spectrometry techniques (MS), LC–MS and proton nuclear magnetic resonance (^{1}H NMR) studies performed by Breton et al. [53] and Salvador et al. [55,56] confirmed this hypothesis and permit to isolate and characterize 2,4,6-ethoxycarbonyl-4,6-ethoxycarbonylmethylenoxycarbonyl-2-hydroxymethyl cyclohexanone and most of its diastereoisomers.

It is noteworthy to mention molecular modelling and conformational studies undertaken by Vangrevelinghe et al. [57,58] on native or eroded (loss of all ethoxycarbonylmethylenoxy moieties) OMM 2.1.2/PMM 2.1.2 (from 5 to 40 MM 2.1.2 monomer units) of various tacticity (*i.e.* syndiotactic or isotactic), in explicit or implicit solvation conditions. Measuring especially its radius of gyration or its end-to-end distance in polar (*i.e.* water) or in apolar (*i.e.* chloroform) solvents, conformation of any native OMM 2.1.2/PMM 2.1.2 chain was shown insensitive to solvent polarity. Inversely, eroded OMM 2.1.2/PMM 2.1.2 shape, though very similar in water and in chloroform, was significantly dependent on the polymer configuration. As anticipated, whatever the configuration of the polymer and the solvent nature, this *in silico* testing showed that the hydrolysis of ethoxycarbonylmethylenoxycarbonyl side chain tended to lengthen the polymer backbone.

3.1.3. Applications

In the late seventies, nanoparticles were described as a promising new drug delivery system to advantageously formulate active ingredients (see [59] as an illustration). To the best of our knowledge, the very first application of poly(alkyl methylidene malonate) nanoparticles was reported by Mbela et al. [39] in 1992. These authors considered poly(diethyl methylidene malonate) (PDEMM) nanoparticles for the formulation and liver delivery of anti-malarial drug primaquine that was entrapped *in situ* into the polymer entanglement or adsorbed onto preformed nanoparticles [39]. Desorption of primaquine from colloidal particles was very slow. The toxicity and the therapeutic activity of these primaquine-loaded nanoparticles were tested in mice after *i.v.* or *i.p.* injection. While primaquine-loaded PDEMM nanoparticle toxicity was not different of free primaquine's one, it was shown that, when compared to free drug, this nanoparticulate formulation significantly increased life expectancy of mice infected with *Plasmodium berghei*. Other types of poly(dialkyl methylidene malonate) were tested for their capacity to form nanoparticles and deliver primaquine [40]. In terms of physico-chemical characteristics and of primaquine release, poly(diallyl methylidene malonate) nanoparticles appeared to be the best of all the poly(alkyl methylidene malonate) nanoparticles tested by Mbela et al. [39,40] in a novel anti-malarial approach.

Then, the first applications of PMM 2.1.2 nanoparticles that were reported was their use in the preparation of anti-CD4 immunonanoparticles [60] and anti-β_2 microglobuline immunonanoparticles [61] both aiming at curing and preventing HIV infection. On the one hand, although the ability of anti-CD4 monoclonal antibodies to inhibit syncitia (*i.e.* giant and aberrant cells resulting from the fusion of several HIV-infected T cells) had been reported earlier [62], their combination with PMM 2.1.2 nanoparticles reduced 100 times their efficient concentration (0.05 μg ml^{-1} vs. 5 μg ml^{-1}). Importantly, this observation was not done with anti-CD4 immunonanoparticles prepared with PIHCA. On the other hand, the approach using anti-β_2 microglobuline immunonanoparticles took benefit of a specific monoclonal antibody recognizing a β_2 microglobuline epitope accessible essentially onto HIV particles [63,64]. Its combination with PMM 2.1.2 nanoparticles strongly potentiated therapeutic and prophylactic anti-HIV activity of the anti-β_2 microglobuline monoclonal

antibody. Here again, PIHCA nanoparticles were shown inefficient because much more toxic than PMM 2.1.2.

These immunonanoparticles, that might also be considered for the development of diagnostic tests, were obtained by mixing PMM 2.1.2 nanoparticles manufactured according to Lescure et al. [41] and Breton et al. [42] with certain concentrations of monoclonal antibodies and bovine serum albumin. These proteins got spontaneously adsorbed onto nanoparticles to formed the so-called PMM 2.1.2 immunonanoparticles.

The interactions between PMM 2.1.2 nanoparticles and proteins forming immunonanoparticles as well as the functionality of the latter were studied extensively according to various methodologies, especially by Surface Plasmon Resonance (SPR) [52,65,66]. In 1996, Breton et al. [52] published data that demonstrated the strong and quasi-irreversible interaction between PMM 2.1.2 nanoparticle surface and proteins (*i.e.* bovine serum albumin and IgG). Approximately, a maximum of 10 μg protein (in the experimental conditions used: approximately 1 μg IgG and 9 μg bovine serum albumin) per mg PMM 2.1.2 might be tightly adsorbed onto nanoparticles. Such observations were further confirmed by Bousquet et al. [66] who undertook SPR studies and evaluated the behavior of human serum albumin and more or less electro-negatively charged substituted human serum albumin [67] incubated with PMM 2.1.2 nanoparticulate systems and who determined interaction kinetic parameters in such systems. It was established that electrostatic interactions were the driving force of strong protein–PMM 2.1.2 interactions that were subsequently stabilized by hydrophobic forces. Contrary to what Breton et al. [52,53] had previously shown, regeneration of sensor chips with chaotropic agents did not seems to involve a nanoparticle solubilization process [66]. This could mean that proteins interacting with PMM 2.1.2 nanoparticles produced according to Lescure et al. [41] and Breton et al. [42] would protect nanoparticles, at least in part, from disintegration. If it could be confirmed, interpretation of earlier reported pharmacokinetic and pharmacodynamic data [41,42] could be somewhat valid since plasma protein would have stabilized PMM 2.1.2 nanoparticles as soon as they were injected in the blood stream?... Clearly, further investigations would now be required. After that kinetic and thermodynamic parameters of the interaction between one of soluble anti-CD4 monoclonal antibodies (*i.e.* IOT4a, clone 13B8-2) and immobilized recombinant and soluble CD4 had been extensively characterized [68], Velge-Roussel et al. [65] also took benefit of SPR technology for "real-time" interaction measurements and demonstrated that anti-CD4 immunonanoparticles, as soluble anti-CD4 antibody did, specifically bound soluble recombinant CD4, or anti-RAM-Fc IgG, covalently anchored to the sensor chip of SPR Biacore™ system.

