Polymeric Nanoparticles

Targeted Delivery of Peptide and Protein Drugs

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General methods for the preparation of nanoparticle systems, such as solvent displacement, salting-out, emulsion diffusion, and solvent evaporation techniques are reviewed. Particular attention is directed to the preparation of nanoparticles loaded with proteic drugs, with specific reference to α-interferon. Nanoparticles for the targeting of α-interferon to hepatocytes are also described.

Controlled drug delivery technology represents one of the most rapidly advancing areas of science in which polymer and pharmaceutical scientists are contributing to biomedical research field. Such delivery systems offer many advantages as compared to conventional dosage forms, including improved efficacy, reduced toxicity, improved patient compliance, and cost effective therapeutic treatment. Indeed, conventional systemic administration of drug is often characterized by unspecific body distribution, which gives rise to negative therapeutic index and unwanted side effects. In particular, controlled release is strongly required for unconventional drugs, such as proteins and oligopeptides. If surface modification is possible, targeted delivery is achievable, resulting in enhancement of the therapeutic efficacy of the dosage forms and lowering of toxic effects. Controlled and targeted drug delivery systems had an incredible impact on nearly every branch of medicine. In 1997, the drug delivery market has reached over 13 billions US$ in the United States alone [1].
Fundamental characteristics of drug delivery systems are ability to incorporate drugs without damaging them, long *in vivo* stability, tunable release kinetics, and targeting to specific organs and tissues. The common concept of these features is related to the system surface properties, both external and internal. *In vivo* stability of the system is strictly connected with external surface properties, and *stealth* surfaces were identified as those not recognized as foreign bodies by the immune system. One strategy consists in the obtainment of a water-like shield, promoted by a strongly hydrophilic coating. PEG polymers are widely used as coatings due to their high biocompatibility and antiopsonizing effect [2]. Normally, particles that are small enough to escape the capillary bed of lungs are quickly scavenged by the reticular endothelial sys-

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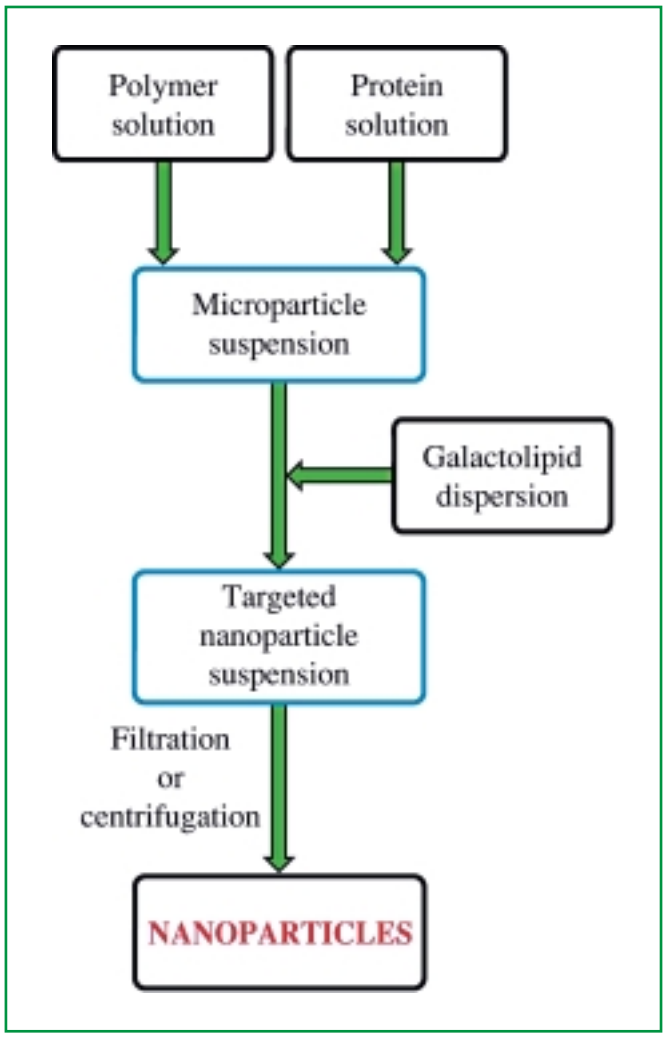

