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Effects of ionizing radiation sterilization on microparticulate drug delivery systems based on poly-α-hydroxyacids: an overview

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Ionizing radiation treatment is particularly advantageous as a sterilization technique for polymeric drug delivery systems. In recent years several authors have investigated this topic with interesting and sometimes controversial results. This overview was aimed at gathering and critically discussing the studies performed on the effect of ionizing radiation sterilization on microparticulate drug delivery systems made of poly-α-hydroxyacids. The results reported in the literature showed that ionizing radiation always led to a decrease in poly-α-hydroxyacids molecular weight. This effect was strictly related to irradiation dose, irradiation conditions, and depended on the starting polymer molecular weight. The presence of a drug and/or an additive inside the polymeric micromatrix could affect polymer behavior upon irradiation and consequently drug release behavior. Electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) proved to be useful techniques to elucidate the radiolytic mechanisms and the drug /polymer interaction upon irradiation.

Key words: Biodegradable polymers – Ionizing irradiation – Microspheres – Poly-α-hydroxyacids.

Two main types of radiation are employed for sterilization purposes: a) non-ionizing and b) ionizing. For example, ultraviolet ray is a non-ionizing radiation.

Ionizing radiation is defined as radiation that has sufficient energy to dislodge electrons from atoms and molecules and to convert them to electrically-charged particles called ions.

Ionizing radiation, called also high energy radiation, can be classified in two main groups: electromagnetic radiations, and high-energy charged particles. Ionizing radiation can be obtained from two different sources: radioisotopes and electrical discharge machines. The most common radioisotopes used commercially are Cobalt-60 and Caesium-137. Both of these radioisotopes are gamma (γ) emitters. Other sources of ionizing radiation are electron accelerators, X-ray machines and positively-charged particle accelerators or ion-beam accelerators.

Gamma radiation produced by a radioisotopic source (Cobalt-60, Caesium-137, X-rays), and electron beam irradiation (β-irradiation) energized by an electron accelerator machine, find several useful applications in the pharmaceutical and medical sectors. They are generally characterized as electromagnetic radiations having the highest frequency and energy and the shortest wavelength within the electromagnetic spectrum: Cobalt-60 and Caesium-137 emit gamma rays of 1,22 and 1,33 MeV of energy and the maximum internationally accepted energy of X rays for sterilization purposes is 5 MeV. The maximum electron beam energy internationally accepted is 10 MeV.

Due to its high energy content and short wavelength, a single incident photon can impart significant damage when absorbed by a living cell. This property leads to the use of ionizing radiation to kill living organisms in the process called irradiation. Applications of this process include industrial sterilization of medical equipment (as an alternative to autoclaves or chemical means), removing decay-causing bacteria from many foodstuffs or preventing fruit and vegetables from sprouting in order to maintain freshness and flavor. In so far as pharmaceutical products are concerned, the rapid proliferation of medical devices unable to withstand heat sterilization, and the concerns about the safety of ethylene oxide, have resulted in increasing applications of ionizing radiation sterilization.

This process is applicable also to drug substances and finished dosage forms giving high levels of sterility assurance. The use of ionizing radiation is referred to in European Pharmacopoeia (Eur. Ph.) and in United States Pharmacopoeia (USP) as being suitable for terminal sterilization [1, 2]. The advantages of sterilization by irradiation include low chemical reactivity and low measurable residues. Moreover, the unique feature of irradiation is the possible full-control of the process by the absorbed radiation dose alone, which can be easily and precisely measured.

Nevertheless, it is well known that high energy radiation can produce ionization and excitation in polymer molecules. Various effects, such as crosslinking and chain scission, can be expected depending on the radiation dose and polymer chemical structure. Certain polymers such as polystyrene, polyimides, polysulphone, aromatic polyurethanes have excellent radiation stability up to 4,000 kGy, while other polymers such as cellulosic esters, fluorinated ethylene propylene, polyacetals have poor/fair radiation stability. For the latter, irradiation sterilization can lead to significant alterations in the treated material, even for doses as low as 4 kGy. Moreover, the presence of additives in a polymer, or in a polymeric device, can modify the radiation resistance of the native polymer. Also the environmental conditions under which radiation process is conducted, such as the presence of oxygen, air, temperature, sample size, must be carefully set up because they can significantly affect the properties of the polymeric material after ionizing treatment [3].

An important application of ionizing radiation sterilization in the pharmaceutical field is the terminal sterilization of polymeric drug delivery systems intended for parenteral administration. The candidates for ionizing radiation sterilization can be either microparticles, nanoparticles, implants or *in situ* gelling implants. These drug delivery systems are morphologically different but they are all designed to control drug release. They consist preferentially of biocompatible and biodegradable polymers containing drugs to be injected or implanted in the human body, therefore they should be sterile.

Among the few biodegradable synthetic polymers studied to formulate these drug delivery systems, e.g. poly-ε-caprolactone, polyphosphazens, poly-α-hydroxyacids are the most successfully used [4-7]. The popularity of polylactide (PLA) and polylactide-co-glycolides (PLGA) can be ascribed to their biocompatibility and biodegradability, to their approval by the US Food and Drug Administration (FDA) for parenteral use in humans, and to the consolidated and successful use as resorbable surgical sutures.

Table I - Microparticulate drug delivery systems investigated under ionizing irradiation (the papers are listed in chronological order).

Polymer	Mw (Da)	Microsphere preparation method	Drug loaded	Ref.
D,L-PLA	10000, 50000, 140000, 300000, 160000	o/w reduced pressure solvent evaporation	Progesterone	56
PLGA 50:50	30000	Phase separation	-	30
PLGA 50:50	34000	Spray-drying	-	31
PLGA 50:50	35000	Spray-drying	Tetracycline HCl	39
PLGA 50:50 PLGA 75:25	34000 17000	Spray-drying	17-β-estradiol	32
PLGA 50:50	8600	w/o/w solvent extraction/evaporation	Leuprolide	52
PLGA 50:50	34000	o/w solvent evaporation/extraction	Ganciclovir	40
PLGA 50:50	75000	o/w solvent extraction	-	37
PLGA 50:50	34000	Spray-drying	Clonazepam	46
PLGA 50:50	34000, 88000	o:w solvent extraction	NSAIDs	33
PLGA 50:50	7500	o/w solvent evaporation	5-fluoro-uracile	34
PLLA + PLGA 50:50	8500-160000 40000-75000	o/o/w solvent evaporation	Etanidazole	36
PLGA 50:50	0.8 dl/g IV	w/o/w solvent evaporation	RhGF-1	53
PLGA 75:25 PLGA 65:35	7000-10000 7000-10000	solvent extraction	-	44
PLGA 50:50	34000	spray-drying	Clonazepam	47
PLGA 50:50	34000	spray-drying	Bupivacaine	45
PLGA 50:50	40000-75000	spray-drying	Paclitaxel	35
PLGA 50:50 PLA	40000, 75000 90000, 120000	phase separation	Etanidazole	50
PLGA 50:50	12000	o/w solvent evaporation	Ibuprofen	26
PLGA 50:50	12000	o/w solvent evaporation	Ibuprofen	27
PLGA 50:50	15000	o/w solvent evaporation	Aciclovir	41
PLGA 52:48	40000	w/o/w solvent evaporation/high pressure homogenizer	Ciprofloxacin HCl	51
PLGA 50:50 PLGA 65:35 PLGA 75:25	6600-7000 6100-10700 7200-7900	o/w solvent evaporation	-	48
PLGA 50:50/PEG	13000	w/o/w solvent evaporation	Ovalbumin	24
PLGA 50:50/PEG	13000	w/o/w solvent evaporation	Ovalbumin	42
PLGA 50:50/PEG	34000	o/w solvent evaporation	Indomethacin	43
PLGA 50:50 PLGA 65:35 PLGA 75:25 PLGA 85:15 PLGA 95:5	8495 10710 9000 11800 9495	o/w solvent extraction	-	49
PLGA 50:50 PLGA 75:25	102900 92000	w/o/w solvent extraction	SPf66 malarial antigen	65

In recent years several authors have investigated the effect of ionizing radiation sterilization on microparticulate drug delivery systems made of poly-α-hydroxyacids (*Table I*) with interesting and sometimes controversial results. This overview is aimed at gathering and critically discussing the studies on this topic.