Another important use of PMM 2.1.2 nanoparticles was recently reported by Qiang et al. [69]. Effectively, studies performed at the University of Toronto, in Prof. Sefton's and Dr. Strauss' laboratories highlighted the interest of PMM 2.1.2 colloids in an *in vivo* adenoviral gene delivery to medial and adventitial layers of vascular compartments. These authors, who prepared smaller nanoparticles according to a new methodology, through the coacervation of pre-formed polymer ($M_w = 10{,}000$ pe), showed that β-galactosidase-encoding adenovirus-covered nanoparticles (Fig. 3) were able to bioadhere to the adventia of rabbit carotid arteries and to promote gene transfection and expression at a much higher level than the adenovirus alone did in both adventia and media of vessels (Fig. 4). This important finding clearly open gates to further investigations in the cardiovascular domain, but also in other key research fields where huge medical unmet need are currently identified.

Finally, it should be mentioned that the successful formulation of different drugs into PMM 2.1.2 nanoparticles was described. Thus, Bru-Magniez et al. [54] have published the preparation of rifampicin, colistin, azidothymidin (AZT), creatine phosphate, or 5-fluorouracil (5-FU)-loaded PMM 2.1.2 nanoparticles.

3.2. PMM 2.1.2 microparticulate systems

3.2.1. Microparticles prepared according to a W/O/W process

3.2.1.1. Preparation and characterization. Like submicronic particles, PMM 2.1.2 microparticles might also have some interest in various pharmaceutical and medical applications. Thus, as soon as in 1996, a group of scientists from Schering AG in Berlin, filed a patent application describing the use of methylene malonic diester derivatives for the preparation of gas-containing microparticles that might

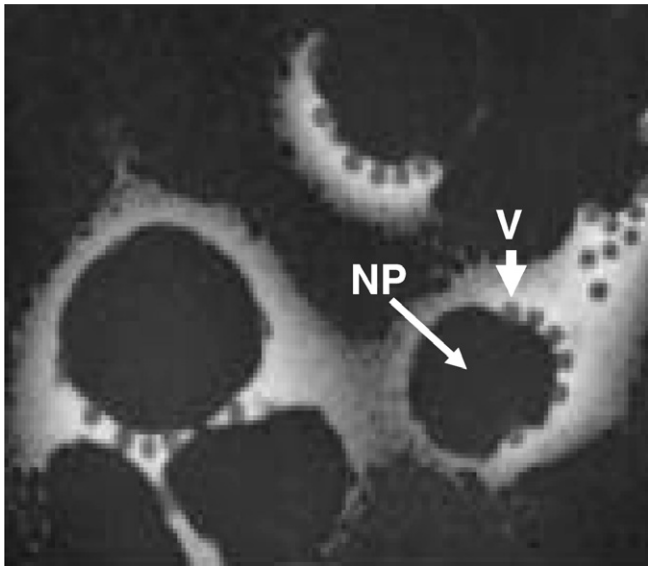

Fig. 3. Transmission electron microscopy pictures showing PMM 2.1.2 nanoparticles (Np) onto which β-galactosidase-encoding adenoviruses (V) got adsorbed. The diameter of pointed nanoparticle (Np) is 200 nm (by courtesy of Qiang et al. [69]).

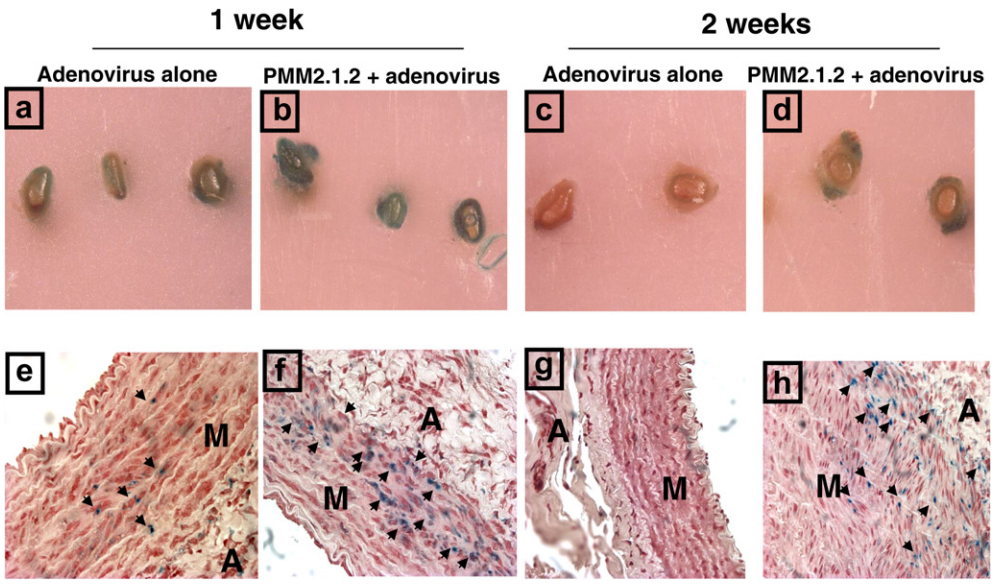

Fig. 4. β-Galactosidase expression 1 and 2 weeks after adenovirus adventitial delivery with and without PMM 2.1.2 nanoparticles. The adenovirus or nanoparticle-adenoviral complexes were directly applied to the carotid arteries of anaesthetized rabbits. After sacrifice the arteries were stained with X-Gal solution. The samples were embedded with paraffin, cut in 5 μm slides and stained with neutral red. The transfected cells were identified by blue-stained nucleus under 40× objective; Upper pictures are low magnification images of paraffin tissue blocks; lower pictures are histological sections. (a and e), adenovirus alone at 1 week; (b and f) adenoviruses adsorbed onto PMM 2.1.2 nanoparticles at 1 week; (c and g), adenovirus alone at 2 weeks and; (d and h) adenoviruses adsorbed onto PMM 2.1.2 nanoparticles at 2 weeks. A means adventitia, M means media. Arrows point out β-galactosidase-expressing cells (by courtesy of Qiang et al. [69]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

