REVIEW

Biodegradable PLGA-*b*-PEG polymeric nanoparticles: synthesis, properties, and nanomedical applications as drug delivery system

Erica Locatelli · Mauro Comes Franchini

Abstract During the past decades many synthetic polymers have been studied for nanomedicine applications and in particular as drug delivery systems. For this purpose, polymers must be non-toxic, biodegradable, and biocompatible. Polylactic-*co*-glycolic acid (PLGA) is one of the most studied polymers due to its complete biodegradability and ability to self-assemble into nanometric micelles that are able to entrap small molecules like drugs and to release them into body in a time-dependent manner. Despite fine qualities, using PLGA polymeric nanoparticles for in vivo applications still remains an open challenge due to many factors such as poor stability in water, big diameter (150–200 nm), and the removal of these nanocarriers from the blood stream by the liver and spleen thus reducing the concentration of drugs drastically in tumor tissue. Polyethylene glycol (PEG) is the most used polymers for drug delivery applications and the first PEGylated product is already on the market for over 20 years. This is due to its stealth behavior that inhibits the fast recognition by the immune system (opsonization) and generally leads to a reduced blood clearance of nanocarriers increasing blood circulation time. Furthermore, PEG is hydrophilic and able to stabilize nanoparticles by steric and not ionic effects especially in water. PLGA–PEG block copolymer is an emergent system because it can be easily synthesized and it possesses all good qualities of PLGA and also PEG capability so in the last decade it arose as one of the most promising systems for nanoparticles formation, drug loading, and in vivo drug delivery applications. This review will discuss briefly on PLGA-*b*-PEG synthesis and physicochemical properties, together with its improved qualities with respect to the single PLGA and PEG polymers. Moreover, we will focus on but in particular will treat nanoparticles formation and uses as new drug delivery system for nanomedical applications.

Keywords Polymeric nanoparticles · Nanomedicine · Polyesters

Introduction

Polymeric nanoparticles

In the last few decades, polymeric nanoparticles (PNPs) have assumed extraordinary importance for the development of drug delivery systems. The aim of drug delivery is the controlled delivery of drugs to their site of action and the release of the same in a time-dependent manner improving the therapeutic efficacy, reducing the dose of drug to be administered and simultaneously minimizing the unwanted side effects. Polymeric nanoparticles are optimal carriers for drug delivery due to their tunable characteristics, small size below 1 μm and for the possibility to entrap,

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dissolve, or attack to these drug molecules or other active principles (McCarron and Hall 2004); using polymeric nanocarriers fundamental problems of traditional medicine can be solved regarding poor drugs solubility in water and consequently low absorption by the body, short in vivo life time due to elevated clearance and un-wanted side effects. Due to their sub-micron size and to their hydrophilicity PNPs can penetrate deep into tissue through capillaries and reach the target tissue where they can be taken up by the cells (Vinagradov et al. 2002). Moreover, nanoparticles can be functionalized above the surface using residual reactive ending-groups with specific proteins, peptides, monoclonal antibodies that are able to selectively bind a site of action or a particular target tissue, without interaction with other cells, minimizing side effects and enhanced drug efficiency. Many efforts have been done to render them adequate for in vitro and in vivo applications: their physical and biochemical properties, like size, nature of the surface, and kind of the polymers was studied and synthetic methods were adjusted to obtain good qualities (Win and Feng 2005; Schmaljohann 2006). For example, size control of nanoparticles for drug delivery applications is a crucial point due to the fact that nanoparticles size affects biodistribution profile: this is organ-specific because is due to the different physical properties of each organ and many works show that it has not a linear relation versus size (Moghimi et al. 1991); very small nanoparticles with mean diameter <60–70 nm will be excreted fast, while larger nanoparticles (200 nm or more) can be sequestrated by liver or spleen, so only nanoparticles with diameter between 70 and 200 nm are optimal for in vivo application (Owens and Peppas 2006). Also nanoparticles charge surface needs to be controlled to ensure stability to the system; zeta potential value measures the electric potential at the surface of the hydrodynamic layer and have been used to establish stability of colloidal suspensions because only particles with high negative or positive zeta potential values (typically −30 or +30 mV) tend to repel each other reducing aggregation (Muller 1991; Zimmer et al. 1991; Dunn et al. 1997).

Polymers for polymeric nanoparticles

A wide variety of synthetic polymers has been used for making nanoparticles, nevertheless not all the materials are suitable for nanomedical applications: they are required by Food and Drug Administration (FDA) since 1990 to be biocompatible, not only with a suitable biodegradation kinetics, good toxicological profile, and drug loading efficacy but also with good mechanical properties and well-characterized structure (Duncan et al. 2006). Synthetic biodegradable polymers including polyamides, polyesters, poly (amino acids), polyurethanes have been used increasingly in the last 20 years due to their controllable properties in terms of molecular weight and shape (linear, branched, dendritic, etc.). Among these, poly(lactic-co-glycolic acid) (PLGA) having already a long history in medicine as material for biodegradable sutures (Ginlding and Reed 1979; Oh 2011), emerged as the most promising material for drug delivery nanocarriers fabrication. Even if many properties of PLGA nanoparticles, such as size and morphology, can be easily controlled by adjusting the parameters during the synthesis, many problems related to the use of simply PLGA remain still unsolved.

PEG (polyethylene glycol) starts to be used for coupling proteins since 1977 (Abuchowski et al. 1977) and immediately arose as the gold standard for stealth polymers in drug delivery field. In 1990, PEGylated adenosine deaminase became the first approved polymer–protein conjugate in USA (Greenwald et al. 2003) and thanks to this, plus the possibility to solve most of other polymers problems in drug delivery, the interest in PEG has grown exponentially. This review will examine the advantages in the use of PLGA-*b*-PEG copolymer to form stable, well-defined, high-quality property nanoparticles for drug delivery applications.

Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA) is a synthetic copolymer obtained by random ring-opening copolymerization of two cyclic dimers of glycolic and lactic acid. Lactic and glycolic acid are linked together by ester linkage in a casual order during copolymerization bringing an aliphatic linear polyester (Astete and Sabliov 2006). Molar ratio of the individual monomer components (lactide and glycolide) in the polymer chain directly influences many properties of PLGA such as degree of crystallinity, mechanical strength, swelling behavior, and capability to hydrolyze that
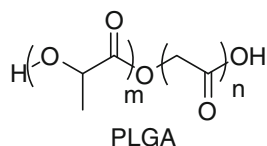
PLGA

Poly(lactic-co-glicolic acid)

m = number of units of lactic acid
n = number of units of glycolic acid

Fig. 1 Chemical structure of PLGA

subsequently affects biodegradation rate: it is well known that PLGA copolymer with a 50:50 molar ratio of the two monomer is hydrolyzed much faster in comparison with the one containing higher quantity of either of two monomers (Li and McCarthy 1999; Park 1994) (Fig. 1).