I. IONIZING RADIATION STERILIZATION FOLLOWING THE PHARMACOPOEIAS

Both USP and Eur. Ph. report the treatment with ionizing radiation as suitable terminal sterilization method. Following the pharmacopoeias, ionizing radiation is recommended for those products that neither withstand moist heat at 121°C for at least 8 min, nor dry heat at 160°C [1, 2]. Sterilization can be achieved by exposing products to ionizing radiation either in the form of γ-radiation, or in the form of electron beam irradiation. The two irradiation processes significantly differ in the dose rate, since for gamma and electron sources of the same strength, the dose rate of the electron source is many times greater than that of the gamma source. Eur. Ph. 6th Ed. states that the reference absorbed dose is 25 kGy, independently of the irradiation process used. USP 31 reports that an effective sterilizing dose, tolerated without damaging effect, should be selected, and the dose could be either lower or higher than 25 kGy. Both pharmacopoeias indicate sterility assurance level (SAL) 10^{-6} or better. USP highlights the importance of validating the efficacy of the sterilization condi-

tions chosen, particularly if exposure levels lower than 25 kGy are used. It is necessary to determine the magnitude (number, degree or both) of the natural radiation resistance (D_{10}) of the microbial population of the product. Both USP and Eur. Ph. use suitable chemical dosimeters either to determine the absorbed minimum and maximum dosage distribution, establish the specific product loading pattern or monitor the radiation absorbed by the product during the sterilization procedure. The Pharmacopoeias do not indicate specifically the chemical dosimeter to be used; they only suggest that the dosimetry procedures should be independent of dose rate. Dosimeters should be calibrated against a standard source at a reference radiation plant on receipt from the supplier and at suitable intervals not longer than one year thereafter. Following USP, the setting of the preferred absorbed dose should be carried out on the basis of pure cultures of resistant microorganisms, employing inoculated products, e.g. with spores of *Bacillus pumilus* as biological indicators. In order to provide a more representative assessment of radiation resistance, the most recent procedures for gamma radiation sterilization base the dose upon the radiation resistance of the natural heterogeneous microbial burden contained in the product to be sterilized. Such procedures are currently being refined but may provide a more representative assessment of radiation resistance, especially where significant numbers of radiation-resistant organisms are present. More detailed descriptions of these procedures are published in the European Guidelines involving the use of ionizing radiation in the manufacturing of medicinal products and medical devices, and the decision trees to select the sterilization method [8-10].

II. POLY-α-HYDROXYACIDS AND THEIR DERIVATIVES OF PHARMACEUTICAL USE

1. Polymer synthesis

Polylactide (PLA) and poly-lactide-co-glycolide (PLGA) are biodegradable and biocompatible thermoplastic aliphatic polyesters with versatile properties and performances.

Polylactide can be obtained through synthetic pathways starting from renewable sources such as corn starch or sugar canes. Bacterial fermentation can be used to produce lactic acid which is oligomerized and then catalytically dimerized to make the cyclic dimer lactide.

The homo- and copolymers of lactic and glycolic acids are synthesized by the ring-opening melt condensation of these cyclic dimers, lactide and glycolide, as shown in *Figure 1*. Because of the D and L stereoisomers, the resulting polymer can be either D, L, or racemic DL.

The polymerization of L-lactide leads to poly-L-lactide (PLLA) that has crystallinity of around 37%, glass transition temperature between 50-80°C and melting temperature between 173-178°C. The polymerization of a racemic mixture of L- and D- lactides results in synthesizing the amorphous poly-D,L-lactide (PDLLA).

The polymerization reaction is usually conducted over a period of 2-6 h at about 175°C. Organo-tin catalysts are normally utilized with stannous chloride and stannous octoate as the most common used catalysts. Lauryl alcohol is often added to control molecular weight during synthesis. The critical parameters in the polymerization are the monomer purity, acidity, and the environmental conditions in the processing area, such as the humidity levels that should be low.

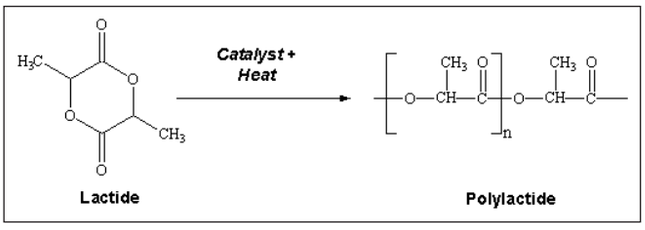

Figure 1 - Scheme of the ring opening polymerization reaction.

2. Physical-chemical characteristics of poly-α-hydroxyacids

The physical-chemical characteristics that mostly affect polymer performances are: a) monomer stereochemistry, b) comonomer ratio, in the case of copolymer polylactide-co-glycolide, c) polymer chain linearity, d) polymer molecular weight, e) crystallinity, f) water uptake and g) the presence of alkyl ester linkages or of free carboxyl groups at the polymer terminus.

These polymers are soluble in common organic solvents such as halogenated hydrocarbons, ethylacetate, tetrahydrofuran, dioxane and are insoluble in water. This last characteristic represents a drawback in the production of drug delivery systems because, due to their toxicity, the organic solvent residuals are strictly limited in the pharmaceutical field [11, 12].

All these polymers have glass transition temperatures (Tg) ranging between 40 and 80°C. Tg values vary depending on the polymer composition and its molecular weight. Tg is the temperature below which the physical properties of amorphous materials are similar to those of crystalline solids (glassy state), and above which the amorphous materials behave like liquids (rubbery state). Above the Tg the secondary non covalent bonds among the polymer chains become weak following thermal motion, and the polymer becomes rubbery and capable of elastic deformation. This behavior can be affected and modified by the presence of water, whose plasticizing effect lowers the Tg value. Referring to drug delivery systems, the polymer physical state can affect the functional properties of the same drug delivery system, such as drug release rate.

If comparing polymers with similar molecular weight, the racemic poly(DL-lactide) (PDLLA) is less crystalline and lower melting than the two stereoregular polymers D-PLA and L-PLA; while the copolymers polylactide-co-glycolides are less crystalline than the two corresponding homopolymers. In addition, the presence of the methyl group makes polylactide more hydrophobic than polyglycolide. All these factors are important because they affect the *in vitro* polymer degradation rate.

3. Degradation and biodegradation behavior of poly-α-hydroxyacids

These polymers degrade by random backbone hydrolysis of the ester linkages. Higher hydrophilicity corresponds to higher water uptake [13] and results in a higher polymer degradation rate. PLGA is always more hydrophilic than PLA, and it is demonstrated that PLGA with free carboxyl groups at the polymer terminus (uncapped PLGA) degrades faster than PLGA with hydrophobic ester linkages at the polymer terminus (capped PLGA) [14]. Moreover, the degradation rate is lowered in the presence of a crystalline phase and enhanced in an amorphous phase, hence the enantiomeric composition of the polymers plays an important role in the degradation process. Other factors affecting the degradation rate are polymer molecular weight (Mw) and Tg value.

Biodegradation behavior and times are the main characteristics that make these polymers a good choice in the production of drug delivery systems. Biodegradation occurs by uptake of water followed by bulk erosion through hydrolytic cleavage of the lactide and glycolide polymer chains to monomeric acids that are eliminated through Krebs cycle. Several factors such as the presence of phosphate ions, the ionic strength, the acidic or basic pH of the surrounding medium can accelerate polymer hydrolysis [15]. Also the presence of enzymes seems to play a role in the *in vivo* polymer degradation [16]. All the factors involved in the *in vitro* polymer degradation affect its biodegradation. The biodegradation mechanism and times depend also on the geometric *in vivo* shape and size of the drug delivery system. As an example, the hydrolytic degradation of massive amorphous poly(D,L-lactic) acid devices was shown to proceed heterogeneously and to go faster inside than on its surface, because in the interior there was a

Table II - Approximate times for biodegradation of lactide/glycolide polymers (from [17]).

Polymer	Biodegradation time (months)
Poly(L-lactide)	18-24
Poly(D,L-lactide)	12-16
Poly(glycolide)	2-4
50:50 poly(D,L-lactide-co-glycolide)	2
85:15 poly(D,L-lactide-co-glycolide)	5

larger contribution of acidic auto-catalysis. In fact, the pH inside the matrix was lowered by an accumulation of oligomers produced by polymer hydrolysis and entrapped inside the same matrix. Thus, drug delivery systems with a large surface area such as microparticles and nanoparticles are expected to degrade faster than pellets, discs and films.

Moreover, the presence of additives and of drugs inside the drug delivery system can affect the polymer biodegradation rate. Therefore, even if the biodegradation times of poly-α-hydroxyacids are reported in the literature, as shown in *Table II*, these times are only indicative values when referring to drug delivery systems because of the different factors that can affect this process.

III. MICROSPHERES OF POLY-α-HYDROXYACIDS AND DERIVATIVES

Microparticulate drug delivery systems for parenteral administration are good candidates for ionizing sterilization. Microspheres are designed to control drug release and they consist of biocompatible and biodegradable polymeric micromatrices whose size can be in the range of 1-1,000 μm. The size parameter must be carefully designed and is strictly related to the parenteral administration route chosen. As an example, the size of microspheres intended as depot drug delivery systems for intramuscular injection usually ranges between 25-50 μm, while microparticles for chemioembolization can be approximately 200-500 μm.

Most studies have been carried out on microspheres containing drugs for chronic disease therapies (e.g. hormones, antitumoral drugs, antibiotics) [4]. The encapsulation of protein drugs into microspheres proved to be a good opportunity to overcome their instability and to enable protein sustained release injections. Extremely prolonged drug releases, up to 28 days or even 2 months, can be achieved with microspheres made of poly-α-hydroxyacids, giving a great advantage in the administration of drugs that are intended for chronic disease therapies and that need constant plasma concentration.

In recent years, several technologies have been evaluated for the production of poly-α-hydroxyacids microspheres [18-21], but at present the preferred ones are the emulsion solvent evaporation/extraction and spray drying methods [18-20, 22].