be used for the ultrasound diagnostics [70]. Although dependent on previous UPSA' patents and/or patent applications, to our best knowledge, this publication was the first that dealt with the use of PMM 2.1.2 in the manufacture of microparticulate systems. On its side, in the mid-1990s, VIRSOL in collaboration with Prof. Couvreur's group started to investigate the use of methylidene malonates for the formulation of biologically active and hydrophilic substances, such as proteins, peptides, DNA or oligonucleotides, into microspheres obtained according to a double emulsion W/O/W (Water in Oil in Water) evaporation process that was patented and published first in 1999 and 2001 [71,72]. Here, it is interesting to underscore that, PMM 2.1.2–made microspheres were elaborated from a high weight average molecular weight ($8000 < M_w < 125{,}000$ pe) pre-formed polymer synthesized, as suggested by earlier data [54], by an addition of 0.1 N sodium hydroxide to a solution of MM 2.1.2 in acetone [72]. Le Visage et al. [72] devoted a lot of efforts to optimize and characterize these microspheres. The influence of the nature of the organic solvent as well as the PMM 2.1.2 concentration and the molecular weight of the polymer on particle morphology (*i.e.* size, appearance, kinetic of release of ovalbumin) were carefully investigated. Mostly sensitive to the organic solvent nature, microparticle diameter was comprised between 2 and 30 μm, with ethyl acetate clearly giving the smallest structures. In these studies, ovalbumin was used as a model protein to evaluate encapsulation efficiency and release from PMM 2.1.2 microspheres incubated in phosphate buffer (pH 7.4). While the former did not exceed 20%, the latter showed a burst of about 10% in the first few hours, probably corresponding to proteins associated to the particle surface, and then a very slow release of no more than 3% of ovalbumin over approximately 90 h. Assuming that the bioerosion of polymer side chains and subsequent polymer solubilization account for the release profile of ovalbumin, it was not surprising to observe that this slow release kinetics correlates with a polymer solubilization which did not exceed 10%, whatever the molecular weight considered, after 8 days incubation in an esterase-containing medium. The low weight average molecular weight polymers (*i.e.* 8000 pe) got eroded and solubilized only slightly faster than high molecular weight polymers (*i.e.* 30,000 pe) [72].

With the aim of oral administration of these PMM 2.1.2 microspheres, further studies were performed by Le Visage et al. [73] to improve ovalbumin release in artificial gastric and intestinal media. In these media, within 2 h, about 90% of ovalbumin was released and mostly degraded from microspheres prepared as described by Le Visage et al. [73]; confocal microscopy studies of fluorescent ovalbumin-loaded microspheres showed that most of these released proteins were located at the periphery of the particles. A slight change in the manufacturing process with the addition of a surfactant (*i.e.* Pluronic F68) into the inner aqueous phase clearly modified the location of ovalbumin into microspheres so that the proteins appeared entrapped in the internal aqueous cavities, protected from early degradation, and their kinetics of release was significantly decreased [73].

A third paper by Le Visage et al. [74] reported the behavior of microparticles prepared with or without polyvinylalcohol (PVA) in the aqueous outer phase, *in vitro*, in contact with Caco-2 cells and, *in vivo*, after oral administration to mice. Whereas PMM 2.1.2 microparticles prepared in presence of PVA and incubated with Caco-2 cells were non toxic up to 1 mg/ml, the ones prepared without PVA showed some significant toxicity starting at 0.4 mg/ml. At subtoxic concentrations, through mechanisms that are, in some extent, energy-dependent, fluorophore-loaded microparticles were closely associated to cells and endocytosis seemed to occur. When given through the enteral route, radiolabeled PMM 2.1.2 microparticles were mostly located in the luminal compartment of the gastro-intestinal (GI) tract over 24 h following the gavage. At this same time point, a larger amount of PVA-free microparticles than of the ones made in presence of PVA appeared to reside (15% vs. 5%) in the GI tract (lumen + mucosa), however, in both cases, histological observations revealed that, though associated to various tissues of mucosa, microparticles were only slightly taken up by Peyer's patches and almost no radioactivity was detected in the systemic compartment [74].

3.2.1.2. Applications. As for nanoparticles [69], PMM 2.1.2 microparticles were evaluated as a potential drug delivery system in the field of vascular gene delivery. Indeed, Du et al. [75] reported the use of ultrasonography (US) to dynamically monitor catheter-based vascular delivery of GFP (Green Fluorescent Protein) gene-loaded PMM 2.1.2 microspheres into femoral arterial walls of pigs. Confirming earlier data from Albayrak and Rössling [70], these authors demonstrated that US is a convenient tool to visualize PMM 2.1.2 microspheres released in vessel walls through the micropores of a catheter-borne balloon. Underlining the great interest of such an imaging technology, immunochemical analysis of GFP expression perfectly correlated with Doppler US pictures. Based on these initial studies, PMM 2.1.2 microparticles represent potent gene vectors that may attract interest of many companies focused on the gene delivery as a treatment or a prevention of cardiovascular disorders.

3.2.2. Microparticles prepared according to an O/W (Oil in Water) process

3.2.2.1. Preparation and characterization. Essentially designed for the formulation of hydrophobic therapeutic drugs, these microparticles were mostly lacking aqueous cavities, and active agents were distributed throughout the microsphere-constituting polymer entanglements. Derived from the methodology described by Bru-Magniez et al. [71], PMM 2.1.2- and drug-containing organic solvent were dispersed in an aqueous medium to form a single emulsion, and then, microspheres were formed after gentle evaporation of the organic solvent.

3.2.2.2. Applications for the formulation of hydrophobic drugs. At our best knowledge, the formulation of paclitaxel (Taxol®), a widely used and highly hydrophobic antitumor drug considered, in particular, for the treatment of ovarian and breast cancers, is currently the only example of PMM 2.1.2 microspheres loaded with a very poorly water soluble drug. This novel and proprietary delivery system were administered intravesically to a mouse model for the treatment of superficial bladder cancer [76]. In recent reports, Le Visage et al. [77,78] described the preparation and characterized paclitaxel-loaded microparticles (diameter: 5 μm). They showed that encapsulated paclitaxel fully retained its cytotoxic effect (Fig. 5) and that Nile Red-loaded fluorescent PMM 2.1.2 microparticles adhered to the urothelium allowing a sustained on-site release profile of the drug (Fig. 6). Within the 24 h-post-administration to mice, whereas more than 90% of instillated free paclitaxel was eliminated in urines, 70% of the encapsulated dosage still remained in the bladder lumen. When instillated into *N*-butyl-hydroxybutylnitrosamine-induced bladder tumor-bearing mice, these novel paclitaxel formulation improved dramatically the animal survival rate which correlates with the lack of hyperplasia. It's noteworthy that paclitaxel was totally absent from the systemic compartment. Thus, unlike other type of commonly used polymers such as poly(L-lactide-*co*-glycolide) acid or polystyrene (Leong, Le Visage, Malavaud, personal communication), PMM 2.1.2 behaved as a valuable and bioadhesive material able to strongly potentiate anti-tumor activity of paclitaxel in contact of bladder epithelium. These remarkable bioadhesive properties of blank and paclitaxel-loaded PMM 2.1.2 microparticles were further studied and characterized by Chan et al. [79] who used a model collagen-made extracellular matrix to determine the Young's modulus, the adhesion energy and the degree of particle deformation occurring in the interaction setting. In particular, they showed that those last two parameters were sensitive to pH and to paclitaxel loading.