The major advantage of using PLGA is that this copolymer undergoes complete biodegradation in an aqueous media. This process occurs via hydrolytic degradation of the ester linkages present in the polymer chain and has an uniform rate in all the polymeric matrix: in this way, the number of carboxylic acids present in the chain increases and this seems to catalyze the biodegradation process; the first biodegradation products of PLGA copolymer are the two original monomers, lactic and glycolic acids that in body can be metabolized and eliminated as carbon dioxide and water or excreted unchanged in the kidney (Muthu 2009).

Due to these excellent properties, PLGA is therefore one of the most utilized polymers for biomedical application since 1970: it possess not only extreme biocompatibility but also good mechanical strength that make it suitable for a large variety of medical artifices such as sutures or fibers (Ginlding and Reed 1979; Lewis 1990). However, the real challenge of PLGA is the ability to self-assemble into polymeric devices like microspheres, microcapsules, nanoparticles optimal for delivery-systems fabrication, because these are water-soluble and can be simply administrated through the body orally, parenterally, or locally. Moreover, these PLGA-derived systems can be loaded with a variety of drugs, proteins, peptides which are released in the body slowly in a time-dependent manner due to the hydrolytic process described above (Lu et al. 2009); for these reasons PLGA has been approved by the Food and Drug Administration for drug delivery use.

Nevertheless these systems present some important disadvantages, first of all the necessity to use a stabilizer which is not always biocompatible, for example, poly(vinyl alcohol), to stabilize nanoparticles and to obtain small diameter with uniform distribution; in addition, PLGA nanoparticles present high-grade recognition by the reticulo-endothelial system (RES) of liver and spleen that remove these from blood circulation and reduce the residence time of nanoparticles drastically in bloodstream thus avoiding the delivery of nanoparticles to the selected organs or tumors tissues (Muthu 2009).

Poly(ethylene glycol)

Poly(ethylene glycol) (PEG) is a non-ionic hydrophilic polyether synthesized by polymerization of the monomer ethylene glycol and nowadays it can be obtained with a wide range of molecular weight from 300 to 100,000 Da. PEG is commonly utilized in medicine for various scopes: it is given orally as a laxative, used in drugs formulation as excipient, and in capsules preparation as coating agent (Cleveland et al. 2001). PEG is not a biodegradable polymer because it is excreted unchanged in kidney and does not undergo some biodegradation process in body; although, this disadvantage of PEG is extremely biocompatible and no accumulation in tissue occurs especially for low molecular weight chains. Thanks to its hydrophilicity, PEG polymer can be used to stabilize nanoparticles in aqueous media, increase solubility, and avoid aggregation of them by steric hindrance in production, storage, and applications. Moreover, as PEG does not involve ionic moiety, no problems occur when it enter in contact with charged biological molecules such as DNA (Knop et al. 2010). The real prerogative of PEG polymer is its ability to suppress the opsonization phenomenon: this means the fast recognition by immune system of foreign agents, like not only pathogens but also drugs and nanoparticles devices, and the consequent elimination of those by the bloodstream; the reduced blood clearance of drugs and nanocarriers by coating with PEG is known as stealth effect and this brings an increasing of in vivo circulation time thus allowing the possibility that drug reaches its site of action. Indeed, many studies demonstrated that PEGylated products undergo slower uptake by the RES in liver and spleen than the non-PEGylated systems, extending circulation in blood

more than 63 % after intravenous injection (Oupicky et al. 2002).

Another important goal of PEGylated products is the enhanced permeability and retention (EPR) effect that consents a passive tumor targeting: tumor or inflamed tissues present an hypervascularization and loosely connected endothelial cells with a decreased lymphatic drainage that allow easy entrance of PEGylated drugs or nanoparticles (Joralemon et al. 2010).

Poly(lactic-co-glycolic acid)-block-poly(ethylene glycol)

Gref et al. first described in 1994 the effect on pharmacokinetics of PLGA microparticles externally coated with PEG: the authors demonstrated that a drastic increase in half-life of micelles occurs when these were covered by PEG polymer and only the 30 % of these were captured by liver after 2 h of injection (Gref et al. 1994). It is clear that PEG coating on micelles core must be stable in in vitro and in vivo conditions, so desorption or displacement phenomena must be avoided: in fact PEG has been found to be more effective in prolonging drug or micelles circulation when it was covalently bond on nanoparticles surface than when it was simply adsorbed on (Bazile et al. 1995); similarly, Mosqueira et al. (2001) demonstrated that high PEG surface density is necessary to produce an increased half-life of the nanoparticles in vivo. All these studies suggest that a coating with some PEG fragments is not enough to ensure stability to the micelles but a real co-polymer with a strong covalent bound is necessary to achieve both high PEG surface density and to prevent desorption phenomenon. Accordingly, PLGA–PEG copolymers were extensively investigated for various applications like implantable materials, pharmaceutical products, drug delivery systems, and nowadays the market are expected to continue to grow as for their precursor PLGA and PEG. Different kinds of copolymers based on PLGA and PEG were synthesized, such as di-, tri-, or multi-block copolymers. Also linear, branched, and mixed copolymers, with or without functional ending-groups, were investigated, but most of all the di-block copolymer (PLGA-*b*-PEG) has attracted particular interest due to special properties, such as amphiphilicity and easier synthesis, in comparison to the others (Huh et al. 2003). PLGA-*b*-PEG copolymer shows quite different properties when compared to each

constituting polymers so it became a new and unique biomaterial: it possess both the biodegradability and biocompatibility of PLGA polymer and the stealth behavior of PEG but more than this PLGA-*b*-PEG can form micelles better than the single PLGA or other type of PLGA–PEG copolymers because of its well-distinct lipophilic portion (PLGA) and its hydrophilic one (PEG) that make the PLGA-*b*-PEG an amphiphilic polymer: this two parts during micelles formation self-assemble generating a system in which the hydrophobic PLGA remains inside the micelles and the hydrophilic PEG goes outside creating a stabilizing shell. In this way, several possibilities are opened: not only easy entrap of lipophilic molecules like drugs or smaller nanostructures in the inner core constituting of PLGA chains but also obtainment of residual functional groups in the outer shell by previous functionalization of the PEG chains that can be used for conjugation of active molecules (proteins, peptides, monoclonal antibodies) for targeting desired tissues: various functional groups can be useful for this and PEG chains of various lengths already functionalized with a wide variety of reactive groups such as amino, carboxylic acid, methoxy, maleimido, etc., are commercially available (Fig. 2).