1. Microsphere preparation by emulsion solvent evaporation methods

The emulsion solvent evaporation methods differentiate in: a) single emulsion, b) double emulsion solvent evaporation method, and c) emulsion solvent extraction method [18-20, 22].

The first method consists in dissolving the polymer in a suitable organic solvent such as methylen chloride and in emulsifying the polymeric solution into an aqueous phase containing a thickening agent and/or a surfactant (e.g. polyvinyl alcohol) to achieve a single o/w emulsion. Subsequent evaporation of the low boiling organic solvent is performed by raising the emulsion temperature under continuous stirring. The loading of a lipophilic drug in the microspheres, by this preparation method, can be achieved by direct drug dissolution in the polymeric solution, while hydrophilic drugs should be loaded through suspension in the polymer organic solution.

The double emulsion solvent evaporation method has been widely studied for loading hydrophilic proteins and polypeptide drugs into microspheres. In these cases, as shown in *Figure 2*, the drug is dissolved in an aqueous phase that is firstly emulsified in the polymer organic solution. The first w/o emulsion is subsequently emulsified in a second aqueous phase making a w/o/w emulsion, from which the organic phase is evaporated as described above.

The emulsion solvent extraction method is a modification of the previous ones where organic solvent elimination is achieved by extraction with another solvent that should be miscible with the first organic solvent but does not solubilize the polymer and the drug. The advantage of this method is that it does not involve temperature increase, but it involves certain restrictions due to the choice of organic solvents [22].

Sometimes a combination of solvent evaporation and solvent extraction method has been used. All the methods offer several advantages such as the possible control of microsphere size and porosity through the set up of stirring and solvent evaporation rate. Moreover, excipients such as polyethylen glycol (PEG), Labrafil and NaCl can be added to stabilize protein drugs from the denaturation caused by the contact with organic solvents, to achieve high payloads and reduce burst release [23-32].

2. Microsphere preparation by spray-drying

Spray drying method, as shown in the scheme of *Figure 3*, consists in dissolving the polymer in a suitable low boiling solvent. The drug substance is added to the polymer solution and the resulting solution, dispersion or emulsion is atomized through the nozzle of a spray drier apparatus in a stream of hot air. The solvent evaporates very quickly leaving solid microparticles that are collected into a separator cyclone. The process presents several advantages, i.e. it is a time-saving process and allows the production of high amounts of product by a one-step procedure. Moreover, process conditions are quite mild and the process can be performed in aseptic conditions or in inert atmosphere [18-20].

Several products based on microspheres made of poly-α-hydroxyacids are commercially available, as shown in *Table III*. Either mixtures poly-α-hydroxyacids with other biodegradable polymers such as poly(orto esters) (POEs) [33], or new derivatives of poly-α-hydroxyacids are investigated in order to optimize microspheres performances above all regarding the loading and controlled release of protein drugs [5-7, 34].

IV. MICROSPHERE IRRADIATION

The final scope of the microparticulate drug delivery systems irradiation is to achieve sterility. Following the European Guidelines, 25 kGy represents the minimum absorbed dose considered adequate for the purpose of sterilizing pharmaceutical products without providing any biological validation [9]. For this reason, the effect of this irradiation dose on poly-α-hydroxyacids and on microspheres has been thoroughly investigated (*Table I*).

The most important polymer physical-chemical properties evaluated are its molecular weight (Mw) and molecular number (Mn) that can be directly determined by gel permeation chromatography (GPC). Differential scanning calorimetry (DSC) is used to obtain information about possible changes of structure, and consequently of Tg or melting temperature (Tm), induced by irradiation. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) are performed to check the possible effect of irradiation on microparticle shape and surface characteristics. The changes upon irradiation of these characteristics are monitored because they can modify the functional properties of microspheres, hence the drug release rate. All authors agree that the interaction of polymers with ionizing radiation leads mainly to a reduction in their molecular weight and the data are consistent with the chain scission phenomena.

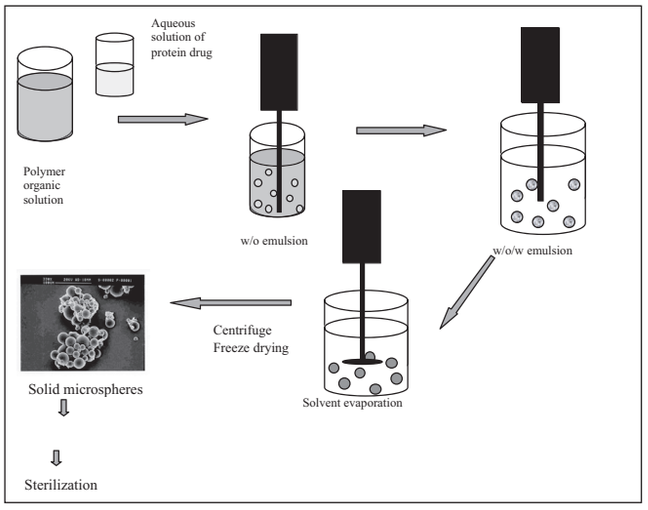

Figure 2 - Scheme of the w/o/w emulsification/solvent evaporation process used to prepare microspheres loaded with a protein drug.

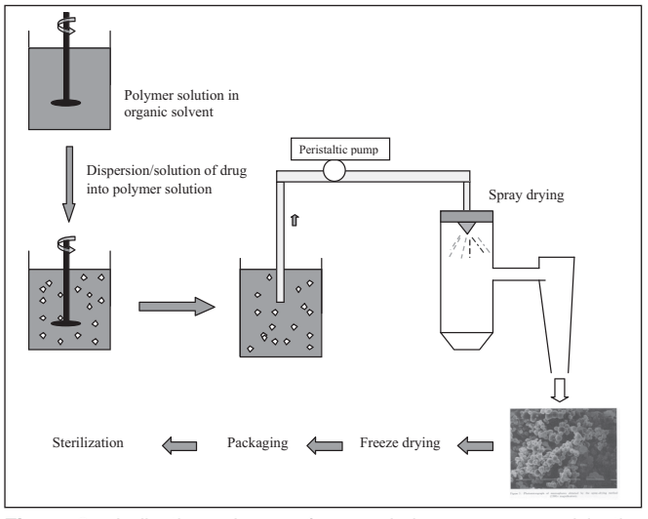

Figure 3 - Indicative scheme of spray drying process used in the preparation of drug-loaded microspheres.

Table III - Pharmaceutical products based on PLA and PLGA microparticles on the market in Europe and US.

Commercial product	Polymer	Drug	Manufacturer
Enantone 3.75, 11.75 (Lupron Depot in USA)	PLA or PLGA	Leuprolide	Takeda
Trelstar Depot	PLGA	Triptorelin	Pfizer
Decapeptyl SR	PLA or PLGA	Triptorelin	Ipsen-Beaufour
Decapeptyl	PLG	Triptorelin	Ferring
Suprecur MP	PLGA	Buserelin	Aventis
Nutropin Depot	PLGA	Growth hormone (HGH)	Genentech
Sandostatin LAR	PLGA-glucose	Octreotide	Novartis
Somatuline LA	PLGA	Lancreotide	Ipsen-Beaufour
Arestin	PLGA	Minocycline	OraPharma
Risperdal Consta	PLGA	Risperidone	Janssen Pharmaceutica

1. Effect of irradiation dose

Investigations have been performed under different gamma irradiation doses starting from 5 kGy up to 25 or 50 kGy [35-40] in order to evaluate the effect of radiation dose and to elucidate the possible chain scission mechanism. Montanari *et al*. [36] investigated two different PLGAs 50:50 with the same molecular weight but different structures: RG503H carries predominantly free carboxylic acid groups on one of the polymer chain ends (uncapped polymer) while in RG503 the carboxylic terminal groups are esterified with an ethyl group (capped polymer). The authors irradiated the polymers and the corresponding microspheres at doses of 5, 15, 25 kGy. They found that radiation effect on Mw can be considered equivalent for the polymer and the related microspheres, both showing a trend in decreasing their Mw as a function of the irradiation dose. Moreover, the capped polymer resulted significantly more stable to irradiation than the uncapped one: for RG503, the decay in molecular weight was always negligible for doses below 15 kGy.

Mohr *et al*. [37] found linear decrease in PLGA 50:50 Mw independent from monomer composition and drug loading, while the almost unchanged Mn value indicates that random chain scission was the preferred chain cleavage mechanism.

Faisant *et al*. [39] investigated the effect of irradiation dose on drug release rate from PLGA 50:50 microparticles loaded with 5-fluorouracile. For this purpose, the *in vitro* release test was performed on microspheres irradiated at 4 and 33 kGy. Results showed that the drug release rate significantly increased with increasing γ-irradiation dose. This effect was evident above all on the initial drug release rate. The authors explained this result with the free volume theory of diffusion. The decrease in average polymer molecular weight caused by irradiation leads to increased polymer molecule mobility. Consequently, the free volume available for diffusion of 5-fluorouracile through the polymeric matrix was enhanced. In this case the dominating drug release mechanism was diffusion. Wang *et al*. [40] investigated the effect of γ-irradiation at the low clinical dose of 0.05 kGy and at higher dose of 10 kGy, on microspheres and discs made of PLGA 50:50 with or without an additive as the D-α-tocopheryl polyethylen glycole1,000 succinate (Vitamin E TPGS), used to improve drug stability. Results showed that no degradation was highlighted for the delivery systems made of PLGA without additive, either after treatment at 0.05 kGy or at 10 kGy. On the contrary, the presence of additive enhanced polymer degradation, but only after irradiation at 10 kGy. The authors also investigated the effect of irradiation dose on paclitaxel release from PLGA discs. They concluded that the drug release profile was modified upon irradiation only in the presence of vitamin E TPGS.