3.2.3. 5-Fluorouracil-loaded microparticles

Whether it was quite obvious to consider the formulation of hydrophobic and poorly hydrosoluble drugs into PMM 2.1.2 microspheres prepared according to an O/W (Oil in Water) process, the successful encapsulation of hydrophilic drugs was also advantageously carried out. Actually, the first, and currently unique, example of the preparation and the use of hydrosoluble drug-loaded PMM 2.1.2 microspheres was provided by the group of Prof. Benoit at the University of Angers (France) who described 5-fluorouracil (5-FU)-loaded PMM 2.1.2 microspheres as a novel potent therapy to fight brain tumors [80–82].

3.2.3.1. Preparation and characterization. Essentially, based on an emulsion/extraction method described earlier by Boisdron-Celle et al. [83] for the preparation of 5-FU-loaded PLGA microspheres, Fournier et al. [80–82] were able to disperse solid 5-FU ground crystals into the organic phase of an O/W emulsion and then to entrap these crystals

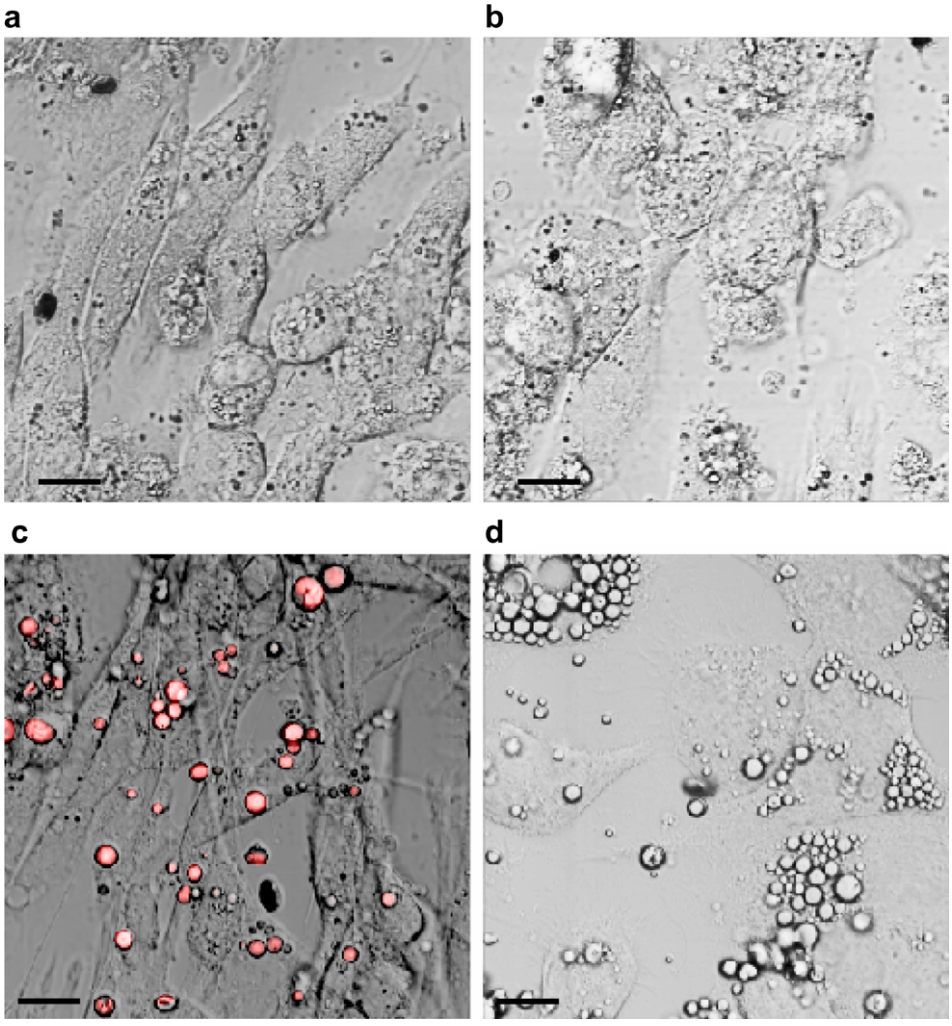

Fig. 5. Confocal microscopy pictures of MBT-2 cells incubated for 3 days in (a) culture medium, (b) paclitaxel-containing medium, (c) blank fluorescent microspheres and, (d) paclitaxel-loaded microspheres. Scale bar represents 10 μm (by courtesy of Le Visage et al. [77]).

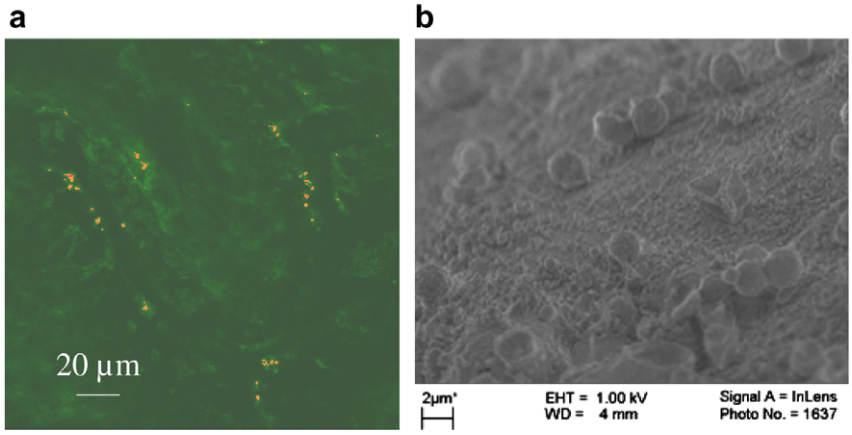

Fig. 6. Microscopic observations of murine bladder sections 48 h after instillation of 50 μl of 50 mg/ml suspension of Nile Red-loaded fluorescent PMM 2.1.2 microspheres. (a) confocal microscopy picture of fluorescent PMM 2.1.2 microspheres, (b) scanning electron microscopy picture of the same microspheres (by courtesy of Le Visage et al. [77]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

into PMM 2.1.2 network (M_w: about 20,000 pe) of microspheres mean diameter of which was established consecutively to extraction of organic solvent.