Figure 1 - Schematic representation of the coprecipitation technique

tem (RES), particularly by liver Kupffer cells. This sequestration represents the major barrier to targeting cells or tissues elsewhere in the body. On intravenous administration, particles are quickly coated by specific blood components (opsonins) and then recognized and captured by RES. The antiopsonizing action of polymers such as PEG consists in the adsorption of a surface water layer to form a hydrophilic barrier. This causes a reduced uptake of the particles by RES, thus enhancing their circulation half-life. PEG-based shields can be obtained by:

1) physical coating of preformed nanoparticles with PEG copolymers [3];
2) PEG residues chemically linked to functional groups on the preformed nanoparticle surface [4];
3) thermodynamic assembly of block amphiphilic copolymers in core-shell nanostructures exhibiting hydrophilic external surface and hydrophobic core. In these cases, the hydrophilic domain constituted by PEG blocks results in an intrinsic shield action [5].

For tissue targeting, drugs are chemically linked to or physically trapped within non-immunogenic polymers, which either will degrade or will be excreted by the kidneys. Depending on different diffusional rates, particle biodistribution drastically changes. Active targeting to specific tissues can be achieved by complexing polymer-drug conjugates with molecules (antibodies, lipids, carbohydrates) that will be recognized only by cell surface receptors of a specific tissue. One issue of this approach consists in finding highly specific non-immunogenic targeting molecules.

Polymeric nanoparticle systems

Liposomes are still certainly the most developed carrier system available [6]. Even though they allow for high drug payloads, they present some disadvantages related to their *in vitro* and *in vivo* instability and difficult surface functionalization [7].

The concept of solid polymeric nanoparticles has been developed since 1976 by using non-biodegradable polymers [8] and since 1979 with biodegradable polymer systems [9]. Nanoparticles (NPs) are solid colloidal particles made of artificial or natural polymers with a diameter ranging from 1 to 1,000 nm and in which the biologically active molecules can be trapped, dissolved, and/or encapsulated. The design of a polymer matrix for NPs formulation do improve material performance in terms of physical chemical characteristics, surface properties and allow for designing targeting systems, as surface engineered NPs. In particular, the size and surface characteristics of nanoparticles, in terms of charge, hydrophilic-hydrophobic balance, and presence of site-specific components, dramatically influence their body distribution and targeting attitude [10]. The use of biodegradable materials avoids the problems related to physiological excretion or mechanical removal of the drug delivery device after drug depletion. Moreover, biodegradable matrices can provide a further control of release rates, by joining the typical diffusive mechanisms with tunable polymer degradation. NPs can be prepared by several techniques, depending on the nature of the polymeric material and the characteristics of the drug to be loaded. The starting can be either monomers or preformed polymers. The second method is usually the preferred one, because it allows for the use of biodegradable polymeric matrices that are not appropriate for emulsion polymerization. Additionally, polymerization residues can be removed from the matrix before NPs formulation. Many reviews focus on polymeric matrices [11] and NPs preparation [12-17].

General methods for the preparation of nanoparticles

Microparticles (MPs) and nanoparticles belong to the class of colloidal systems, multiphase systems in which one or more microphases are dispersed in a continuous matrix of different composition or physical state. The main characteristic of colloidal dispersions is their extremely large interface area between the dispersed and the continuous phase. Colloidal dispersions are metastable or unstable, since minimization of interface free energy between two different phases is dictated by thermodynamic constraints. However, in some cases colloids display significant kinetic stability that prevents their aggregation in macrophases. Hence, production of MPs and NPs relies essentially upon the chemical production of colloidal dispersions, their kinetic stabilization, and effective recovery of the final formulates. Polymeric materials are constituted by large molecules whose peculiar solution characteristics often allow for the preparation of stable and size-controlled colloidal dispersions, which in turn can be converted into MPs and NPs. In addition, several polymers can be used as good stabilizers of colloidal dispersions, since they provide a surface coating of the metastable microphase, thus lowering its tendency to phase-aggregation.

The common feature of all methods for the preparation of MPs and NPs is the externally-induced separation of at least two phases: a colloid-rich phase and a colloid-poor phase. This process is better known as *coacervation*, and it may be promoted by a number of different techniques. Furthermore, these procedures have been adapted in order to load the MPs and NPs with active principles of different nature.

