Albumin nanocarriers, γ - irradiated crosslinked, combined with therapeutic drugs for cancer therapy

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Abstract

Background: The use of biodegradable polymeric nanoparticles (NPs) for controlled drug delivery has shown significant therapeutic potential. Concurrently, targeted delivery technologies are becoming increasingly important as a scientific area of research. They have been the platform of choice for nanoparticle-based cancer drug delivery applications over the past decade, but extensive research has revealed some limitations of these drug delivery carriers. This new class of gamma irradiated-nanoparticles, aimed at combining the advantages of proteins like albumin, and gamma irradiation techniques that leaves a clean, innocuous and biodegradable NP. *Methods*: This review introduces biophysical (size, Z potential, microscopy) and biochemical (drug release, cytotoxicity) methods used to synthesize and characterize albumin NPs, discussing what influences in the final diameter size as well as the strategies used to treat cancerous cell-lines with NPs decorated with folic acid or similar compounds. *Results*: A novel way to design albumin – based nanoparticles by radiation synthesis is presented, alongside with the evaluation of the most efficient protocol to target the aforementioned nanoparticle with folic acid as a ligand. The utility of such system is discussed by preliminary tests and literature on the matter. *Conclusion*: These protein nanoparticles made of serum albumin possess several characteristics making them highly suitable for drug delivery. Nevertheless, as discussed in these review, there are some challenges that must be overcome to realize the full potential of albumin NPs in vitro and pre-clinical trials.

Keywords: albumin, nanoparticles, radiation induced crosslinking, cancer cell-lines, therapeutic-drugs, desolvation.

1. INTRODUCTION

Research and applications in the field of nanotechnology has soared up in recent years. The advantages, especially in medicine, seem to grow optimistically. The use of nanotechnology in medicine, also called nanomedicine, is based on the preparation of nanostructures, such as nanoparticles (NPs), which have particular physicochemical characteristics able to be easily detected. In addition they can load therapeutic drugs in the same entity, combining therapeutic and diagnostic functions. Targeting these nanostructures to specific tumors tissues will improve their pharmacokinetics, pharmacodynamics and enhanced intracellular activation. Nanoparticles are made of biological material like lipids, phospholipids,

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proteins, lactic acid among others, or can be engineered as carbon, silica, polymers and metals aggregates [1].

There are huge consensuses, based on biological results, that a particle diameter with a maximum size of 500 nm is a crucial parameter to be internalized by a cell [2, 3]. Many authors consider the zeta potential as the second relevant parameter in the preparation of NPs. This parameter is valuable to characterize NP coating with low-molecular weight molecules or synthetic polymers. However, considering biological applications, this parameter it not enough to predict the stability of NPs in a complex media.

The chosen size of the NPs used in anti-cancer therapy varies between 10-100 nm. An advantage of the use of NPs in such therapies is that the tumor vasculature has higher permeation for macromolecules. In addition to the poor functionality of lymphatic system in the surround media, NPs accumulate in tumors leading to phenomenon known as 'Effect of enhanced permeability and retention' also called EPR [4, 5].

The surface of the NPs has a pivotal role in the final destination within the body. Its role relays on the interaction between the NP and the local environment [6]. Also, it has been recently recognized that the surface of the NPs and their interaction with serum proteins is very complex [7]. NP decoration by covalent attachment of targeting ligand gives rise to specific interactions with target cells. This functionality will allow the NP to enter the cell via receptor-mediated endocytosis. However, there are still non-specific interactions through other proteins of the media which strongly reduce the NP enrichment in the target.

One of the most successful strategies to overcome the strong interaction between NP and unwanted serum proteins is the coating of the NP with an inert polymer. One of the most used polymers for this purpose is polyethylene glycol (PEG) [8-12]. Depending on the density of the PEG coating the NP, the system can be captured by several organs in mouse such as, liver, spleen, kidney, lung and heart. This phenomenon establishes a relationship between the physicochemical properties of the NPs and their pharmacokinetics and biodistribution profile [13].

Albumin, as many globular proteins, has a very short stability range. Small changes in the temperature, pH or solvent denature this very precise structure. Also these modifications have been used to prepare albumin aggregates in the pharmaceutic industry. However, these aggregations have not kept the functional activity of the native structure. There are some exceptions such as the use of cold ethanol to recovery native albumin from plasma.