3.2.3.2. Applications in a brain cancer therapeutic approach. Expected to release their payload on a longer period of time than equivalent drug-loaded PLGA microspheres

did [84], 5-FU-loaded PMM 2.1.2 microspheres were first tested as a new therapeutic approach against brain cancer in syngeneic Fischer F344 female rats bearing an experimental F98 glioma. It was shown that these drug-loaded PMM 2.1.2 microspheres were able to significantly increase the median survival of animals having received them (*i.e.* about 34 days versus 23 days for control animals) [80]. Such an efficacy was confirmed in another experimental model using C6 glioma cells implanted in the brain of Sprague–Dawley rats [81]. With the aim of optimizing these novel pharmaceutical form, Fournier et al. [82] undertook a development process and especially focused on 2^3 factorial design to evaluate and control the influence of (i) the polymer molecular weight, (ii) the polymer concentration and (iii) the O/W emulsion preparation time on the 5-FU release profile. With these 3 parameters maintained at their optimal values and with the same objective, the solvent extraction time was also modulated appropriately. Adapting and fine tuning of all these parameters allowed to obtained microspheres (40–50 μm in diameter) which, *in vitro*, showed a reduced 5-FU initial burst release and which released the drug over a period of more than 43 days. Interestingly, these microparticles could be sterilized by γ-irradiation with no alteration of their native physico-chemical characteristics. As PMM 2.1.2 microspheres degrade slowly *in vivo* and could constitute an interesting long-term delivery system for the treatment of neuro degenerative diseases, Fournier et al. [85] evaluated the biocompatibility and the brain tissue response to 5-FU PMM 2.1.2-based microspheres and to their degradation products. In this first study, the interesting slow degradation rate of loaded microspheres was confirmed. However, when the polymer started to degrade, an inflammatory and a specific immune response developed. Further experiments are now required to identify the potential toxic breakdown products and to elucidate the mechanisms involved in the immunological reaction.

4. MM 2.1.2-based materials as drug reservoirs and implants

4.1. Peptide and polypeptide formulation and delivery

Whereas PMM 2.1.2 and OMM 2.1.2 were mostly considered for the preparation of colloidal and microparticulate systems involved in drug delivery approaches, these polymeric and oligomeric materials were also at the basis of new implantable drug reservoirs which could advantageously release their payload locally over a long period of time. These implants could be designed to hold various physical and mechanical characteristics such as softness/hardness, elasticity, flexibility, resistance or tackiness. In this particular field of investigation, two major applications were reported by Virsol scientists and their academic coworkers.

The first pertained to the vast market of tissue repair after wound, trauma, ulcer, neurodegenerative diseases or other tissue impairments where there is still a huge medical unmet need. The results published by Désiré et al. [86,87] and patented by Bru-Magniez et al. [88] should draw attention of researchers engaged in this type of research programs. These authors showed that growth factors such as Fibroblast Growth Factor-1 or Fibroblast Growth Factor-2 (FGF1 or FGF2) together with heparan sulfate (HS), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β), sphingosylphosphorylcholine (SPC), GM-CSF and/or Nerve Growth Factor (NGF) could advantageously be formulated into biocompatible blends of PMM 2.1.2 ($M_w > 6000$ – 40–80% w/w) and OMM 2.1.2 ($M_w < 6000$ – 20–60% w/w), also containing eventually MM 2.1.2. When tested *in vitro* on various cell lines, 2–5 mm thick films of optimal oligo-polymeric compositions and their soluble degradation products showed a limited cytotoxicity (Fig. 7a). These drug-loaded films were able to improve the 21-day storage stability at 37 °C of embedded cell growth factors and almost totally preserve their functionality over a 21-day sustained release period (Fig. 7b and c). Considering that one of the major drawbacks of using recombinant growth factors as therapeutic agents may rely on their lack of stability and bioavailability [89–91], such proprietary MM 2.1.2-based formulations might be regarded as a very promising approach for local, safe, scalable and efficient delivery of biologically active peptides and polypeptides.

4.2. Corticoid formulation and delivery

Another important application of MM 2.1.2-based implants that was recently investigated by Felt-Baeyens et al. [92,93] was their use in ophthalmology to deliver corticoids over several months through the sclera in order to treat pathologies like proliferative vitreoretinopathy, uveitis or retinitis. Based on data from Mora et al. [94] who first showed that triamcinolone acetonide (TA) can go through human sclera, TA-loaded disc implants were prepared with a PMM 2.1.2 ($M_w = 100{,}000$–$150{,}000$) matrix associated with plasticizers such as OMM 2.1.2 (Fig. 8a). Sterile scleral implants which were manufactured by a modified compression-molding method [95] and which held appropriate mechanical characteristics were tested in a rabbit model. When placed in contact of rabbit eye sclera, no inflammation or other type of adverse event was observed (Fig. 8b), and studies conducted over several weeks have provided the evidence that these scleral implants were absolutely biocompatible.

In term of TA delivery and intravitreal availability, High Performance Liquid Chromatography assays showed that TA had been detectable in the vitreous up to 9 weeks after surgical implantation, while therapeutic concentrations had been measured up to 5 weeks into this eye compartment. Concentrations of TA found in vitreous was between 300 and 1800 ng/ml, whereas those reported in the sclera was between 50 and 500 ng/ml, depending on the manufacturing specificities of the PMM 2.1.2-based implants.