The solvent displacement method is a patented straightforward procedure [18] in which polymer, drug and, if necessary, lipophilic stabilizer are dissolved in a semi-polar water-miscible solvent, such as acetone or ethanol. The organic solution is then poured or injected into an aqueous solution containing a stabilizer under magnetic stirring. NPs are formed instantaneously by rapid solvent diffusion, and the organic solvent is then eliminated from the suspension under reduced pressure. Even though *precipitation* or *nanoprecipitation* are often used to define this method, it is important to stress that the formation of NPs is due to polymer aggregation in stabilized emulsion droplets. Apparently, nucleation and growth steps are not involved. NPs of poly(ε-caprolactone), PLA homopolymers, PCL-PLA and PLGA copolymers loaded with conventional drugs were prepared by this method, by using acetone as organic solvent [19-21]. The major limit to the application of this technique is represented by the difficulty of finding a drug/polymer/solvent/nonsolvent system in which NPs are formed and the drug efficiently entrapped. In addition, this method is not suited for the encapsulation of water-soluble drugs, which quickly diffuse into water from the organic phase [20, 21].

The salting-out technique, firstly applied to pseudolatexes, was adapted to the preparation of drug-loaded biodegradable nanoparticles [22]. This method is based on the separation of a water-miscible solvent (acetone) phase from aqueous solutions promoted by a salting out effect. Water is added to the emulsion obtained by addition of an acetone solution of poly-

Table 1 - Methods for the preparation of nanoparticles loaded with conventional drugs

Method	*Polymer*[a]	*Drug*	*Particle size (nm)*	*Ref.*
o/w	PLA, PLA-*b*-PEG	Lidocaine	247-817	30
o/w	PLA-PEG-PLA	Progesterone	193-335	31
o/w	PLGA	Dexamethasone	109-155	32
o/w	PEO-*b*-PBLA	Doxorubicin	37	33
solv-disp	PMLAiPr	Halofantrin	150-160	34
solv-disp	PMLAnHe	Halofantrin	90-190	34
solv-disp	PLGA	Vancomycin	160-170	20
solv-disp	PLGA	Procaine Hydr.	<210	21
solv-disp	PCL, PLA, PCL-*co*-PLA	Nimodipine	81-132	19
o/w	PEO-*b*-PCL	Enalapril maleate	50	35
o/w	PLA	Lidocaine	115-123[b]	36

[a] PBLG = poly(g-benzyl L-glutamate); PBLA = poly(b-benzyl L-aspartate); PMLAiPr = poly(b-malic acid *iso*-propil ester); PMLAnHe = poly(b-malic acid *neo*-hexil ester); [b] dimension of unloaded particles.

mer and drug emulsified under vigorous stirring in an aqueous gel containing the salting-out agent and the colloidal stabilizer. As a consequence of the dilution, acetone diffuses into water resulting in NPs formation. Solvent and salting-out agents are then eliminated by cross-flow filtration. This procedure allows for the incorporation of large amounts of drug with excellent yields, and procedure scale-up is fairly easy. Indeed, once optimized the solvent/salting out agent/stabilizer system, it is not necessary to search for specific proportions to obtain drug-loaded NPs. However, this technique can be used only for loading lipophilic drugs.

The emulsion diffusion method is a slight modification of the salting-out technique. It differs mainly because the organic solvent is only partially miscible with water, and it is previously saturated with water, in order to reach an initial thermodynamic equilibrium between water and organic phases. After addition of water, solvent diffusion is observed, and a nanoparticle suspension is formed.