Furthermore, challenging entrapment of hydrophilic moieties is possible: by modifying the general nanoparticles formation process, water-soluble micelles with good diameter, surface charge, drug-
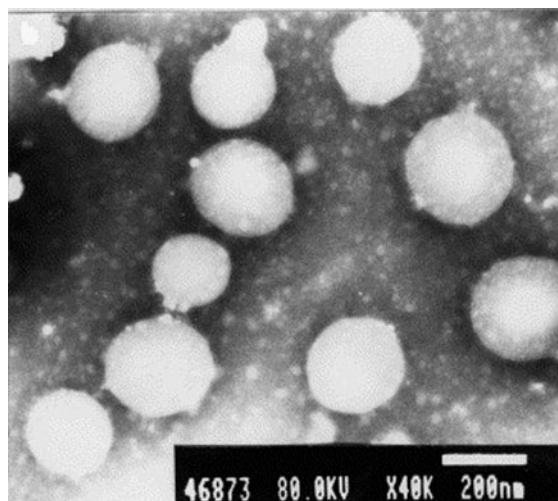

Fig. 2 TEM image of PLGA-*b*-PEG nanoparticles (Li et al. 2001)

loading efficacy and stability can be obtained (Zambaux et al. 1998).

Synthesis of PLGA-*b*-PEG copolymer

The synthesis of PLGA-*b*-PEG was first described by Gref et al. (1994) and consists of the covalently linkage between a PLGA chain that brings a free carboxylic acid at one side (PLGA-COOH) and a PEG chain doubly functionalized at the two ends with an amino- and another reactive group (X) that, in this specific case of this study, is a methoxy group ($_2$HN-PEG-OMe). In a general procedure, PLGA-COOH is first activated using dicyclohexylcarbodi-imide (DCC) and *N*-hydroxysuccinimide (NHS) in dichloromethane, then the product washed several times with the non-solvent diethylether; the so-activated PLGA-NHS is linked with the $_2$HN-PEG-X using an organic base like *N*,*N*-diisopropylethylamine (DIPEA) in chloroform: an amido-group is so formed in the middle of the copolymer that, after washings with ether and water, is stable if kept in freezer for months (Farokhzad et al. 2006; Comes Franchini et al. 2010a, b) (Fig. 3).

Other coupling agents can be used instead of DCC like EDC (1-ethyl-3-(3-dimethylaminopropyl) carbo-diimide) or CDI (1,1′-carbonyldiimidazole) with same results.

Important is to remove all the un-coupled PEG that can remain entrap between PLGA-*b*-PEG chains. Coupling is confirmed by NMR analysis and yield of reaction is estimated about 83 % (Cheng et al. 2007).

Other synthetic routes are reported in the literature for the synthesis of PLGA–PEG copolymer, such as the copolymerization of lactic and glycolic acid in the presence of mPEG, but these methods lead to a different kind of copolymer, the so-called (AB)$_n$-type multi-block copolymer, and not to the AB-type di-block one, so they avoid the scope of this review (Beletsi et al. 1999; Dong and Feng 2004; Kumar et al. 2001).

This simple synthesis, instead ring-opening copo-lymerization or multi-steps reaction with strong con-dition necessary for the production of other kinds of PLGA–PEG copolymers (for example PLGA–mPEG), is one of the keys of the success that PLGA-*b*-PEG reached during these last years.

Preparation of micelles for entrapment of lipophilic moieties

Several methods exist for the manufacture of PNPs (Hans and Lowman 2002) we describe in this review two of this that fit well for PLGA-*b*-PEG copolymer: nanoprecipitation (NP, also called solvent deposition) and the oil-in-water (O/W) emulsion methods. Both of these are suitable for the entrapment into PNPs, during the formation process, of any kind of lipophilic drugs, molecules, or small nanoparticles, so they can be utilized for the creation of nanostructured drug delivery systems.

The nanoprecipitation is commonly applied to several polymeric systems, with or without amphiph-ilicity or charge chains because this technique leads to the formation of PNPs due to the nucleation of small aggregates of macromolecules followed by aggrega-tion of this nuclei: the process is not an ordered assembling and does not require specific abilities of the polymers involved but brings to really good colloidal suspensions with reproducible and control-lable properties. The process requires the dissolution of the polymer in a water miscible organic solvent like acetone, tetrahydrofuran, dimethylformamide,

Fig. 3 Synthesis of PLGA-*b*-PEG copolymer
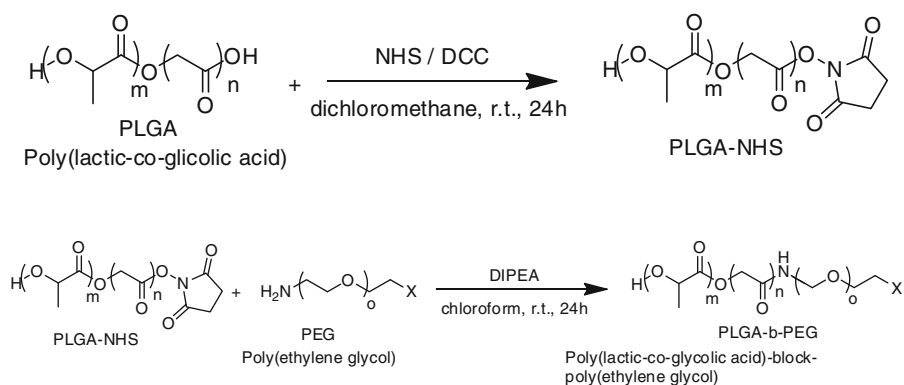
acetonitrile, dimethyl sulfoxide, and so on. As well as the polymer also the lipophilic drugs or any other organic-soluble active principles are also dissolved or dispersed in the organic solution. This organic mixture is poured under vigorous stirring into water, a polymer non-solvent, which may contain surfactant or stabilizing agents. The polymer immediately begins the nucleation process just put in contact with water and the growth of the nanoparticles stops when stability is reached. Organic solvent remained into PNPs matrix is then removed from the colloidal suspension by evaporation (Kreuter 2004). PLGA-b-PEG forms a core shell structure due to the presence of the two blocks with different solubilities: PLGA chains remain inside the nanoparticles and form the micelles core containing the lipophilic drugs, while PEG chains are oriented to the water phase so that the functional groups, presented at the end of PEG chains, are exposed on the surface of nanoparticles. Particles formed with this technique can be obtained with small diameter (50–250 nm) and good polydispersity index (PDI) (Schubert et al. 2011).

Oil-in-water emulsion methods is particularly good for amphiphilic polymers such as PLGA-b-PEG due to their micelle-like behavior that consents the formation of ordered spheres with the hydrophilic chains addressed to the water phase; the polymers also must have good mechanical strength because strong energy is involved in the process that can disrupt a non-robust polymer structure: the process begins with the dissolution of the polymer and of the lipophilic moieties that must be entrapped in a volatile, water-immiscible organic solvent like chloroform, dichloromethane, ethyl acetate, and others. This mixture is emulsified using an ultrasonicator in an aqueous phase so nanosized droplets can be formed: with this strong energy the oil phase is dispersed in nanodroplet in the aqueous phase and the polymer dissolved in it acts as a surfactant, self-assembling into micelles with the nanodroplet of oil in inner core in contact with the hydrophobic portion and the hydrophilic part oriented to the external water phase. Equally to the previous method, the solvent is removed from the nanoparticles by evaporation giving nanospheres with lipophilic drugs entrapped with good PDI and diameter in the range 100–500 nm (McCarron and Hall 2004).