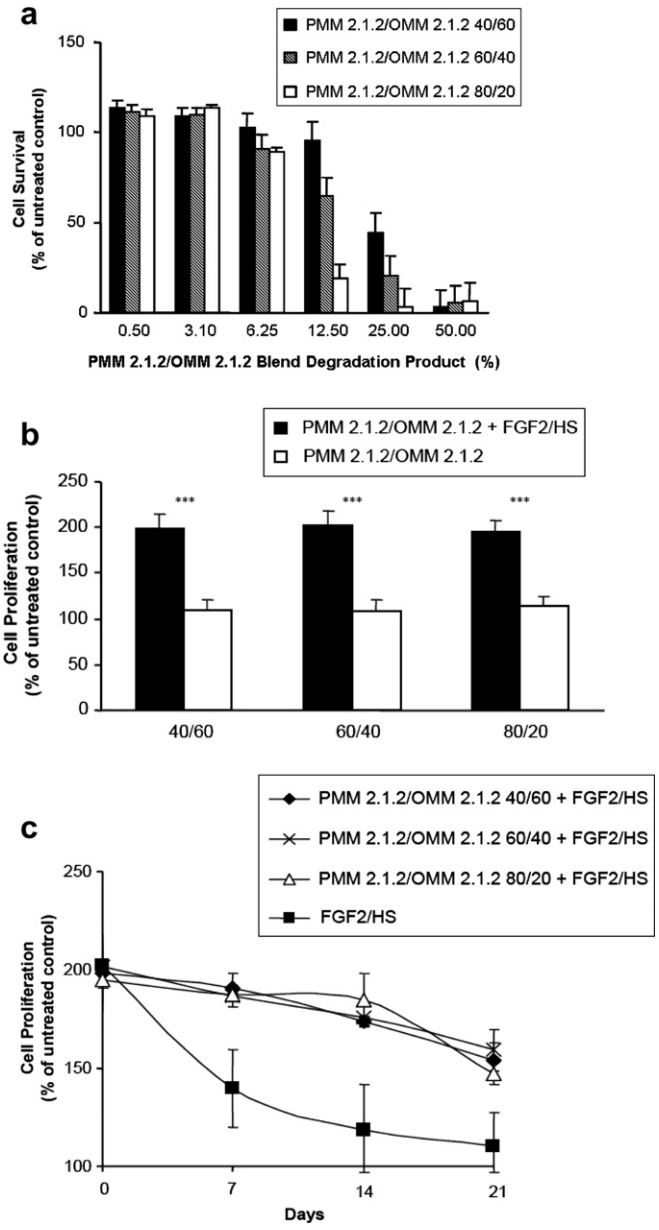

Fig. 7. The changes in PMM 2.1.2/OMM 2.1.2 blend composition that determine physico-chemical characteristics and degradability profile do not influence FGF2 bioactivity. (a) PMM 2.1.2/OMM 2.1.2 films of various compositions underwent degradation in a conditioned cell medium for 24 h and increasing aliquots of incubation supernatant were added to BP-A31 cells. Cytotoxicity was determined with an MTT assay. MTT values of untreated cells are set as 100%. (b) an FGF2/HS complex was added or not to 3 various PMM 2.1.2/OMM 2.1.2 blends and the bioactivity of these growth factor formulations was monitored on BP-A31 cell proliferation after poly- and oligomeric films were incubated for 24 h in a conditioned cell medium to undergo degradation. For fibroblast proliferation testing, 0.5% degradation products were added to BP-A31 cell culture medium. (***$p < 10^{-4}$ by Mann–Whitney test) Wells containing untreated cells displaying 100% growth served as control in all experiments. (c) Mitogenic activity of FGF2 released from different PMM 2.1.2/OMM 2.1.2 blends. At day 0, FGF2/HS was formulated or not into PMM 2.1.2/OMM 2.1.2 composition and all preparations were stored at 37 °C in similar conditions until assayed for FGF2 bioactivity on BP-A31 cell growth, at the indicated time, after 24 h incubation in conditioned cell medium. For fibroblast proliferation testing, 0.5% degradation products were added to BP-A31 cell culture medium (by courtesy of Désiré et al. [86]).

These first set of data obtained in this particular application opened interesting and promising avenues for the convenient delivery of drugs in the posterior segment of the eye, while minimizing or overcoming the drawbacks of conventional routes of administration.

5. MM 2.1.2-based materials as potent wound suture devices

One particular and attractive application of MM 2.1.2-based materials was their use as a wound suture device as described and patented in 2003 by Bru-Magniez et al. [96].

Prior to this innovation, only alkylcyanoacrylate-made products (*e.g.* Dermabond® from Ethicon) had been described and developed and several of them had been marketed for the suture of benign skin wounds [97]. These products, which have represented an alternative to stitches, staples or adhesive strips, have held several relative advantages, among them; their easiness to be implemented and the absence of pain when applied. Nevertheless, their use has required (i) bringing in close contact the wound edges and, (ii) the careful application of a thin layer of liquid acrylic monomer on the top of the wound. While polymerizing within a very few minutes, this monomer forms a film that strongly adhere to the skin and maintain the wound firmly closed. Probably, the main features which characterized these acrylate-based compositions has been the requirement to not have them introduced into the wound.

On the contrary, MM 2.1.2-based materials, which may contain poly(lactide-*co*-glycolide) or polyethylene glycol, were advantageously and conveniently applied, with no running off, inside model wounds practiced in guinea pigs. Followed until complete healing, scars from glued wounds were compared to non-resorbable threads. Data showed that synthetic MM 2.12-based glue yielded scars aesthetically and significantly better than the ones obtained from wounds sutured with conventional stitches.

Thus, these results were sufficiently convincing to justify the development of a new MM 2.1.2-based product which could strongly compete with Dermabond® and other alkylcyanoacrylate-based glues.

6. MM 2.1.2-based copolymers

As shown in the present review, proprietary methylidene malonate and poly(methylidene malonate)-based materials provide numerous development opportunities in the field of drug delivery systems and medical devices where they could represent valuable alternatives to existing products. Thus, these molecular and macromolecular species, alone or in combination with other biocompatible polymers, appear as a technological platform which could be cleverly expanded in order to offer new and potent drug formulating tools to pharmaceutical technologists.

Indeed, that was the main objective pursued in the design and synthesis of MM 2.1.2-based copolymers which could be advantageously implemented in various and potential drug delivery strategies.

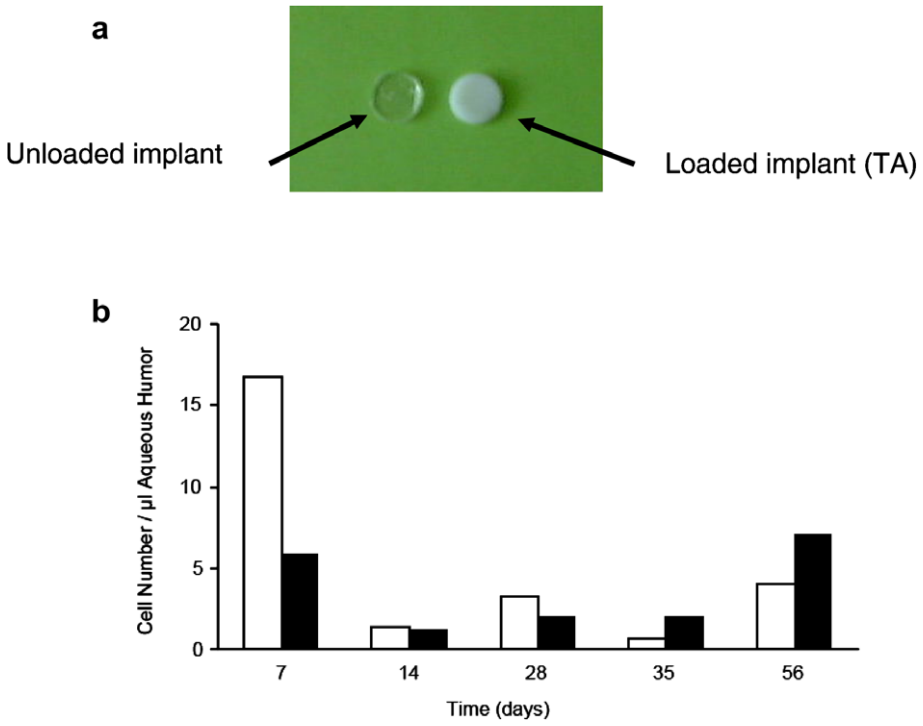

Fig. 8. (a) Loaded and blank PMM 2.1.2-made scleral implant. (b) number of inflammatory cells in eye aqueous humor of rabbits at days 7, 14, 28, 35 and 56 after implantation of PMM 2.1.2-made device; (□) control eye; (■) implanted eye ($n = 2$–3) (by courtesy of Felt-Baeyens et al. [92]).