The solvent evaporation method is a patented well known technique [23] that basically consists in the formation of a bi-(o/w) or tri-phase emulsion (w/o/w). The inner phase is constituted by a polymer solution in organic solvent in the biphase procedure, and a water in oil emulsion in triphase method. In both cases, the continuous phase is an aqueous solution in which the polymer is insoluble. The resulting emulsion is then exposed to a high-energy mixer, such as ultrasonic devices, homogenizers, colloid mills, and microfluidizers to reduce the globule size. Removal of the organic solvent, by heat, vacuum, or both, results in the formation of a fine aqueous dispersion of NPs, which can be collected and purified by lyophilization or other procedures. This method is widely used for the preparation of micro and nanoparticles made of polysaccharides, aliphatic polyesters, such as PLA, PLGA, PGA, and other synthetic polymers such as PEG copolymers. Hydrophobic drugs are finely dispersed or dissolved in the organic polymer solution, and the aqueous phase contains the emulsifier. If the drug is hydrophilic, it is first dissolved in water and then added to the organic solution. The resulting emulsion in then added to the emulsifier water solution. Solvent evaporation method may present some drawbacks. In fact, toxic chlorinated solvents, such as chloroform and methylene chloride are often used because of their water insolubility, easy emulsification, solubilizing properties, low boiling point. Moreover, the evaporation step can result in agglomeration of microparticles. To avoid some of these problems, a modified procedure was developed for the preparation of PLGA nanoparticles [24].

Mixed techniques

Nanoparticles may be produced also from natural macromolecules. Denatured albumin nanoparticles were obtained by heating an oily emulsion of albumin in aqueous solution [25]. Magnetic NPs incorporating magnetite particles [26-28] were prepared by this method, which of course can only be applied to drugs that are not heat sensitive. Recently, NPs were prepared by gelification of alginate solutions with calcium ions [29]. After strengthening the resulting microgels with poly(L-lysine), NPs of well defined sizes that presented an unusual high surface hydrophilicity were obtained.

Table 1 summarizes some examples of the preparation of micro and nanoparticles loaded with conventional drugs.

Protein-loaded micro and nanoparticles

Protein drugs constitute a very important class of new therapeutic agents [37], but their administration suffers from structural lability in hostile environment (temperature, pH, ionic strength, denaturing agents), which may determine loss of biological activity; as a consequence, parenteral administration is the most common route. Because of protein short half-life, frequent administration is necessary, which may cause fluctuation of protein concentration and accumulation in plasma, sometimes giving rise to serious adverse effects. Alternate protein delivery routes as nasal, oral, rectal, ocular, and transdermal were developed, but they are not very effective because of uncompleted systemic drug absorption and because of high and sometimes toxic local drug concentration. At present, controlled delivery systems represent the best approach to protein drug administration. Liposomes and biodegradable MPs and NPs are the most utilized protein carriers. Encapsulation of protein drugs in liposomes results in a friendly procedure, guaranteeing for the maintenance of biological activity [38, 39]. An inherent limitation of liposomes is their short *in vivo* half-life and consequently, they can only deliver drugs over limited periods.

Biodegradable MPs and NPs allow for sustained and controlled release of proteins, but the development of protein-specific techniques for their preparation is necessary. Matrices based on PLA/PGA have been widely utilized, because of their good biocompatibility, non toxicity, and biodegradation characteristics [40]. It is known, however, that PLGA matrices

Table 2 - Preparation of micro and nanoparticles loaded with protein drugs[a]

Method	*Polymer*	*Protein*	*Particle size (nm)*	*Ref.*
w/o/w	PLGA	L-asparaginase	196-226	45
w/o/w	PLGA	BSA	ms	46
w/o/w	PLGA	BSA	100-200	32
w/o/w	PLGA, PLA	BSA	ms	47
w/o/w	PLGA blend PLA	BSA	ms	48
w/o/w	PLGA, PCL	BSA	20-1,000	49
w/o/w	PLA	Protein C	230-340	50
w/o/w	PLGA	FITC-BSA FITC-HRP	ms	51
w/o-o/w	PLGA	TRH	250-800	52
w/o/w	PLGA	IL-1a+BSA	ms	53
o/w	PLGA	Rism. porcine	ms	54
o/w	Biod. polym.	Peptides	-	55
	PLGA	BSA	300-600	56
w/o/w	PLGA	rhBMP	ms	57
w/o/w	PEG-PLGA	BSA	-	58
w/o/w	PEG-co-PBT	BSA	ms	59, 60
w/o/w	PLGA-b-PEO	BSA	ms	61