These two methods had applied to different polymers and co-polymers like poly(ε-caprolactone), PLA (polylactic acid), PLGA, PLGA-b-PEG and many

parameters like polymer concentration, polymer molecular weight, organic solvent, water miscibility of solvent and water/solvent ratio had been studied to ensure good properties and stability to the colloidal solutions.

In the case of PLGA-b-PEG, Cheng et al. (2007) studied all the above-cited parameters and they established correlation between polymer concentration, solvent used, solvent/water ratio, and mean diameter of the nanoparticles formed. They initially found that an increase of the polymer concentration in the initial organic solution leads to an increase of the resulting mean particle volumetric size. Subsequently, they investigated hardly on the correlation between solvents—water miscibility and final nanoparticles size—specifically an increase in water miscibility of the solvent used for nanoprecipitation technique leads to a decrease in the size of particles thus dimethylformamide result the best solvent to obtain small particle diameter; also, acetone leads to good results and generally it is chosen due to its reduced toxicity and lower boiling point. Not a linear correlation was found for solvent/water ratio and nanoparticles diameter but good and reproducible result was obtained with the ratio of 1/10.

Gentili et al. (2009) used this methodology for the entrapment into PLGA-b-PEG nanocarrier of metal nanoparticles, such as gold nanorods (GNRs): the originally water-soluble nanoparticles were rendered lipophilic by a coating with an organic ligand, then the so obtained lipophilic GNRs were entrapped into the PNPs using the oil-in-water emulsion method giving a promising drug delivery system both for imaging and for therapeutic applications.

More than one metallic nanoparticle have been entrapped contemporaneously in a recent paper: GNRs and silver NPs using nanoprecipitation technique were entrapped being lipophilic and linked after an organic "click" transformation. In this way, a theranostic nanostructure based on PLGA-b-PEG N7Ps has been obtained (Locatelli et al. 2011) (Fig. 4).

Preparation of micelles for entrapment of hydrophilic moieties

A separate discussion should be done for the manufacturing of micelles that have to entrap hydrophilic drugs or moieties because the methods described above are not suitable for this challenging purpose;
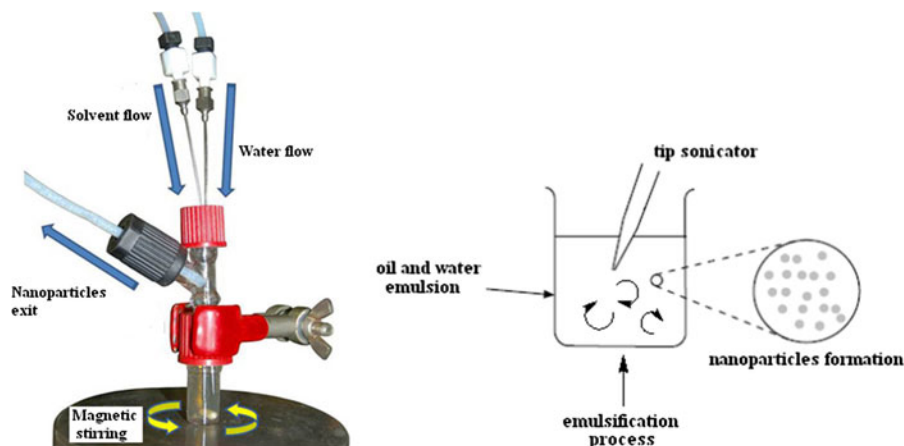
Fig. 4 Schematic representation of nanoprecipitation (*left*) and oil-in-water emulsion (*right*) technique

entrapment of drugs, proteins, peptides, an especially DNA or RNA fragments that are water-soluble assumed extreme importance in last decades because it allows the formation of systems for gene delivery therapy applications. To perform this, a modification of the previous oil-in-water emulsion methods is necessary: the process is named the water-in-oil-in-water (w_1/o/w_2) double emulsion solvent evaporation (DESE) method and permits the formation of water-soluble PNPs with entrapped in the inner core an hydrophilic moiety (Alex and Bodmeier 1989; Iwata and McGinity 1992).

The first step in this process requires the dissolution of the hydrophilic drug in the aqueous phase (w_1); this is emulsified by sonication with a volatile water-immiscible organic solvent like dichloromethane or chloroform in which the polymer or co-polymer are dissolved forming the first w_1/o emulsion. The organic phase is in the majority compared to the aqueous phase, so an opposite situation respect to the nanoprecipitation and the oil-in-water emulsion is created and the water phase with the drugs dissolved-in remains inside the micelles core, while the oil phase constitutes the external environmental. Now a bigger amount of a second aqueous phase (w_2) is added to the previous emulsion and the entire system is re-emulsified to obtain the w_1/o/w_2 double emulsion: the secondary aqueous phase always contains surfactants or stabilizing agents such as polyvinylalcohol (PVA), polyvinylpyrrolidone (PVP), poloxamers, or other agents.

During this secondary emulsion, the oil phase remains entrapped between the two aqueous phases,

the w_1 in the inner core and the w_2 that constitutes the external ambient: the organic solvent can be now removed by evaporation and the final system, dispersed in water with water in the inner core, can be obtained.

PLGA and PLGA-*b*-PEG has been largely used to obtain this kind of micelles and to entrap hydrophilic moieties such as proteins, drugs, or RNA fragments. Feczkò et al. (2008) investigated several parameters for the synthesis of micro- and nanoparticles with the double emulsion method using PLGA polymer: they optimized the kind of surfactant agent comparing PVA, PVP, and polaxamer, their concentration versus nanoparticles diameter and also protein encapsulation efficiency (Feczkò et al. 2008, 2011).

PEGylated PLGA nanoparticles were first investigated for the entrapment of proteins or drugs due their capability to form small-sized nanoparticles and especially to the enhanced circulatory half-life and the best biodistribution profile (Li et al. 2001).

In the last decade, PLGA–PEG copolymers has been used not only for proteins or drug entrapment but also in particular to generate system for gene delivery therapy due to the possibility to entrap with the double emulsion solvent method water-soluble RNA fragments: encapsulation yield using this copolymer is higher than using other polymer and no unwanted degradation or release of the entrapped RNA occurs (Bouclier et al. 2008).

Moreover, PLGA-*b*-PEG can be functionalized on the PEG chains with different reactive ending-groups as well as for the nanoprecipitation or O/W methods
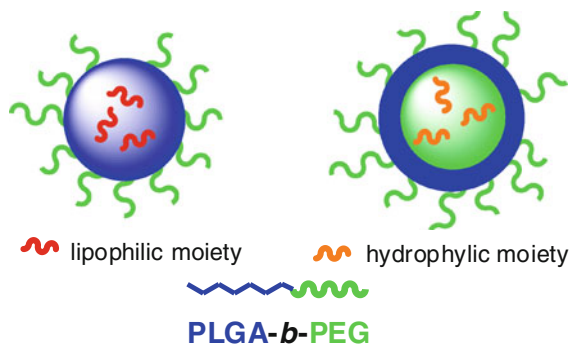
Fig. 5 Formation of PLGA-*b*-PEG nanoparticles for the entrapment of lipophilic moiety (*left*) or hydrophilic moiety (*right*). (Color figure online)

described above and thanks to its amphiphilicity allows the exposure of this groups in the external aqueous media: this enables the possibility to use this reactive groups for conjugation of active targeting moieties as will be discussed later (Luo et al. 2010) (Fig. 5).