6.1. Amphiphilic copolymers

6.1.1. Synthesis

An hydrophobic polymer, PMM 2.1.2, as underscored earlier, can undergo a bioerosion of its ester-bearing side chains to become progressively an anionic, hydrophilic and hydrosoluble polymer. Considering this biochemical feature as an intrinsic asset, the preparation of amphiphilic PMM 2.1.2-based block copolymers which could turn into fully hydrophilic and hydrosoluble was undertaken. Thus, as the synthesis and use of poly(ethylene oxide)-*block*-poly(D,L-lactide-co-glycolide) copolymers were previously reported [98], Larras et al. [99] and Bru-Magniez et al. [100] described and patented novel poly(ethylene oxide)-*block*-poly(methylidene malonate 2.1.2) (PEO-*b*-PMM 2.1.2).

These novel tensio-active macromolecules were synthesized in a control atmosphere and under drastic conditions allowing, by an anionic mechanism, the successive polymerization of ethylene oxide and MM 2.1.2 to yield block copolymers of various HLB values (Hydrophilic Lipophilic Balance). Classically, copolymers made up of approximately between 80 and 250 ethylene oxide units and 10–50 MM 2.1.2 residues could be obtained and characterized. Poly(ethylene oxide) (PEO) was maintained under its "living" and highly reactive form until addition of MM 2.1.2 [99]. Alternatively, a preformed and well-characterized hydroxylated PEO could be turned into an alcoholate which promptly reacted and initiated MM 2.1.2 polymerization. Based on the same chemical mechanism, triblock copolymers PMM 2.1.2-*b*-PEO-*b*-PMM 2.1.2 were successfully prepared from dihydroxylated PEO transformed into a dialcoholate PEO just prior addition of MM 2.1.2 monomers [100].

Bru-Magniez et al. [100] also reported synthesis examples for which MM 2.1.2 was used in combination with or replaced by other methylidene malonate monomers (*i.e.* MM 3.3 or MM 2.3.2) to form a statistic and hydrophobic poly(methylidene malonate) sequence,. These authors also mentioned the synthesis of PEO-*b*-PMM 2.1.2 by coupling of both preformed homopolymers appropriately α-functionalized.

The preparation of grafted block copolymers was described [100]. Such copolymers were obtained either by transesterification of PEO monomethylether onto PMM 2.1.2 side chains or by radical and statistical copolymerization of MM 2.1.2 and PEO methacrylate (macromonomer).

For drug targeting purposes, Studer et al. [101] prepared and described the synthesis of PEO-*b*-PMM 2.1.2 bearing a mannosyl residue at the PEO end chain. Using 2-aminoethanol or 3,3-diethoxypropan-1-ol as initiators of ethylene oxide polymerization, these authors were first able to introduce an α-amino or an α-acetal functions at the PEO end chain of PEO-*b*-PMM 2.1.2. By acidic treatment, α-acetal PEO-*b*-PMM 2.1.2 copolymer were further transformed into an α-aldehyde PEO-*b*-PMM 2.1.2 copolymer. In aqueous media, all α-amino PEO-*b*-PMM 2.1.2, α-acetal PEO-*b*-PMM 2.1.2 and α-aldehyde PEO-*b*-PMM 2.1.2 formed micelles of about 56 nm in diameter. In solution in organic

solvents (*i.e.* THF or DMF) or as a micellar solution in water, in mild experimental conditions, α-amino and α-aldehyde functions can easily react with (4-isothiocyanatophenyl)-α-D-mannopyranose and (4-aminophenyl)-α-D-mannopyranose, respectively, to yield α-mannosyl-functionalized PEO-*b*-PMM 2.1.2 copolymers. The yields measured were higher with copolymers dissolved in organic solvents than with copolymers organized in micelles. In addition, in the experimental conditions used, coupling α-aldehyde functions with (4-aminophenyl)-α-D-mannopyranose gave better yields than coupling α-amino with (4-isothiocyanatophenyl)-α-D-mannopyranose. Micellization of α-mannosyl-functionalized PEO-*b*-PMM 2.1.2 copolymers lead to a monodisperse micelle population with an average size of 56 nm.

Finally, Studer et al. [102], with the aim of preparing series of macromolecular species available for the development of drug targeting and delivery strategies, recently reported the synthesis of allyl end-functionalized PEO-*b*-PMM 2.1.2. The allyl function of block copolymers were subsequently modified to yield carboxyl or amino functionalized and biodegradable block copolymers. These chemical modifications were performed with good yields, either in organic media or in aqueous micellar solutions. Resulting carboxyl- and amino-bearing macromolecules can then easily be substituted by various structural moieties/ligands which can ensure targeting of drug delivery systems containing them.

6.1.2. Potential applications as new surfactants

New PEO-*b*-PMM 2.1.2 amphiphilic copolymers hold tensio-active properties and behave as surfactants able to significantly reduce water surface tension and to stabilize W/O emulsions. They were able to form micelles from 30 to 90 nm in diameter that could potentially carry hydrophobic drugs entrapped in their core [99,100].

Considering the results obtained earlier by Breton et al. [52] and Bousquet et al. [66] showing the strong interactions which occurred between PMM 2.1.2 nanoparticles and proteins, one of the most interesting applications was probably their use to prevent protein adsorption onto PMM 2.1.2 materials. Indeed, as described by Spenlehauer et al. [98] and Bazile et al. [103] for poly(ethylene glycol)-covered poly(D,L-lactide-co-glycolide) nanoparticles, PEO-*b*-PMM 2.1.2 copolymers were conveniently implemented to prepare drug-loaded and long-circulating (''stealth'') MM 2.1.2-based nanoparticles. Such formulations, known to avoid uptake by reticuloendothelial system and to increase dramatically the plasmatic half-life of drugs and of regular nanoparticulate drug reservoirs after i.v. administration, were described by Bru-Magniez et al. [100]. These authors prepared stealth MM 2.12-based nanoparticles (diameter: 160–240 nm) containing, for instance, cyclosporin A or doxorubicin.

Potentially, the implementation of α-mannosyl-functionalized PEO-*b*-PMM 2.1.2 copolymers [101] in the preparation of particulate systems (*i.e.* micelles or ''stealth'' nanoparticles) might successfully achieve drug targeting of specific cells or tissues expressing mannose-binding lectins.

6.2. Cationic copolymers

In the late 1990s and early two thousands, gene therapy had gained a growing interest as an innovative approach to treat and prevent various types of disease. In this arena, nucleic acid delivery and expression of transfected genes were a major challenge. Though certainly the most efficient vehicle to deliver and express DNA or RNA into target cells, viruses hold major drawbacks such as immunogenicity, which prevents repeated administrations, and safety [104–106]. To circumvent these important issues, a lot of efforts has been deployed to try and develop non-viral delivery systems mainly based on synthetic and lipidic cationic molecules or macromolecules able to complex nucleic acids to form so-called lipoplexes which, thus, ensure the stabilization and the uptake of messengers by target cells and tissues [106]. Although a few successes were reported, the gene expression level obtained through a non-viral strategy was constantly lower and more transient than the one observed with viral approaches.