Lee *et al*. [41] irradiated double walled microspheres made of PLLA and PLGA 50:50 loaded with etanidazole at the clinical dose of 0.05 kGy and at the sterilizing dose of 25 kGy. They found that the impact of 0.05 kGy dosage on the physical properties of polymeric carrier was negligible, while a dose of 25 kGy resulted in leftward shifts in Tg and Tm that could be correlated to a decrease in polymer molecular weight.

Dorati *et al*. [42] evaluated the effect of different γ-irradiation doses on PEGd,lPLA and PEG-PLGA multiblock copolymers, and compared the behavior to irradiation of the multiblock copolymers to that of PLA, PLGA polymers. The polymers were irradiated at 5, 15, 25 and 50 kGy total dose by using a Cobalt-60 irradiation source, in the presence of air and at room temperature. The dominant effect of γ-irradiation on all polymer samples was chain scission, as highlighted by the results of GPC and infrared absorption spectrophotometry. PEGd,lPLA presented higher sensitivity to irradiation treatment with respect to PLA, probably due to the presence of PEG in the matrix. Moreover, the effect of γ-irradiation was shown to continue over a much longer period of time after the treatment has been performed, leading to further polymer degradation with time. The authors suggested that the material reacts with oxygen to form peroxyl free radicals,

which may further undergo degradation reactions during storage after irradiation.

1.1. Bioburden determination

Some authors [37, 43] have performed microsphere bioburden evaluation for radiosterilization dose selection. This appears an interesting point to be investigated since it has been highlighted that a sterilizing dose of 25 kGy leads to a significant reduction in polymer Mw and indirectly modifies the functional properties of microspheres in terms of drug release rate. The choice of sterilization dose depends on the initial microbiological contamination (bioburden), radiosensitivity of the micro-organisms and the required sterility assurance level (SAL).

Geze *et al*. [43] determined the bioburden and the main source of contamination of PLGA50:50 microparticles prepared by emulsion/extraction process in non aseptic conditions. Following the ISO method [44], the study was conducted on 18 batches of microspheres chosen as representative samples of the product. The results showed that batch bioburden varied from 6.3 to 55.5 CFU in 110 mg of microspheres. From these data, the ISO 11137 method recommends a sterilization dose of 19.61 kGy to achieve a SAL of 10^{-6}. This value represents a radiation dose reduction of 20% if compared to 25 kGy. The authors demonstrated that the radiation dose reduction could lead to a 14% gain in polymer Mw compared with the effects of the Eur. Ph. reference dose. Moreover, the authors found that the main source of contamination was Gram-positive micro-organisms probably originated from the human commensal flora. They checked also the bioburden of the water/methylen chloride solutions and confirmed that the presence of methylen chloride had an antimicrobial effect on the polymeric phase. The authors concluded that, considering the microbiological results obtained, and providing certain precautions, 19.6 kGy was suitable as the upper limit of the radiosterilization dose.

Mohr *et al*. [37] determined the changes in bioburden due to the processing conditions and consequently set-up the sterilization dose of PLGA 50:50 microspheres prepared by spray drying. Moreover, they checked the D_{10} value of the official test micro-organism for irradiation sterilization, *Bacillus pumilus*, microencapsulated into PLGA polymer. They applied the standard overkill procedure using 25 kGy, which implies a 10^{-6} SAL: this irradiation dose corresponds to about eight times the *Bacillus pumilus* D_{10} value. The maximum bioburden, that fulfills the microbiological requirements for commercially available polymers and synthetic drug substances, is commonly 10^2 CFU/g. Additional microbiological contamination of microparticles was caused by spray drying process. An increasing microbiological quality was detected with increasing processing time. Limiting the initial bioburden to a maximum value of 10^2 CFU/g, reliable sterility was achieved with the lowest irradiation dose of 5.1 kGy. These findings demonstrated the need to limit bioburden of the starting materials, and to establish and validate the equipment cleaning procedure also from microbiological perspective.

The microencapsulation of the bioindicator *Bacillus pumilus* and its subjection to γ-irradiation demonstrated that spores were not further protected by the process, and confirmed the literature data of the respective D_{10} value of 2.4 kGy.

2. Effect of process conditions

Temperature, air and moisture are the process conditions involved in the irradiation treatment. Most authors [32, 35, 37, 40, 41, 45-47] performed irradiation in dry ice, indicating that low temperature is needed to reduce ionizing phenomena generated by γ-irradiation.

Fernandez-Carballido *et al*. [32] irradiated at 25 kGy PLGA 50:50 microspheres loaded with indomethacin and containing Labrafil as an additive, and the corresponding raw polymer. The process was performed in the presence of air, in dry ice, or without dry ice (room temperature). They analyzed the microspheres by GPC, SEM, X-ray diffraction, before and after irradiation. Their results showed that similar decay in Mw was observed despite the process conditions used, both for raw polymers and microspheres. The release rate of indomethacin was similar for both non-sterilized and sterilized microspheres at low temperature, while a significant increase in drug release rate was observed in microspheres sterilized without temperature control. The results suggested that the change in indomethacin release rate upon irradiation at room temperature could be caused by radiation sensitivity of Labrafil. The authors concluded that, considering that the Tg of poly-α-hydroxyacids ranges between 40 and 80°C, the irradiation process should be performed at temperatures lower than the polymer Tg value. Moreover, the temperature increase during irradiation is strictly correlated to irradiation dose rate, which depends on the characteristics of Cobalt-60 source. In so far as the presence of air is concerned, the authors performed the irradiation process in air [29, 36, 38, 48-52], or under vacuum [53, 54] or under nitrogen or argon atmosphere [37, 45], but only a few authors [55] have investigated whether the presence of air during the irradiation process could affect polymer degradation behavior in terms of Mw reduction or Tg shift.

Dorati *et al*. [55] irradiated PEGd,lPLA, PEG-PLGA, PLA and PLGA polymers using a Cobalt-60 irradiation source in air and under vacuum at 25 kGy total dose. After irradiation the polymer samples underwent a stability study under different storage conditions involving either the presence of vacuum or air. The authors evaluated the Mw and Mn changes of polymer samples by GPC. Thermal behavior was investigated by DSC. The results showed higher stability for those samples irradiated under vacuum and stored at + 4ºC, 40% RH in the absence of oxygen.

All authors nevertheless agree in performing irradiation processes on dried or even freeze-dried microparticles in order to minimize moisture content.

3. Effect of polymer composition and additives

It has been demonstrated, and evaluated by several authors, that poly-α-hydroxyacids can react differently to γ-irradiation, depending on their composition and Mw [36, 54, 56]. Montanari *et al*. [36] investigated the effect of ionizing radiation on microspheres made of two types of PLGA 50:50 (RG503 and RG503H) with the same Mw (34,000 Da) but different chemical structures, and consequently different hydrophilicity, as previously reported. The samples of raw polymers and microspheres were irradiated at 5, 15, 25 kGy in air and at room temperature and were characterized by GPC and DSC before and after irradiation. GPC results (*Tables IV and V*) showed that both the raw polymer (P) and microspheres (Ms) presented a decrease in their Mw at time 0 as a function of the irradiation dose. The decay in Mw for RG503 was always negligible for irradiation doses below 15 kGy, while it became around 10% for 25 kGy. After 150 days of storage at 4°C and 75% RH, the Mw decay was 20% in the raw RG503 and 25% in the corresponding microspheres. It was not possible to evaluate the radiation effect, at different storage times, on RG503H and the corresponding microspheres, because this polymer resulted to be highly unstable. In all cases, the Tg values of both raw polymers and microspheres did not change after irradiation up to 25 kGy and remained stable for up to 150 days of storage.

Bushell *et al*. and Williams *et al*. [54, 56] evaluated the effect of irradiation on PLGA with different lactide:glycolide molar ratios and comparable Mws.

In particular, Williams *et al*. [56] performed the study on five samples of both raw PLGA and microspheres with comparable molecular weights and different composition in terms of lactide:glycolide molar ratios which were 50:50, 65:35, 75:25, 85:15, 95:5. The samples were irradiated at 30 kGy in air and at room temperature and were characterized by size exclusion chromatography (SEC), DSC and for their water content (Karl-Fisher volumetric titration), before and after irradiation. The results showed that the exposure significantly decreased the mo-

Table IV - Weight average molecular weights (Mw) of PLGA RG503 polymer (P) and of PLGA RG503 microspheres (Ms), before and after irradiation (from [31]).