[a] ms = microsize; CP = coprecipitation; FITC-BSA = fluorescein isothiocyanate-labeled BSA; FITC-HRP = fluorescein isothiocyanate-labeled horseradish peroxidase; IL-1a = recombinant human interleukin-1a; rhBMP = recombinant human bone morphogenetic protein-2

can adversely affect protein stability, especially as a consequence of the low pH consequent to matrix degradation [41, 42]. Furthermore, the hydrophobic nature of PLGA is responsible for the poor compatibility between protein and matrix, resulting in protein adsorption at the polymer surface, denaturation, and aggregation, thus negatively affecting biological activity and release kinetics [43]. Most of the adopted techniques are modifications of standard methods, adapted to the specific protein properties, in order to maintain the protein activity and to realize the proper release kinetics. The double emulsion method is the most widely utilized procedure. In fact, water-soluble proteins cannot be effectively encapsulated by the o/w process due to the partition of the protein in the aqueous medium that results in low encapsulation efficiency. Very recently, a new procedure for the obtainment of BSA loaded reservoir-type microspheres has been reported [44].
Typical examples of MPs and NPs loaded with proteic drug are reported in Table 2.

Nanoparticle suspensions for the targeted release of protein drugs

The design of new injectable dosage forms for the administration of peptide or protein drugs requires, for the realization of targeted release NPs systems, the selection has to be done of three fundamental ingredients consisting of:

1) compatible polymeric matrices soluble in water or water/organic solvents provided with structural functionality suitable to interact with protein drugs and protein stabilizers without any adverse effect;
2) stabilizing component whenever the polymer matrix of choice does not exert a stabilizing effect on the trapped protein drug;
3) structurally defined components able to establish interactions with specific receptor sites of the target organ.

Alkyl hemiesters of alternating copolymers of maleic anhydride and vinyl ethers of monomethoxyoligoethylene glycols were selected as biocompatible matrices for the formulation of NPs. Those materials displayed a high versatility to combine with proteins in different proportion and to provide hybrid bioerodible matrices without any adverse effect on the structure and activity of proteins [62]. The synthetic polymers as well as their combinations with human serum albumin/α-interferon mixtures did not give any negative response to *in vitro* and *in vivo* biocompatibility tests, including platelet aggregation, complement activation, acute toxicity, and acute thromboembolic potential [63].
The choice of the protein stabilizer stemmed from the necessity of coating the hybrid formulates by a strongly hydrophilic shell, in order to minimize opsonization by blood proteins. In this respect, modified β-cyclodextrins grafted with the glycidyl ethers of protected polyols (glycerol and pentitols) appeared rather promising components for their amphiphilic character, connected to the presence of an hydrophobic pocket and an external hydrophilic shell with an amplified number of hydroxyl groups [64].
Targeting of the new dosage form to the asialoglycoprotein receptors located on the liver hepatocytes [65] was achieved by inclusion of galactolipids, such as digalactosyldiacylglycerol (DGDG), the corresponding fully hydrogenated derivative (HDGDG), and dihexosylceramide sphingolipid (DHCS), in the hybrid formulation [66].

Preparation of nanoparticle suspensions
Two different procedures, based respectively on *slow solvent evaporation* and on *co-precipitation* [67, 68] a new proprietary method developed during the fulfillment of an RTD research, were tested for NPs formulation.
The *slow solvent evaporation* technique afforded milky dispersions. Dynamic light scattering analysis of the dispersions highlighted a rather heterogeneous dimensional distribution that in most cases resulted bimodal. Depending on the suspension chemical composition, particles with an average diameter ranging between 0.1 and 3.4 μm were obtained. No effect of the polymer/protein/lipid weight ratios on the dispersion stability was detected, at least in the investigated range. SEM micrographs of lyophilized samples evidenced the presence of a dispersion of particles of comparable size embedded in a homogeneous matrix.
By taking into account these difficulties, a new procedure for

Figure 2 - Picture of two nanoparticle suspensions loaded with α-interferon (left) and myoglobin (right) after two months storage