Purification and storage of PLGA-b-PEG nanoparticles

Purification process can affect size of the already formed nanoparticles especially if no stabilizing agent has been used during the synthesis: centrifugation is usually used to recover and washing nanoparticles but only 10 min of high-speed centrifugation (10,000 rpm) brings to an increase in diameter of 20–30 %. This problem can be avoided using the low-speed ultrafiltration technique: commercially available centrifuge filtration devices allow purification and simultaneously concentration of colloidal solution with control of the particle size (Kreuter 2004).

Nanoparticles with high PLGA/PEG ratio are not stable during ultrafiltration process because these have not good steric stabilization and can collapse on the membrane of the device: solution of this problem is pre-conditioning of the membrane of the device with a 5 % water solution of polyethyleneglycolbisphenol A epichlorhydrin copolymer for 2 h and use the device for no more than 5 cycles.

Storage of nanoparticles solutions also presents some problems due to the hydrolysis that in water or in buffered media starts to occur shortly; micelles constituting of PLGA–PEG copolymers were investigated and the loss of lactate and mPEG in phosphate buffered saline solution at different incubation times were evaluated: micelles containing good amount of PEG show increased stability in aqueous media during time and also in the electrolyte solution that in other cases can induce aggregation of the preformed nanoparticles (Avgoustakis et al. 2003). However, storing frozen in solid state is more suitable because it avoids all the problems concerning aqueous storage: it has been demonstrated that addition of 10 % lyoprotectant such as sucrose consents complete recover of the frozen nanoparticles with similar size as the originally formulated (Kreuter 2004).

Advantages and disadvantages

PLGA-*b*-PEG PNPs are deeply appreciate from the scientific community especially for their biocompatibility, that allow to use these in bio-medical applications. Also other materials are already been used for preparing nanoparticles for drug delivery therapies but most of these needs FDA approval yet; otherwise, PLGA and PEG are already obtained this award and this allows using them for in vivo applications and preclinical tests. Moreover PLGA-*b*-PEG copolymer, as already described, is able to form micelles easily, with well controllable size and good encapsulation efficiency also without the helping of surfactant agents, which are often toxic or not-well tolerate by cells and organism.

Despite these extremely appreciate properties, PLGA–PEG nanoparticles present also some disadvantages when compared to others; one of the most disadvantages is the cost of the starting material: both PLGA and PEG are synthetic polymers and so they require industrial process to be provide, purification steps, expensive building block; this is not the case of other polymers, like the natural ones, for example, chitosan (Duceppe and Tabrizian 2010), that derived from natural waste material and do not require long artificial process to be prepared. Related to this aspect there is another disadvantage in using PLGA–PEG nanoparticles: the use of synthetic compounds for bio-applications could lead to several problems especially regarding starting material purity. Heavy metals used during polymerization of lactic and glycolic acid, such as stannous, have to be completely removed from the polymer chains otherwise they accumulate in the body and cause toxicity or organs damages.

For these reasons in the last years the interest in natural material is starting to grow; chitosan is not only an example but also natural fatty acid, triglycerides, cholesterol were investigated for nanoparticles production and used in bio-applications, also to avoid the injection into body of foreign material, like the synthetic ones, that can be easily recognized and eliminated from blood circulation than the natural ones (Mehnert and Mäder 2012).

Drug loading, determination, release

Ways to load drugs in PNPs and loading efficiency is a crucial point for the development of a successful drug delivery system. A promising nanoparticle systems have to possess high-loading capability to reduce the quantity of carrier that have to be administrated to achieve therapeutic effect (Soppimath et al. 2001). Plenty of works described methods to bound drugs to the nanoparticles, including incorporation of drug into the nanoparticles matrix during nanoparticles formation as previously described, covalent or electrostatic binding of the drug on the nanoparticles surface or surface adsorption of the drug by incubating nanoparticles already formed in a drug solution (Kreuter 1994).

In general, the loading method is determined by the physico-chemical properties of the drugs, such as hydrophilicity or lipophilicity, incorporation is suitable for lipophilic products while chemical linkage or adsorption is best for water-soluble drugs if the w/o/w method cannot be used. It is evident that a large amount of water-soluble drug can be entrapped by covalent-binding respect by surface adsorption or by common incorporation methods; Yoo and Park (2001) conjugated PLGA nanoparticles with doxorubicin and estimated a loading efficiency of 96.6 % (w/w) in comparison with the 6.7 % (w/w) of the common incorporation method.

In the case of lipophilic drug, the incorporation methods during nanoparticles formation are the best: thanks to this strategy, drugs can be loaded with high efficiency and especially without having to use several reaction steps, purification passages, energy consumption, and so on. Using this method with the PEG-coated nanoparticles, and in particular with PLGA–PEG copolymer, a drug-loading efficiency of about 75–95 % can be reached. Peracchia et al. (1997) demonstrated this percentage adjusting reaction

Table 1 Drug-loading capability of di- and multi-blocks copolymers (Peracchia et al. 1997)

Copolymer	Drug loading (maximum 20.0)	Drug loading percentage (%)
PLGA-*b*-PEG (5 kDa)	18.0	90
PLGA-*b*-PEG (12 kDa)	18.4	92
PLGA-*b*-PEG (20 kDa)	17.8	89
PLA–mPEG (5 kDa)	15.0	75
PLA–mPEG (12 kDa)	13.6	68
PLA–mPEG (20 kDa)	12.6	63

parameters and studying a lot of polymers, such as polycaprolactone (PCL), polysilamine (PSA), PLA/PLGA, and their PEGylated di- and multi-blocks copolymers; they also found that for the multi-blocks copolymer the loading efficiency is lower (maximum 75 %) than that obtained with the two di-blocks copolymer; their results, regarding di- and multi-blocks copolymers are summarized in Table 1.

Determination of the drug content into PNPs is another crucial point for drug delivery applications: it is mandatory to give a good and precise estimation of the concentration of drug in the nanoparticles solution before administration both for in vitro and in vivo studies. This can be a problem due to the colloidal nature of the carrier than in some cases can affect the measurement for example absorbing radiation in the ultraviolet range and making thus impossible determination by UV detector (Magenheim and Benita 1991); a good strategy that has often been used require the disruption of the polymeric carrier using an organic solvent like not only dichloromethane or chloroform but also acetonitrile: the prolonged contact with the solvent generates the re-opening of the polymers chains already assembled in the micellar state freeing the drug and permits the determination of the same in the resulting solution by common analytical methods like HPLC (Redhead et al. 2001; Cheng et al. 2007).