Storage time (days)	Radiation dose (kGy)							
	0		5		15		25	
	P	Ms	P	Ms	P	Ms	P	Ms
0	19800	19100	18800	19000	18700	17800	17900	17500
15	-	19300	18300	18500	18000	17400	16900	16700
30	-	19200	18600	20400	18000	18400	16700	16200
60	18700	19400	18700	18900	16800	17600	17000	16100
150	18400	18500	17300	17500	15900	15900	15600	14600

Table V - Weight average molecular weights (Mw) of PLGA RG503H polymer (P) and of PLGA RG503H microspheres (Ms), before and after irradiation (from [31]).

Storage time (days)	Radiation dose (kGy)							
	0		5		15		25	
	P	Ms	P	Ms	P	Ms	P	Ms
0	19000	20700	19900	19500	18300	18200	16500	16300
15	-	-	18300	18700	18000	17000	16600	16900
30	-	-	18900	19300	16800	17900	16500	16900
60	18200	19500	18600	18700	16000	17100	16000	15700
150	10200	8900	12500	13000	11100	10900	8500	9300

lecular weight of the samples with lactide:glycolide molar ratio up to 75:25; higher lactide content resulted in an insignificant decrease in the average Mw upon irradiation. Both the average molecular number and average molecular weight were comparably affected by irradiation, supporting the random chain scission degradation mechanism of PLGA. No significant changes in Tg or water content of the PLGA samples were highlighted after irradiation. The long term stability of the samples was affected by γ-irradiation with a greater decrease in molecular weight and Tg value, and a significant increase in water content of the irradiated samples on storage in comparison to the non-irradiated samples. The microspheres proved to be more stable than the corresponding raw polymer. This result was explained by the authors with a reduced hydrophilicity of the microspheres with respect to raw polymer, caused by washout of water-soluble species during the microsphere preparation process. The authors concluded that the lactide:glycolide ratio of PLGA copolymer played an important role in controlling the stability characteristics of both raw polymers and the microspheres in the solid state.

Considering PLGAs of different composition in terms of lactide:glycolide molar ratio, Bushell *et al*. [54] focused on electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) as a technique to characterize the radiolytic behavior of polymers and microspheres submitted to irradiation. For this reason this paper is more extensively discussed in the chapter dedicated to EPR and ENDOR techniques (see 5.7).

Yip *et al*. [57] investigated the effect of γ-irradiation on microspheres made of different PLA/PLGA blends prepared by polymer phase separation. Their study was performed through microsphere characterization by GPC and DSC upon irradiation at 0.50 and 25 kGy. The authors found that only the 25 kGy dose was responsible for polymer degradation. Moreover, they suggested that the presence of two polymers (PLA and PLGA) with different degradation rates caused an heterogeneous degradation process with the outer layer and the inner matrix of the microspheres degrading at different rates. Even though several additives may be used in the formulation of poly-α-hydroxyacid microspheres such as poly(ethylene glycol) (PEG) or gelatin, only a few authors have expressly investigated whether the presence of an additive could change the effect of γ-irradiation on the microspheres.

PEG is one of the most studied additives. Fernandez-Carballido *et al*. added PEG (Labrafil) to microsphere formulations loaded with anti-inflammatory drugs, such as ibuprofen [31, 32] or indomethacin [49], in order to modulate drug release rate. This additive was widely investigated as a stabilizer of protein drugs loaded into microspheres [23-29, 48].

Dorati *et al*. 2005 [29] prepared, with the w/o/w emulsion solvent evaporation method, ovalbumin loaded microspheres using different blends of PLGA 50:50 and PEG 400 or PEG 4000 as additives. The microspheres were irradiated at 25 kGy in air and characterized before and after irradiation in terms of ovalbumin and PEG content and *in vitro* ovalbumin release. The authors found that small amounts of PEG inside the microspheres prevented the changes in protein release rate from microspheres that were highlighted, after irradiation, in the microspheres without PEG and in those with high amounts of PEG (15, 30%).

The effect of the presence of additive in the polymeric micromatrix was investigated by Dorati *et al*. 2006 [48] using atomic force microscopy (AFM). Results showed significant changes in surface roughness after irradiation of PLGA microspheres, while no changes in surface roughness were highlighted in PLGA/PEG microspheres indicating that this additive exerted a protective effect towards γ-irradiation (see also IV.4).

Dorati *et al*. [42] in a previously commented paper (see IV.1) evaluated the effect of different γ-irradiation doses on PEGd,lPLA and PEG-PLGA multiblock copolymers, and compared the behavior to irradiation of the multiblock copolymers to that of PLA, PLGA polymers. The authors highlighted how the extent of Mw degradation caused by γ-irradiation was more prominent for polymers with high molecular weight. Moreover in the same molecular weight range, the presence of PEG in the multiblock copolymer resulted in higher sensitivity to irradiation treatment with respect to the corresponding homopolymer.

Bozdag *et al*. [58] prepared ciprofloxacin HCl-loaded PLGA nanoparticles by w/o/w emulsification solvent evaporation followed by high pressure homogeneization. They investigated the effect of freeze-drying with different cryoprotectants and of γ-irradiation, on the physicochemical characteristics of the nanoparticles. Freeze-drying was performed in the presence of 5% w/v mannitol, trehalose or glucose, with 5 or 15% w/v dextran. Irradiation was performed at 25 kGy. The authors found that the freeze-drying process induced a significant increase in particle size, excepted when mannitol was

used. γ-irradiation affected neither particle size nor zeta potential but the reconstitution of nanoparticles suspension was more difficult. Concerning drug release, γ-irradiation significantly reduced the drug release rate in all nanoparticles samples with the exception of nanoparticles treated with 5 and 15% glucose and dextran, demonstrating the protective effect of these sugars.

4. Effect of ionizing radiation on microsphere morphology and size

Morphologic analysis of microspheres is performed to highlight shape and surface properties and to have an idea of microsphere size and size distribution. The morphologic analysis of microspheres after irradiation and the comparison with their feature before irradiation plays an important role because changes in morphology can be related to the irradiation process and can affect microsphere performances. Moreover, when microspheres are intended for parenteral administration, their size must be in a well-stated range, according to the administration route, e.g. for intramuscular administration it is recommended not to exceed 50 μm, for intra-arterial route (chemioembolization) sizes between 200-500 μm are required.

Scanning electron microscopy (SEM) is the most common technique used to analyze microsphere morphology. Almost all the authors considered (see *Table I*) used this technique. The information showed that PLGA and PLA microspheres were usually spherical with a smooth surface and internal porosity [36, 41, 46, 52]. In some cases, SEM highlighted the presence of drug crystals within the core of microspheres [41] or the characteristic double wall morphology of microspheres made of polymer blends [33].

Calis *et al*. [38] reported that the presence of drugs inside the microsphere changed their morphology in terms of increased porosity.

Dorati *et al*. [29, 48] investigated the effect of γ-irradiation on microsphere morphology both by SEM and atomic force microscopy (AFM). The study was carried out on microspheres made of PLGA/PEG blends prepared by double emulsion solvent evaporation. The microspheres were irradiated at 25 kGy with a Cobalt-60 source, in air at room temperature. SEM analysis showed that irradiation always led to microsphere aggregation up to incipient fusion among the particles. Moreover, when the starting microparticles had porous structure, they broke up very easily upon irradiation. This behavior was highlighted both for placebo and drug-loaded microspheres containing PEG as additive (*Figure 4*).

AFM analysis showed that the surface of microspheres prepared without additives was dramatically scraped after irradiation, while the surface of microspheres containing PEG did not show evident change after irradiation. The quantitative comparison of the mean roughness values (rms) derived from the image analysis before and after irradiation (*Table VI*) suggested that the presence of PEG protected the microsphere surface from damage by irradiation. Montanari *et al*. 2003 [51] used AFM analysis to show that surface roughness of irradiated microspheres increased upon γ-irradiation with respect to electron beam treatment.

Both authors concluded that these results represented an interesting and innovative application of AFM as a technique for surface analysis of microparticulate drug delivery systems.

Size distribution of microspheres is often determined by instrumental methods such as light scattering (Malver Master sizer), light blockage (HIAC-ROYCO) and electric sensing zone methods (Coulter Counter).

Granulometric analysis performed before and after irradiation gave controversial results. It appeared that irradiation treatment could sometimes cause aggregation and breakage of microspheres. This phenomenon could not be generalized and was probably connected to microsphere composition, size and structure. Microspheres of larger size and higher porosity seemed to be more subject to aggregation up to fusion, as highlighted by Dorati *et al*. 2005 [29].