The lack of efficiency of existing cationic agents was, in part, due to a low release of nucleic acids from lipoplexes entrapped into phagosomes and lysozomes after their target cell internalization. With the objective of improving the intracytosolic release of free nucleic acids able to migrate into the cytosol to be translated (for RNA), and into the nucleus to be transcripted (for DNA), Limal et al. [107] combined into new and proprietary cationic copolymers, amino-bearing units (ABU), some of them being new and patented by these authors (Fig. 9), with MM 2.1.2 units. The roles of amino-bearing and MM 2.1.2 comonomers associated in these hydro- or organo-soluble macromolecules were, respectively, to complex nucleic acids through electrostatic interactions and to ease their release after cellular uptake through electrostatic repulsions consecutive to the bioerosion of MM 2.1.2 side chains generating an increasing amount of negatively charged carboxyl residues.

6.2.1. Synthesis

Whereas various ABU and polyABU (PABU) were prepared and patented by Limal et al. [107,108], many types of random (*i.e.* poly(MM 2.1.2-*r*-ABU)) and block (*i.e.* PMM 2.1.2-*b*-PABU) copolymers were described [107]. Generally, ABUs were synthesized according to well-known methods and processes by coupling an appropriately functionalized methacrylate, vinylic or allylic derivative with a molecule bearing one or several protected or free ionizable amino groups.

Random poly(MM 2.1.2-*r*-ABU) copolymers were obtained through a radical polymerization using an initiator such as cyclohexyl percarbonate or α,α′-azoisobutyronitrile (AIBN). Block copolymers such as PMM 2.1.2-

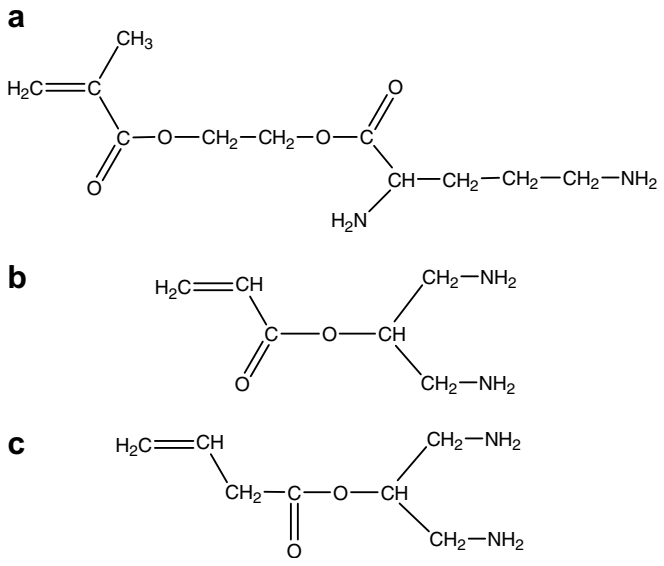

Fig. 9. Molecular structures of new and proprietary ABU bearing two ionizable primary amine functions [106]. (a) Ornithyl hydroxyethylmethacrylate, (b) 1,3-diamino-2-hydroxypropyl acrylate, (c) 1,3-diamino-2-hydroxypropyl vinyl acetate.

b-PDMAEMA (PDMAEMA: poly(dimethylaminoethyl methacrylate)) were prepared on a vacuum ramp by a group transfer anionic polymerization (GTP). First, PDMAEMA was synthesized in presence of *tetra-n*-butyl ammonium bibenzoate (TBABB) as a catalyzer. Then, an adequate volume of MM 2.1.2 was added to form the PMM 2.1.2 segment of the diblock copolymer.

Proton nuclear magnetic resonance (^{1}H NMR) was the main technique to characterize the copolymers. According to the relative length of the PMM 2.1.2 and PABU sequences and to the pH the resulting cationic copolymers were more or less hydrosoluble.

6.2.2. Potential applications in the field of gene delivery

The patent application by Limal et al. [107] provides *in vitro* preliminary results in the field of biomolecules delivery, especially in gene delivery. To ease DNA encapsulation, certain poly(MM 2.1.2-*r*-ABU) were implemented in the preparation of PMM 2.1.2 microparticulate systems obtained through a W/O/W multi-emulsion process. With an organosoluble copolymer such as poly(MM 2.1.2-*r*-HCl,GlyEMA), the DNA encapsulation rate was improved from about 21%, in absence of random MM 2.1.2-containing polycationic copolymers, to 37–56% in presence of various concentration of these cationic copolymers. Poly(MM 2.1.2-*r*-HCl,GlyEMA) or poly(MM 2.1.2-*b*-DMAEMA) was also used to prepare nanospheres on which plasmids were subsequently adsorbed.

Gene transfection experiments were carried out on cell cultures using GFP (Green Fluorescent Protein)-, luciferase- or β-galactosidase-encoding reporter genes. BHK-21 hamster kidney cells as well as SRDC, a novel murine spleen dendritic cell line [109] were used in this set of experiments. While much less cytotoxic, certain MM 2.1.2-containing polycations were shown at least as efficient as polyethylenimine (PEI) in transfecting BHK-21 cells. Interestingly, results obtained on SRDC demonstrated that unlike lipofectamine or DOTAP, common commercially available gene vectors, a poly(MM 2.1.2-*b*-DMAEMA), in specific experimental conditions, enabled the gene transfection/expression into this dendritic cell line. It is worthwhile to note that dendritic cells are quite well-known to be very difficult to transfect with non-viral gene vectors.

7. Conclusion

As new proprietary and biocompatible materials, MM 2.1.2-based oligomers, polymers and copolymers were extensively studied by Laboratoires UPSA and Virsol, together with a large panel of internationally acknowledged scientists. From this research consortium, fundamental and key data, original macromolecular species, and novel drug delivery systems, which further allowed the identification of various valuable biomedical applications, were obtained. The present review tried and summed up the different research and development efforts which were undertaken in medical fields, such as cardiology, tissue repair, ophthalmology or oncology where it remains major unmet needs and where MM 2.1.2-based materials might provide some interesting benefits in the future.

It is believed that the pool of data that was generated under the sponsoring of Virsol clearly provided a very robust basis of knowledge for further industrial developments of several MM 2.1.2-based products which could certainly be launched in the upcoming years and serve large unmet medical needs. The authors sincerely hope that biopharmaceutical company(ies) will pursue past Virsol endeavors.

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