Drug release profile is also important to determine the successful of a drug delivery system: this must be constant during time and possibly durable enough to enable the reduction of the administrations number of the drug formulation to a patient. The release rates depend on multiple factors such as desorption from the surface in the case of surface-bound drug, diffusion of drug trough the nanoparticles matrix in the case of incorporated drug, polymer matrix biodegradation, or

combination of both of these. Several methods have been developed to study the in vitro release: most of this involved typical purification procedures like the use of artificial or biological membranes, dialysis, ultrafiltration, or ultracentrifugation techniques but nowadays difficulties in a good determination of the release profile are still present (Washington 1990).

In general, the process starts with a rapid release of drug contained into nanoparticles that consists of the fraction of drug not really incorporated but only adsorbed to the nanoparticles matrix and then there is an exponential release rate due to the factors described above (diffusion and biodegradation). The way to load drug in nanoparticles has shown to have an effect on the release profile: drugs that are only adsorbed on the surface have a too fast release, while chemically linked drugs present a too slow release against the incorporated ones that in generally ranges in a time of a few days; thus the incorporation methods allow to obtain the best release profile (Yoo et al. 1999). It was also found that another factor that may influence the release rate is the volume of the nanoparticles surrounding media: the presence of bigger amount of the aqueous media brings to acceleration in the drug release profile (Magenheim et al. 1993) (Fig. 6).

PLGA-*b*-PEG nanoparticles show good release profile due to PLGA biodegradability in biological medium. Dhar et al. (2008) well described the releasing properties of this kind of nanoparticles loaded with the antitumor drug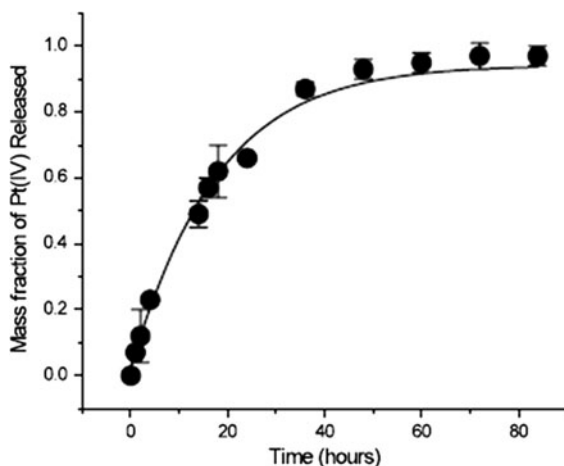 cispatin: at the beginning there is a 12 % of drug released immediately, probably due to the adsorption portion, then a controlled exponential release is established and after 24 h the 66 % of the drug loaded was released; after 60 h the release is still present though with a reduced rate. Avgoustakis et al. (2002) also investigated on the release profile of PLGA–PEG nanoparticles testing different PLGA/PEG ratios; they found that an increase in the PEG content leads to an increase in the rate of drug release and also the fraction of drug released in the initial rapid phase is higher than corresponding with low PEG amount: PLGA–PEG nanoparticles may allow thus a controllable drug release profile by adjusting the two polymers ratio. No effect on the drug release profile is observed changing the drug-loading concentration suggesting that no correlation elapses between these two parameters.

Targeting

As already mentioned, the main purpose in the development of nanocarriers for drug delivery application is certainly drug targeting to the desired site of action and controllable biodistribution into the body. This enables the possibility to have high concentration of the drug at the target site, to reduce administrated quantity and also unwanted side effect. This challenging goal can be obtained exploiting natural passive targeting that always occurs into the body or altering this process by a modification of the nanocarriers in order to achieve the so-called active targeting (Wanga and Thanoua 2010) (Fig. 7).

Fig. 6 In vitro release kinetics of encapsulated cisplatin from PLGA-*b*-PEG nanoparticles at 37 °C (Dhar et al. 2008)
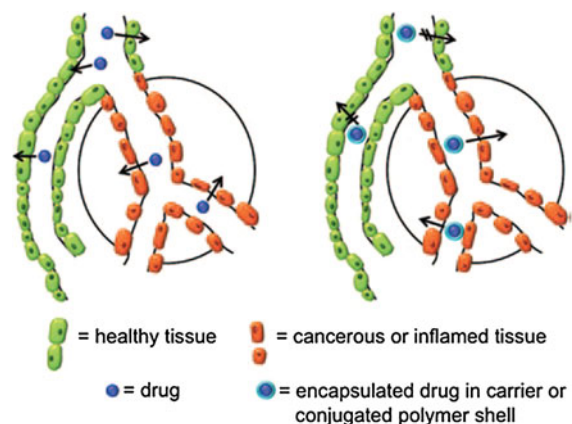

Fig. 7 Schematic representation of the EPR effect (Knop et al. 2010). (Color figure online)

Passive targeting also called EPR effect takes advantage from the natural tendency of the particles to accumulate in some particular site of the body: solid tumors or inflamed tissues possess hypervascularization and a leaky vasculature that allow nanoparticles to enter in the site easier that in the healthy tissue and a poor lymphatic drainage that consents to the same particles to remain inside (Knop et al. 2010). To satisfy this condition, a long circulation of the particles in the blood is necessary to reach the tumor or the inflamed tissue and particles must not be removed before by opsonization phenomena. Not PEGylated nanoparticles show a too rapid clearance from the blood circulation and accumulation in the RES organs, such as liver and spleen, due to the high activation of the immune system, while the presence of PEG chains can drastically reduce this phenomenon. Beletsi et al. (2005) have long investigated the effect on the biodistribution of nanoparticles composed of different amounts of PEG in PLGA/PLGA–PEG mixtures. They studied blood and tissue distribution in mice by radiolabelling PNPs with ^{125}I; they found that the blood residence of PLGA/PLGA-b-PEG nanoparticles increases as their PLGA-b-PEG content increases reaching the maximum of longevity when these are composed only of the PLGA-b-PEG portion; moreover, they calculated the radioactivity percentage after 3 h after injection in liver, spleen, and other RES organs and they found that this decreases when PEG amount increases, demonstrated that the increased of PEG density on the nanoparticles surface results to the formation of a steric barrier inhibiting nanoparticles opsonization and enables the possibility to have high passive targeting (Fig. 8).