5. Effect of ionizing radiation on drug-loaded microspheres and on drug release

An important point to be evaluated is the behavior of the drug loaded in a microparticulate drug delivery system submitted to sterilization by ionizing irradiation. The stability of drug under irradiation conditions and the changes in drug release properties are parameters involved in the quality assurance of drug delivery systems. Several papers have evaluated these parameters [29,31,32,37,38,40,46,47,52,57,59-65] showing that drug release kinetics from the irradiated biodegradable drug delivery systems very frequently presented positive or negative deviations from non-irradiated controls depending on the drug, matrix type and irradiation dose. As an example, the random chain scission of PLGA and PLA upon irradiation treatment could generate an increase in the number of polymer carboxylic end groups that interacted by the ionic interaction with the positively charged amine groups of proteins. This factor could contribute to the reduction in protein release that was frequently observed after microsphere irradiation [64]. Anyway, it is almost impossible to define a common mechanism describing the effects of irradiation on drug loaded into microspheres, and each drug should be investigated individually. Moreover, several authors

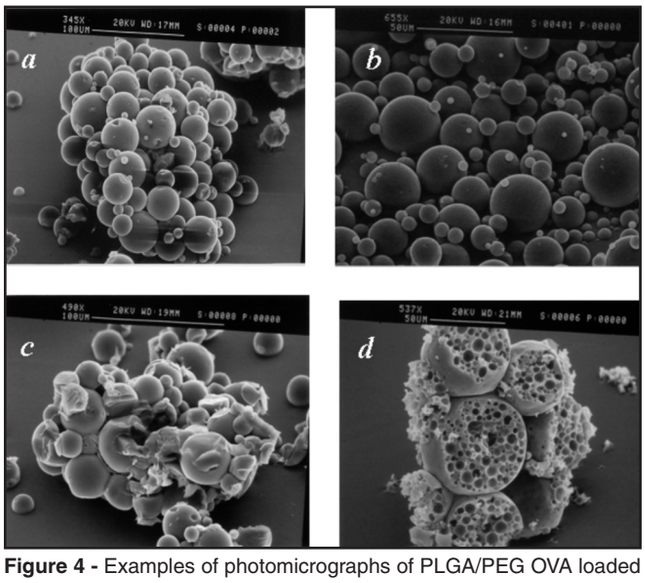

Figure 4 - Examples of photomicrographs of PLGA/PEG OVA loaded microspheres before irradiation (a and b) and after irradiation (c and d) (from [24]).

Table VI - Microsphere rms roughness values before and after γ-irradiation (M ± SD) (from [42]).

Batch composition	PEG content	rms before irradiation (nm)	rms after irradiation (nm)
PLGA/OVA	-	6.83 ± 0.84	14.04 ± 6.01
PLGA/OVA/NaCl	-	3.59 ± 1.99	7.24 ± 0.99
PLGA/PEG/OVA	4.36 ±0.32	6.10 ± 0.66	6.05 ± 0.73
PLGA/PEG/OVA/NaCl	0.48 ±0.07	3.11 ± 0.75	2.75 ± 0.77
PLGA/PEG/OVA	9.22 ±0.21	4.22 ± 1.05	4.47 ± 1.40
PLGA/PEG/OVA/NaCl	4.32 ±1.05	3.21 ± 0.69	3.38 ± 0.62

have investigated the effect of γ-irradiation on the formation of free radicals in the drug and polymer using electron paramagnetic resonance (EPR) spectroscopy technique. This technique provided interesting and unique results concerning the radiolytic mechanisms of drugs and polymers and is discussed in a dedicated chapter (see IV.7).

Mohr *et al*. [37] investigated the effects of γ-irradiation on 17β-estradiol loaded into PLGA 50:50 microspheres obtained by spray-drying. The drug formed a solid solution in the polymeric matrix. The microparticles were irradiated with irradiation doses ranging from 5.1 to 26.6 kGy at low temperature (dry ice), in air. The authors found that 17β-estradiol as drug substance showed excellent stability against γ-irradiation in the investigated dose range, whereas the microencapsulated estradiol seemed to be converted to conjugation products with PLGA and to partially degrade into the degradation product 9,11-dehydroestradiol. *In vitro* drug release studies showed accelerated kinetics with increasing irradiation doses due to dose dependent polymer degradation.

Bittner *et al*. [45] evaluated PLGA microspheres loaded with tetracycline-HCl obtained by spray-drying. Their work was addressed mainly to investigate the effect of γ-irradiation on the formation of free radicals in the drug and polymer. In so far as the tetracycline-HCl content in the microspheres is concerned, it was not affected by exposure to γ-rays.

Herrero-Vanrell *et al*. [46] used the o/w solvent extraction method to prepare PLGA microspheres loaded with ganciclovir intended for intraocular administration. The microspheres were sterilized by γ-irradiation at 25 kGy in dry ice. No significant differences in the amounts of the encapsulated drug and dissolution profile were highlighted before and after γ-radiation exposure.

Montanari *et al*. 2001 [52] evaluated the effect of γ-irradiation on the stability of PLGA microspheres loaded with clonazepam. This work was mainly addressed to evaluate the influence of clonazepam on PLGA radiolysis mechanism and to identify possible irradiation marker using the EPR spectroscopy technique. The microspheres were prepared by spray-drying and irradiated at 25 kGy in air and under vacuum. The microspheres irradiated under vacuum were stable in the storage time evaluated (180 days), whereas the clonazepam release rate increased by 10% immediately after irradiation in air, and did not change further in the following storage period. Faisant *et al*. [39] investigated the effect of different γ-irradiation (4-33 kGy) on the release kinetics of 5-fluorouracil (5-FU) from 5-FU loaded PLGA microspheres. Drug release was found to depend significantly on the applied γ-irradiation dose. The release profiles were always biphasic with a rapid initial drug release rate followed by a slower, approximately constant drug release phase. The effect of γ-irradiation was an increase in the initial burst release. The resulting drug release rate could be related to the irradiation dose. The authors concluded that diffusion seemed to be the dominant release controlling mechanism in the case of 5-FU. No degradation of drug upon irradiation was highlighted.

Calis *et al*. [38] investigated the behavior to irradiation of two non-steroidal anti-inflammatory drugs, namely naproxen sodium and diclofenac. The drugs were loaded in microspheres made of PLGA of two different molecular weights (34,000 and 88,000 Da) and submitted to γ-irradiation at 5, 15, 25 kGy doses, in air and at room temperature. The authors found that drug loadings of the irradiated and non-irradiated microspheres were essentially the same. Drug release rates of both anti-inflammatory drugs evaluated increased with increasing irradiation doses, independently of the polymer molecular weight.

Lee *et al*. [41] and Yip *et al*. [57] investigated the release behavior of etanidazole (2-nitroimidazole-1-acetamide, N-(2-hydroxyethyl) a nitro-imidazole hypoxic radiosensitizer, encapsulated in microspheres made of different PLGA/PLA blends and irradiated at 0.05 and 25 kGy. Application of the low clinical dose of 0.05 Gy is justified because it is the total dosage during a radiotherapy treatment period.

Lee *et al*. [41] entrapped etanidazole into double walled microspheres made of a PLGA core surrounded by a PLLA wall. Due to its hydrophilicity the drug was mostly encapsulated in the more hydrophilic core. The drug release rate significantly changed after irradiation at 25 kGy. Indeed, sustained release for more than three weeks was highlighted in the irradiated microspheres, compared to 80% of drug released over 10 days in the non-irradiated samples. The change in drug release was evident above all in the intermediate release phase. The authors correlated the onset of sustained release to the increased polymer degradation upon irradiation as detected by the lowering of their Tg and Tm values. They concluded that the onset of a sustained release phase was due to a change in the release mechanism from diffusion controlled to degradation controlled mechanism.

Yip *et al*. [57] loaded etanidazole into microspheres made of different PLA/PLGA mixtures and prepared by the polymer phase separation method. The authors found that at dosage of 0.05 kGy there was no apparent effect on the tri-phase drug release profile. On the contrary, after treatment at 25 kGy, the release profiles of drug carriers were drastically modified. The faster erosion of the polymer with high dosage irradiation was considered responsible for the hastened drug release. The behavior of microparticles to irradiation treatment was shown to be dependent also on their polymeric composition since the two polymers PLA and PLGA have different degradation rates.

Wang *et al*. [40] prepared PLGA microspheres and discs loaded with paclitaxel, an anticancer drug and radiosensitizer; PEG, isopropilmyristate (IPM) and D-α-tocopheril polyethylene glycol 1000 succinate (vitamin E TPGS) were added as excipients in the formulations. Microspheres and discs were irradiated at 0.05 and 10 kGy. Results showed that the irradiation at 10 kGy caused the increase in the paclitaxel release rate from the drug delivery systems after 18 days of the *in vitro* release test, and the highest enhancement effect was detected in the presence of the additive Vitamin E TPGS. The authors noticed that paclitaxel could be degraded during drug delivery system preparation process and *in vitro* release period.

Martinez-Sancho *et al*. [47] studied the effect of γ-irradiation on PLGA/gelatin microspheres loaded with aciclovir. The microspheres were prepared by solvent evaporation method and sterilized at 25 kGy at low temperature (dry ice), in the presence of air. The controlled release profiles of aciclovir-loaded microsphere, as determined for 73 days, were not altered upon exposure to γ-irradiation. IR spectroscopy, DSC and X-ray diffraction showed no modification of the bulk properties of the microspheres and their components. However, GPC measurements showed a decrease in polymer Mw after the microsphere irradiation. The authors concluded that the maintenance of the properties of aciclovir loaded PLGA microspheres upon irradiation could be due to three factors: the use of a low molecular weight PLGA, low temperature during irradiation exposure and the drug incorporation as a suspension.