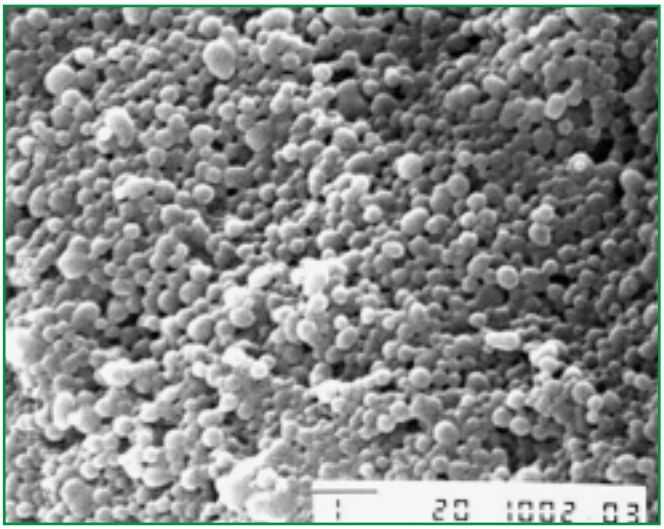

Figure 4 - SEM micrograph (10000 X) of a centrifuged nanoparticle dispersion containing 5% of functionalized βCD

NPs preparation by *co-precipitation* was set-up. This technique does not imply the use of chlorinated solvents and of a vigorous shear mixing, which are both known to cause appreciable protein denaturation [69, 70].

The co-precipitation technique was based on the dropwise addition of a solution of the synthetic polymer in a solvent mixture to an aqueous protein solution under gentle magnetic stirring (Figure 1). The progressive interaction between the water insoluble polymer and the protein gave rise to NPs formation. The glycolipid was then added as an aqueous dispersion to the resulting suspension. No sedimentation was observed after several weeks of storage at room temperature (Figure 2).

Protein concentration, polymer/lipid ratio, and type of solvent used to dissolve the polymer were varied in order to establish the best experimental conditions. The best results, as far as dispersion homogeneity and stability are concerned, were obtained when using the *n*-butyl hemiester (PAM14) of the maleic anhydride copolymer and a 10:1:2 polymer/lipid/protein weight ratio.

Dimensional analysis of the different suspensions indicated that the nanoparticles had an average diameter of 150 nm and a polydispersity index of 0.1-0.3 (Figure 3). The use of β-cyclodextrins functionalized with the glycidyl ether of di*iso*-propylidenribitol (GDR-βCD) as a dispersion stabilizer caused a slight increase of the average size to 200-300 nm.

SEM micrographs of the lyophilized nanoparticle suspensions evidenced a rather homogeneous distribution of spheroidal particles having diameter lower than 1 μm embedded in a continuous matrix. The NPs suspensions were then purified by three centrifugations at 8000 rpm and resuspension in bidistilled water. Homogeneous distributions of almost spherical NPs completely free from the embedding polymer matrix (Figure 4) were obtained from samples prepared by using 5% of functionalized βCD. Reconstitution of the original dispersion was very easily attained by suspending the pellet either in water or in phosphate buffer solution.