Despite interesting behavior, the target sites that can be reached using passive targeting are very limited, and also targeting efficiency is quite low; in addition, the nanoparticles that reach the tumor tissue with passive targeting are then internalized by the cells just in a small amount. Active targeting offers the possibility to enlarge the number of accessible sites, to enhance the quantity of nanocarriers that reach the site and at the same time to increase the drug concentration once the target site is reached, thanks to an increased internalization by the cells. To perform active targeting, a modification of the nanoparticles surface is requested: specific ligands, able to selectively recognize a specific site on cells, must be linked on the surface in the form of decorating moieties: this ligands may be active molecules, proteins, peptides, monoclonal antibody, or any other moiety that have a specific receptor system located on the tissue cells that have to be treated. A number of possibilities exist to obtain the decoration and the most commons are alteration of the surface charge, adsorption to the ligands on the surface or direct attachment of a specific biomolecules (Kreuter 2004). The first two methods are not appreciable because the linkage between nanoparticles and ligand is not strong enough to survive for long period once injected into the body. The most promising approach is the third option that permits to have a covalent linkage with the specific ligand that can survive in the body conditions until the site of action is reached. Accordingly, it is necessary to have a functional group exposed on the nanoparticles surface to make possible a coupling reaction with the ligand. PLGA-b-PEG offers the possibility to functionalized nanoparticles surface with a large variety of reactive group, such as amino, carboxylic acid, and maleimido, by simply modifying the end of PEG chains during synthesis steps and nowadays most of these PEG-derivatives are commercially available. As most of active ligand (especially peptides and proteins) possesses free amino groups, a suitable reactive group on the nanoparticles surface may be a carboxylic acid: through the connection of these two groups a strong, durable, and hard amido-group is generated. Once formed, nanoparticles are water-soluble and the functional groups also result dispersed in water so the chemistry used to bind ligands to the surface must be in aqueous phase: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a coupling reagent suitable to perform this kind of reactions because it works well in water in its hydrochloride form and at a neutral pH (generally between 4 and 7); it can also be used with or without N-hydroxysulfosuccinimide (Sulfo-NHS) to increase coupling efficiency.

We recently performed (Locatelli et al. 2012) in vitro active targeting of cancer cells of glioblastoma (U87MG cell line) developing a PLGA-b-PEG-COOH nanocarrier functionalized on the surface, using EDC chemistry, with a specific proteins (Chlorotoxin) that is able to recognize this kind of tumor cells: we demonstrated that the quantity of drug (silver nanoparticles in our case) that are internalized by each cell is almost 8.4 times higher using the active targeting than without it.
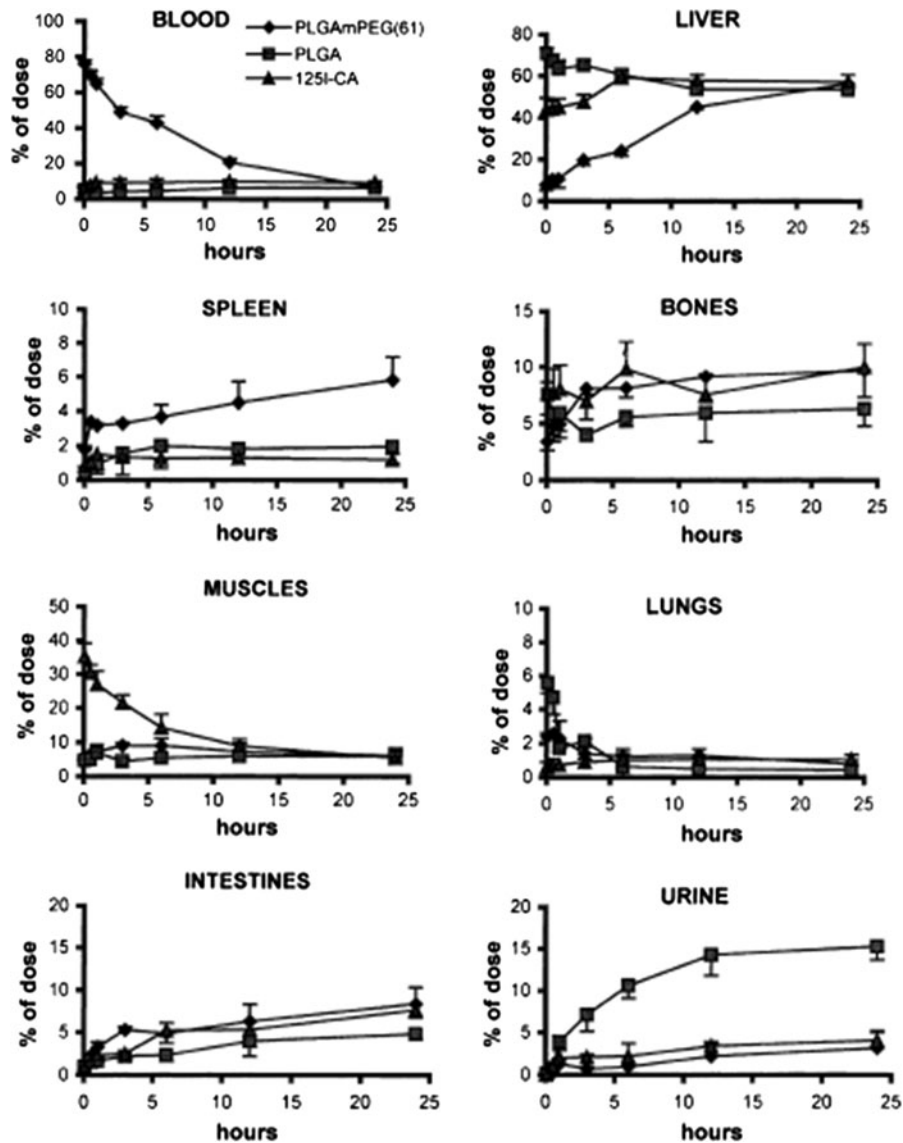
Fig. 8 Example of biodistribution of ^{125}I-labeled PLGA and PLGA–PEG nanoparticles in mice (Avgoustakis et al. 2003)

Zhao and Yung (2007) used doxorubicin-loaded PLGA-*b*-PEG nanocarriers targeted with folate as folate receptor is overexpressed in a wide variety of human tumors. They found that the targeted nanoparticles have higher in vitro cytotoxicity on cancer cells than on healthy cells, demonstrating that with active targeting a specific recognition of the tumors cells can be achieved: with 45–60 % of folate amount on the micelles it was possible to kill cancer cells without interfering with the normal cells.

These results suggest the importance of good active targeting for the development of a drug delivery system but having long circulating particles with no opsonization phenomena remain also fundamental.