Fernandez-Carballido *et al*. 2004 [31, 32] prepared PLGA ibuprofen loaded microspheres containing the additive Labrafil by the solvent evaporation method. The additive was included to modulate drug release rate. Microspheres were sterilized by γ-irradiation at low temperature (dry ice). Results showed that the sterilization procedure employed did not alter the physicochemical properties of the formulation. Dissolution profiles of the formulation overlapped before and after sterilization and no significant changes in polymer Mw were revealed by size exclusion chromatography analysis. Moreover, the sterilized microspheres were stable for 1 year stored in a desiccator at 4°C. The authors concluded that the employment of low Mw PLGA (13137 Da) and suitable sterilization conditions were unavoidable to maintain microsphere properties upon irradiation.

Shameem *et al*. [59] investigated the effects of ionizing irradiation on the physicochemical properties of microspheres made of PLGA 50:50 (Mw 8600 Da) with free carboxylic end groups, and loaded with the peptide drug leuprolide. The microspheres were prepared by solvent extraction /evaporation method, and in part formulated by suspension

in a vehicle of 1% carboxymethyl cellulose (CMC) and 2% mannitol before freeze-drying. The unformulated and formulated microspheres were irradiated at 10, 15, 25 kGy. HPLC analysis based on extraction of peptide from the microspheres showed that peptide content was lowered upon irradiation and the reduction was more pronounced in formulated microspheres. The authors attributed the peptide loss inside the microspheres to the catalytic effects of polymer or formulation vehicle. Both formulated and non-formulated microspheres after irradiation released more drug *in vitro* than the non-irradiated form. The physicochemical properties of polymer where affected by irradiation with significant lowering of Mw, Mn and Tg. At any rate the overall efficacy of the formulation was not compromised *in vivo*.

Carrascosa *et al*. [60] entrapped the recombinant human insulin-like growth factor-I (rhIGF-I) in PLGA microspheres using the w/o/w solvent evaporation technique. The microspheres were irradiated at 25 kGy and the stability of the released protein was investigated by circular dichroism (CD) and sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE). Results showed that the drug loading remained essentially the same after irradiation. However, rhIGF-I aggregation was detected by electrophoresis and the *in vitro* drug release from the irradiated microspheres showed an increased burst effect. Meanwhile a shift of polymer Tg value towards lower values was observed as a consequence of γ-irradiation. As observed by other authors, this phenomenon is a consequence of radiolytic events leading to chain scission [30, 46]. The authors concluded that formation of soluble dimers appeared to be the most predominant form of covalent aggregation for rhIGF-I, confirming previous studies performed on peptide and protein molecules subjected to irradiation in the solid state [61, 62].

Igartua *et al*. [65] evaluated the effects of γ-irradiation on the biopharmaceutical properties of PLGA microspheres containing SPf66 malarial antigen. The microspheres were prepared by the w/o/w double emulsion solvent extraction method using PLGA 50:50 and PLGA 75:25. The obtained microspheres, either placebo or loaded with SPf66, were irradiated using a Cobalt-60 γ-radiation source at a dose of 25 kGy. *In vitro* characterization of microspheres by SEC, DSC and SEM did not reveal a marked effect of γ-irradiation on the microsphere system. *In vitro* release rate of the microencapsulated antigen was slightly faster after γ-irradiation. Subcutaneous administration of irradiated and non-irradiated microspheres into BALB/c mice induced a similar immune response expressed as IgG, IgG1, IgG2a levels and it was comparable to that obtained with SP166 emulsified with Freund's complete adjuvant. Following these results the authors suggested the applicability of γ-irradiation as a method of terminal sterilization of PLGA microparticles loading chemically synthesized antigens.

Dorati *et al*. 2005 [29] studied the effect of γ-irradiation on the release rate of ovalbumin (OVA) loaded into PLGA microspheres. In this work the effect of the presence of PEGs of different Mws (400, 4,000 Da) and in different amounts was evaluated, as discussed above (see IV.3). In so far as the protein is concerned, the authors highlighted that, in the absence of additives, the release rate of OVA was significantly reduced by γ-irradiation. The authors concluded that the changes in OVA release rate could be ascribed to concomitant phenomena that are the modification of microsphere physical structure, the polymer degradation and its interaction with the protein (see also IV.7).

6. Comparison between γ and electron beam irradiation

Raw materials or final products are typically sterilized using γ-rays emitted from a nuclear source (Cobalt-60 or Caesium-137). Nevertheless, electron beam irradiation is a promising sterilization technique offering several advantages with respect to γ-irradiation since it is faster, easier, cheaper, and does not involve the environmental risk of nuclear irradiation. Although the main interaction with matter is basically the same for γ-rays and high energy electrons, minor differences between the two modes remain. γ-rays are a form of electromagnetic radiation characterized by high penetration into matter but a very low dose rate (kGy/h). On the contrary, electron beam irradiation is a form of corpuscolar radiation characterized by low penetration into matter but at very high dose rate (kGy/s). These peculiarities of electron beam irradiation and γ-rays can modify the performances of irradiated drug delivery systems. For the same administered dose, electron beam irradiation and γ-rays treatment does not cause significant heating of the material, while γ-ray treatment could prolong the peroxidative radiolytic mechanism due to exposure time. Montanari *et al*. 2003 [51] compared the effects of γ- and electron beam irradiation on the stability of microspheres made of PLGA 50:50 (RG503) and loaded with different amounts of bupivacaine between 10% and 40%. The microspheres were prepared by spray-drying method and they were treated either by γ- or electron beam irradiation at 25 kGy total dose, at room temperature and in the presence of air. The irradiated microspheres were characterized for their morphology (SEM, AFM) and physicochemical properties (DSC, FTIR). The functional properties were tested by an *in vitro* dissolution test. Results showed that electron beam irradiation and γ-rays modified the performances of bupivacaine loaded microspheres in a significantly different way as regards surface morphology, drug content and drug release pattern. In particular the microspheres resulted to be more sensitive to γ-irradiation which caused an increase in the *in vitro* drug release in the following 90 days of storage. The authors concluded that the choice of irradiation type could be considered a critical parameter to be carefully evaluated in the presence of oxidable and/or thermosensitive materials.

7. EPR investigation

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy is based on concepts analogous to nuclear magnetic resonance (NMR) principles, the difference being that in EPR spectroscopy, electron spins are excited instead of atomic nuclei spins.

The technique is useful for studying chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals, or inorganic complexes possessing a transition metal ion. When an atom or a molecule with an unpaired electron is placed in a magnetic field, the spin of the unpaired electron can align either in the same direction or in the opposite direction as the applied field. These two electron alignments have different energies and the application of a magnetic field to an unpaired electron lifts the degeneracy of the ±1/2 spins of the electron.

Like all high energy radiations, γ-irradiation, is often connected with the formation of radical species promoting related phenomena such as polymer degradation. EPR technique has been advantageously applied to γ-irradiated polymers and microspheres thereby explaining the radiolysis mechanism related to polymer degradation. Moreover, the evaluation and quantification of radicals can be interesting either from a toxicological and analytical point of view as an irradiation index.

Montanari *et al*. 1998 [36] used EPR to investigate the presence of free radicals in polylactide-co-glycolide, RG503 and RG503H, and related microspheres. Those two polymers differ in their structure because RG 503H carries predominantly free carboxylic acid groups on one of the chain ends, and is therefore more hydrophilic. The authors irradiated both polymers and the microspheres in a Cobalt-60 source, at 77 K, under vacuum, with a total dose of 25 kGy. They found that the EPR spectra of the two polymers were identical (*Figure 5*), suggesting that the structural differences in the chain ends play a minor role in the overall radiolysis mechanism. Nevertheless, the concentration of radiation induced free radicals was always higher in RG503H polymer and microspheres, and they were more stable than the free radicals species observed in the case of polymer RG503. Alterations and/or

production of new radicals were observed on exposure of RG503H microspheres to the light. A radiolytic mechanism was suggested based on the assumption of a prevalence of the ionic component, due to the polarity of the polymers (*Figure 6*). The results led the authors to conclude that radiolytic degradation of RG503 and RG503H polymers under vacuum was characterized by a prevalence of chain scission events leading to a decrease in the average Mw. Some crosslinking events could occur mainly in the post irradiation stage through the decay and coupling of the hydrogen abstraction radicals C·(CH_3).