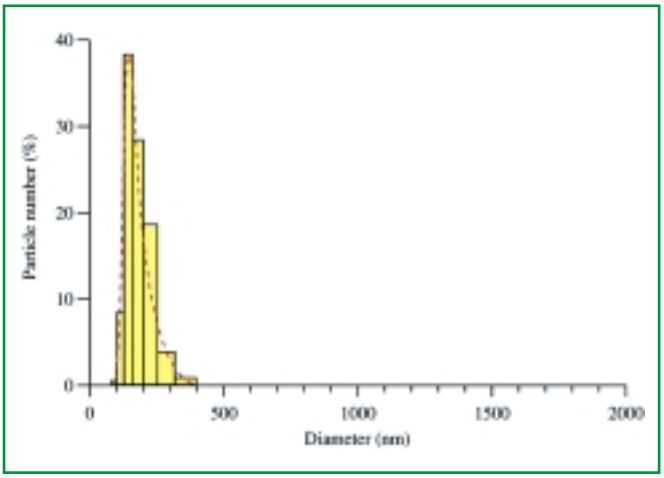

Figure 3 - Typical size distribution of the prepared nanoparticles

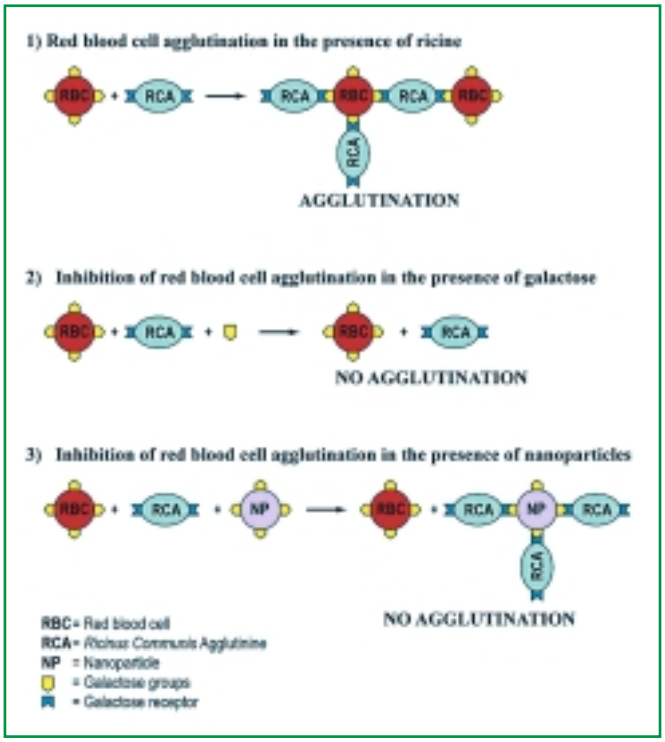

Figure 5 - Schematic representation of the hemoagglutination test

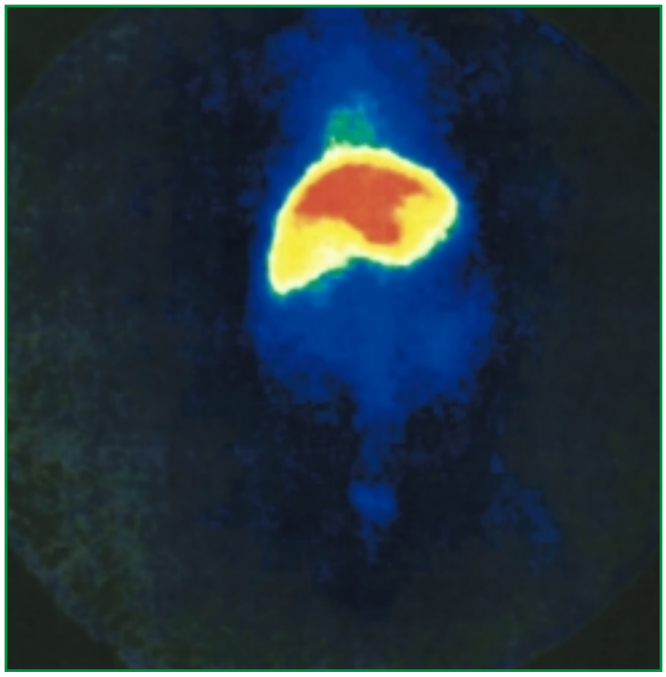

Figure 6 - Gamma camera image of the biodistribution of radiolabelled nanoparticles in rabbit

Bioactivity and targeting efficacy of the prepared nanoparticles

The presence of exposed galactosyl residues on the NPs surface was evidenced by *in vitro* haemagglutination inhibition test (Figure 5). This test is based on the agglutination of blood red cells induced by ricine, a lectin from *Ricinus communis* characterized by a strong affinity towards galactose [71]. Galactosyl groups present on the surface of centrifuged NPs dispersions containing DGDG effectively inhibited the haemagglutination process by competitively interacting with ricine receptors.

In order to test the ability of galactose-labeled NPs to actively target hepatocytes, some preliminary experiments were carried out by flow cytofluorimetry (FACS). Experiments were performed on rat hepatocyte primary cultures by using NPs containing fluoresceinated human serum albumin (HSA-FITC). The best results were obtained for suspensions containing a combination of DGDG and HDGDG.

Preliminary information gained on the *in vivo* biodistribution of radiolabelled nanoparticles recorded on rabbit nicely confirmed the *in vitro* test carried out on a primary cell line of rat hepatocytes [72]. Indeed, almost all radioactivity was concentrated in the rabbit liver (Figure 6) [73].

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