Drug delivery applications

One of the most logical applications of biodegradable PNPs for drug delivery is the treatment of human solid tumors (Vinod Prabhu et al. 2011; Park et al. 2008; Grobmyer and Moudgil 2010). Research started early in this field due to high mortality of this pathologies in our society, to the lack of focused medical care and to the enormous side effects of the existing ones, such as

chemotherapy, and nowadays encouraging results have been reached: the development of a biodegradable, long circulating copolymer, such as PLGA-*b*-PEG, for the fabrication of nanocarriers is part of these results. Nanotechnology for cancer therapy exploits the same drugs that are normally utilized in common invasive therapy but taking advantage by the drug delivery principles. This is the case of cisplatin, a drug used to treat a wide variety of solid tumors: cisplatin possess high efficacy against cancer cells and it is probably the most potent anticancer agents known, but its extreme toxicity and acquired resistance pose many limitation to its application in chemotherapy. Many papers had appeared in the literature during the last decade with the aim of cisplatin selectively delivery only to cancer tissues and not to the healthy ones. Dhar et al. (2008) utilized PLGA-*b*-PEG copolymer for the fabrication of a cisplatin-loaded nanocarrier and conjugated this with a specific ligand, an aptamer that targets the prostate-specific membrane antigen (PSMA). They demonstrated, using fluorescence microscopy, the successful target to this nanocarriers only in the cancer cells and not in the normal ones and the 10-times enhanced cells mortality using the targeted nanocarrier than the non-targeted one. Also, Graf et al. (2012) demonstrated the targeting of cisplatin prodrug encapsulated in PLGA-*b*-PEG nanoparticles to the transmembrane protein $\alpha v\beta 3$ integrin, that is generally upregulated in cancer cells, using a specific cyclic pentapeptide: they found that the entrapped prodrug displayed enhanced cytotoxicity in vitro as compared to cisplatin conventionally administered; in vivo studies demonstrated that the nanoparticles inhibited tumor growth of a human breast carcinoma xenografted in nude mice and contemporary they also exhibited reduced nephrotoxicity when compared to cisplatin. These results can finally lead to the development of a highly effective anticancer drug, but minimizing its devastating side effects.

In a similar manner, Farokhzad et al. (2006) developed a PLGA-*b*-PEG nanocarrier loaded with docetaxel, another well-known antitumor drug, and targeted with an aptamer, for the recognition of PSMA, they found that this nanocarrier can not only selectively bind the prostate cancer cells but also enhanced the in vitro citotossicity of docetaxel. Moreover, they tested it in vivo on xenograft nude mice bringing the tumors and they found, after only one injection of the nanocarrier, a complete reduction of the tumor volume and a 100 % degree of survival of treated mice after 109 days in comparison to the 14 % of the non-treated ones.

In the same way, Danhier et al. (2009) in an attempt to improve the therapeutic index and devoid of the adverse effects of the drug paclitaxel, a major anticancer drug isolated from the bark of *Taxusbrevifolia*, incorporated the drug in a PLGA–PEG nanoparticles: they found that cells viability of Cervix carcinoma was lower after exposure to the formulated nanocarrier than to the commercially available Taxol®(IC50 5.5 vs 15.5 μg/ml). Also during in vivo studies paclitaxel-loaded nanoparticles showed greater tumor growth inhibition effect compared with Taxol® suggesting that these nanoparticles may represent another effective anticancer drug delivery system for cancer therapy.

Another important application of this kind of nanoparticles is the early diagnosis of cancer using already existing imaging techniques or developing new expertises. The diagnosis of cancer at the cellular level will be greatly improved with the development of techniques that enable the delivery of imaging probes directly into cells. Metal nanoparticles due to their unique optical properties has been already exploited for this purpose: as described above metal nanoparticles can be transfer from the original aqueous phase, in which they are generally synthesized, to an organic solution by changing their surface properties through a coating with an organic ligand; this passage makes these suitable for entrapment into PNPs, as well as in the case of lipophilic drug entrapment, leading to the fabrication of an imaging-probe delivery system: using active targeting is then possible to selectively bind cancer tissues and detected them with imaging techniques also in the early stages of the disease, enhanced the normal sensitivity of the common diagnosis methods (Liu et al. 2007).

The possibility is to obtain a system able to detect cancer cells and contemporary to kill them, the so-called theranostic (therapy and diagnosis) system is one of the most challenging goals for the research in this last years.

In this field, we recently encapsulated GNRs in a PLGA-*b*-PEG nanocarrier using the previously described methodology (Comes Franchini et al. 2010a, b): GNRs have already been studied for their particular capability both as imaging probe and as "drug" because they can give hyperthermic effect and

Table 2 Recent research progress of PLGA-*b*-PEG nanoparticles

Fabrication method	Drug	Targeting	Entrap. efficiency	Release profile	In vitro study	In vivo study	Major achievements
O/W (Esmaeili et al. 2008)	Docetaxel	Folic acid	87 %	≈20 % in 24 h	Uptake/cytotoxicity in cancer cells	/	Huge increase of uptake
w/o/w DESE (Chen et al. 2011)	Vincristin sulfate	Folic acid	≈47 %	70 % in 24 h	Uptake/cytotoxicity in cancer cells	/	Huge increase of uptake Strong reduction of IC_{50} value
NP (Liang et al. 2011)	Paclitaxel	Folic acid	95 %	40 % in 12 h	Uptake and cytotoxicity in endometrial cancer cell	Antitumor activity	Inhibition of tumor growth, increased survival rate in mice
O/W (Guo et al. 2011)	Paclitaxel	DNA aptamer	≈45 %	80 % in 12 d	Cytotoxicity test	Biodistribution Antitumor activity	Accumulation in tumor Tumor growth inhibition Prolonged survival of rats
Solvent displacement method (Yu et al. 2012)	/	/	/	/	Mucous penetration	/	Capability to penetrate into mucous, for no i.v. administration
Dialysis method (Ashjari et al. 2012)	Insulin	/	≈60 %	≈50 % in 2 d	/	/	No initial burst in drug release profile
w/o/w DESE (Chen et al. 2012)	Vincristin sulfate	Folic acid and R_7 peptyde	≈45 %	50 % in 8 h	Uptake/cytotoxicity/cell cycle analysis	/	Increase of uptake Induction of apoptosis in cancer cells
O/W (Khalil et al. 2013)	Curcumin	/	>70 %	57 % in 9 d	/	Drug plasmatic level after oral administration	Non i.v. administration Increase of curcumin bioavailability

/ Data non present or non-reported in the paper

burn cancer cells, so they represent a perfect thera-nostic instrument. In this study, we demonstrated the possibility to obtain a biodegradable nanocarrier containing GNRs and we evaluated its low cytotox-icity with in vitro tests but above all we obtained satisfactory optoacoustic signal at a non-cytotoxic concentration of GNRs, proving the fabrication of a promising contrast agent for cancer imaging.

These examples are just to show the enormous possibilities deriving from these kind of copolymer but other important, more recent researches and achievements are summarized in Table 2.

Conclusion

Drug-loading capability, possibility of chemical tar-geting and improvement of the circulation time are important hallmark of the PLGA-*b*-PEG PNPs, which make this material highly attractive in the drug delivery field. Although these good properties, many efforts have still to be performed to better control nanoparticles size, polydispersity, charge surface, and to render these features easily reproducible during synthesis steps. Several parameters are under investi-gation to improve drug-loading capability and drug release profile. Especially, improvement of distribu-tion into tumor tissues remains a challenging goal and this would also be possible, thanks to the development of new and more specific targeting moieties. In conclusion, PLGA-*b*-PEG NPs are well-established nanocarriers for nanomedicine applications. For this reason, scientists worldwide are testing them both in vitro and in vivo to treat cancer.

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