Bittner *et al*. [45] performed EPR spectroscopy on PLGA (RG503) microspheres and on PLGA microspheres loaded with tetracycline-HCl (TCH). The microspheres were prepared by spray-drying, and irradiated at 26.9 kGy and at 54.9 kGy using a Cobalt-60 source, at -80°C under N_2 atmosphere. EPR analyses revealed that γ-irradiation induced free radicals within TCH loaded microspheres, while unloaded PLGA microspheres did not contain radicals under the same conditions. The authors explained these findings by the low Tg value of polymer (37-39°C) that allowed sufficient mobility of the polymer chains. Under these conditions the radicals formed were able to recombine and undergo further reactions to diamagnetic species. In agreement with this finding, TCH proved to be the main component of EPR spectrum of TCH loaded microspheres. In order to determine the mechanism of polymer degradation, the authors co-encapsulated in the microspheres the spin trap tert-butyl-phenil-nitrone (PBN) and the spin probe 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPOL). They analyzed the EPR spectra of TEMPOL loaded microspheres and PBN loaded microspheres, before and after irradiations and correlated EPR results with the results of GC MS, GPC and HPLC analyses.The study showed that γ-irradiation of PBN loaded microspheres resulted in the formation of a lipophilic spin adduct, indicating that a polymeric radical was generated by random chain scission, while possible diamagnetic reaction products of low Mw were not detected in TEMPOL loaded microspheres. The authors concluded that γ-sterilization induced the formation of free radicals in PLGA microspheres and that incorporation of compounds could influence the radical generation process. The radical life-time was determined by the properties of the surrounding matrix. Radicals might be very stable in dry conditions in matrices with a high melting point and high crystallinity, but they could decay rapidly in the presence of water. In the case of amorphous PLGA matrix with low Tg, the radicals should be mobile enough to recombine or participate in further reactions leading to diamagnetic products. Therefore, the free radicals themselves did not influence microsphere degradation in the aqueous buffer solution. Moreover, the authors underlined the usefulness of combining more than one analytical technique in order to clarify the behavior of polymer under γ-irradiation.

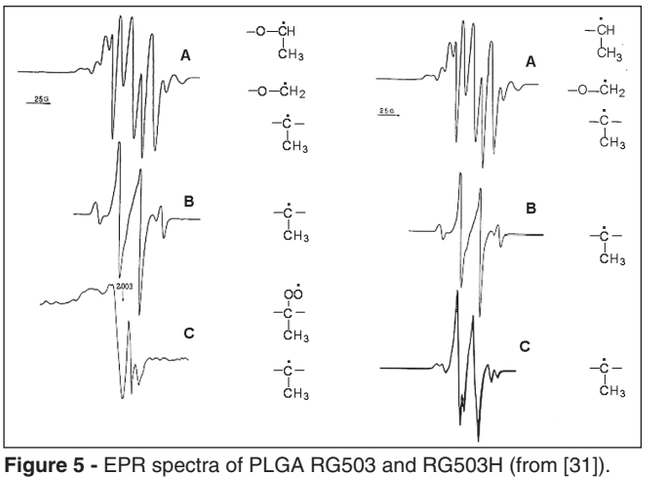

Figure 5 - EPR spectra of PLGA RG503 and RG503H (from [31]).

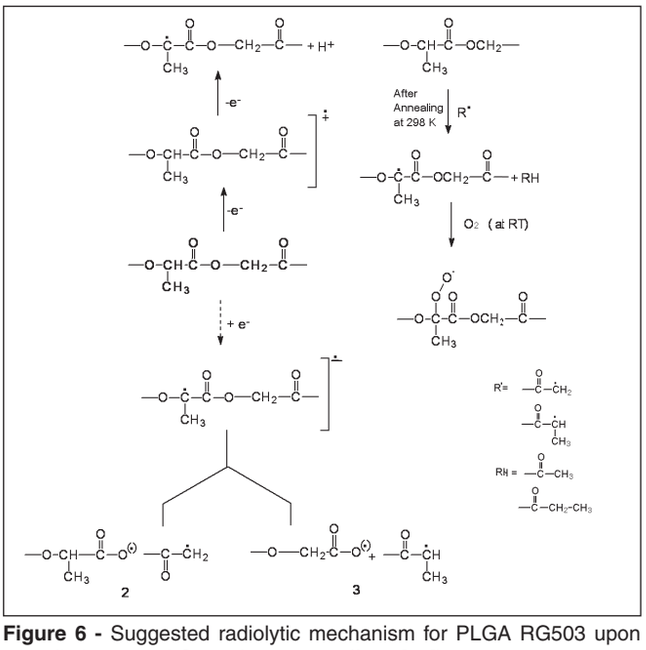

Figure 6 - Suggested radiolytic mechanism for PLGA RG503 upon γ-irradiation at 25 kGy under vacuum (from [31]).

Montanari *et al*. 2001 [52] and Faucitano *et al*. [53] investigated the radiolysis mechanism of clonazepam (CLO) loaded PLGA microspheres irradiated at 25 kGy using a Cobalt-60 source. The study was conducted by matrix EPR spectroscopy, in the temperature range of 77-298 K. Results showed major drug-polymer interactions leading to significant deviations of the G (radicals) from the additivity law. In particular polymer/CLO spin transfer reaction suggested that CLO had a radio-stabilizing effect on the polymeric matrix, testified by a decrease in the overall polymer radical yield which was accompanied by an increase in the drug radicals yield. The authors attributed these effects to the radical scavenging properties of the nitro group with respect to electron and polymer radicals, and they concluded that these findings could be applied to all pharmaceutical formulations containing drugs bearing nitro groups in their chemical structure.

Claybourn *et al*. [50], Bushell *et al*. [54] used EPR and electron-nuclear double resonance (ENDOR) spectroscopy to characterize free radicals generated, after exposure to γ-and electron beam radiation. ENDOR technique is complementary to EPR technique and is especially useful for identifying nuclei that interact weakly with the electron spin. It can provide detailed information about atoms involved in a paramagnetic site. Samples were irradiated under vacuum and at room temperature. The study was performed on a series of PLGA raw polymers and microspheres whose lactide:glycolide composition was 75:25, 65:35, and 50:50. A dose of 26.6 kGy was applied in the case of γ-irradiation with a Cobalt-60 source, while a dose of 32.1 kGy was applied with a 4.5 MeV electron beam accelerator. Both sets of irradiated samples resulted in the formation of similar types of paramagnetic radical species formed through chain scission and subsequent H abstraction free radicals reactions, thus achieving analogous EPR spectra. Computer simulation of EPR spectra along with ENDOR analysis showed overall systematically higher radicals concentrations as the lactide content in the PLGA the raw polymer and microspheres increased (i.e. 75:25 > 65:35 > 50:50) for both γ- and electron beam irradiation. The relative concentrations of free radicals were similar in the raw polymer samples after exposure to γ- or electron beam irradiation. On the contrary, a significant difference was found for the microsphere samples, where an approximate doubling of the radical content was found in the γ-irradiated PLGA microspheres compared to the identical electron beam irradiated microspheres.

Dorati *et al*. 2005 [29] investigated by EPR spectroscopy the radiolytic mechanism of microspheres made of mixtures of PLGA 50:50

and PEG and containing the model protein ovalbumin. Microspheres with different composition in terms of amounts of PEG (either 15% or 30% of total microsphere weight) were prepared by the w/o/w double emulsion solvent evaporation method. The irradiation was performed at 25 kGy under vacuum at 77 K. The EPR spectra of the microspheres, after annealing at room temperature, were compared to EPR spectra of raw materials PLGA, PEG, and ovalbumin irradiated in the same conditions. Results showed that the most developed radical products either at low temperature or at room temperature were the ones usually detected in the spectra of pure irradiated PLGA. However, when the spectra were recorded at higher power, after annealing at room temperature, gzz and gyy features of the ovalbumin RS S. perthyl radical were clearly identified. A significant difference between the two spectral series was represented by the lower intensity of the perthyl radical signal detected from samples with higher PEG content. The authors concluded that PEG had a perturbation effect on the perthyl radicals yield in ovalbumin loaded PLGA/PEG microspheres leading to formation of molecular products by radical coupling. This mechanism could contribute to the change in ovalbumin release rate highlighted after microsphere irradiation.

Dorati *et al*. 2008 [55] investigated the EPR behavior of PEGd,lPLA, PEG-PLGA, PLA and PLGA polymers irradiated by using a Cobalt-60 irradiation source, under vacuum, at 25 kGy total dose. The results led the authors to conclude that the presence of PEG units in PEGd,lPLA and PEG-PLGA copolymer chains had two major consequences on EPR spectra: a) a decrease in the radiolytic yield of radicals -C(=O) O-C·(CH_3)-O- at 77 K and b) a decrease in the radical trapping efficiency and faster radical decay rates at 290 K.

*

The papers gathered and commented demonstrate that the effect of ionizing radiation sterilization on poly-α-hydroxyacids and microspheres has been thoroughly investigated in recent years.

All authors agree that ionizing radiation leads to decrease polymer Mw due to a prevalence of chain scission events caused by irradiation. Degradation follows the random chain scission mechanism. The effects of ionizing radiation are strictly related to irradiation dose and conditions, and they also depend on the starting polymer Mw. Bioburden control can be an useful tool to suitably reduce irradiation dose in order to minimize the effects of irradiation dose on the same polymer.

The presence of a drug inside the polymeric micromatrix can affect polymer behavior upon irradiation. Also the presence of additives may perturb polymer and drug interaction with ionizing irradiation. These interactions can lead to significant change in drug release from microspheres.

Irradiation was also shown to affect the long term stability of microspheres with a greater decrease in Mw and Tg value.

The sensitivity to γ-irradiation depends greatly on polymer composition, and should be always investigated for new poly-α-hydroxyacids derivatives.

These findings can be useful when choosing a suitable polymer or polymer blends to manufacture drug delivery systems whose sterilization is a compulsory requirement.

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