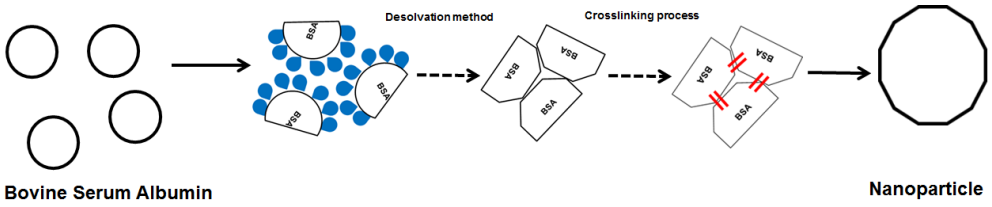

FIGURE 1: General approximation of the NP experimental designed and obtention process.

Our laboratory has reported the preparation of albumin – based NPs stabilized by radiation-induced crosslinking [14]. During this process, the potential of ionizing radiation for generating nanostructures in a simple and straightforward manner has been demonstrated. This review aims at the discussion of different albumin NP assemblies, the factors affecting the final structure and possible applications.

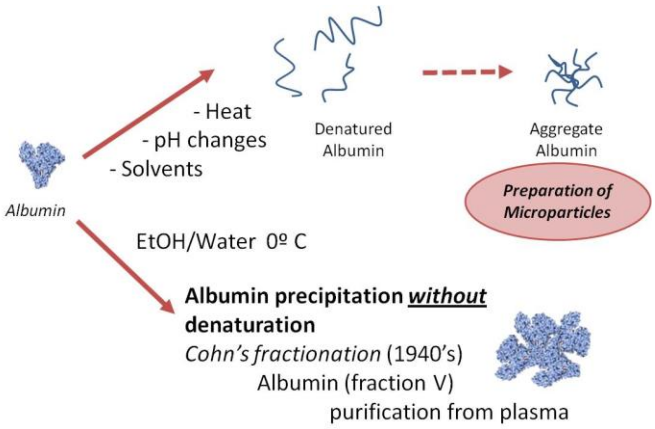

FIGURE 2. Experimental details of NP obtention process.

1. THERAPEUTIC NANOCARRIERS IN CLINICAL

Nowadays several therapeutic nanocarriers have been approved for clinical use but few protein based unless a combination of lipids and proteins. However, there are only a few clinically approved nanocarriers that delivery selectively molecules targeting cancer cells [15]. Even though there is a lot of research nowadays and all of them pulls out the very important advantages of all of the anticancer drug carriers, there are only a few of them that have been approved by the FDA. Roughly, up to date there are only two polymer-protein conjugates, around five liposomal formulations and one polymeric nanoparticle available in the market. This should be contrasted with the sixteen FDA approved monoclonal antibodies. Nonetheless, researchers continue the efforts in this field and right now, in consideration there are several polymer-protein and polymer-drug conjugates, many other liposomal and immunoliposomes formulations, and other polymeric micelles and last but not least some nanoparticles [16].

The first nanoparticles used for delivery of cancer chemotherapy were the liposomal nanocarriers. These nanoparticles were under 100 nm in size and consisted of several lipid bilayer containing

hydrophobic drugs as well as a water core that could host hydrophilic drugs. Nowadays, almost all of the clinically approved formulations are liposome-based and those includ Doxil and Myocet [17]. Doxorubicin was the first efficiently entrapped drug into a liposome-designed drug delivery system. It was FDA approved, based on its *in vivo* efficiency by means of its pharmacokinetics and biodistribution. Doxorubicin has cardiotoxic secondary effects. Even though, the Doxorubicin–liposome system still presents cardiotoxicity, its improved drug concentration found in tumors, together with an increased ovarian and breast antitumor activity, is prioritized. Afterwards, other clinically approved polymeric nanoparticles includde Adagen, Genexol PM, Eligard and Copaxone [17].

Combined Drug Delivery Systems (DDS)			
Polymers	***Lipids***	***Therapeutic Drugs***	***Cancer Model***
PLGA	DSPE-PEG	Doxorubicin, combretastatin A4	Melanoma and Lewis lung carcinoma (B16F10 and Lewis lung carcinoma cells)
HPESO (hydrolyzed polymer of epoxidized acid 30 : 70 w/w soybean oil)	Tristearin–stearic	Doxorubicin, Elacridar (GG918)	Multi-drug resistant breast cancer (MDA435/LCC6/ MDR1 cells) breast cancer (EMT6/WT cells)
HPESO	Myristic acid	Doxorubicin, mitomycin C	Multi-drug resistant breast cancer (MDA-MB 435/ LCC6/MDR1 cells)
PLGA	Soybean lecithin, DSPE-PEG	2′-Deoxy-5-azacytidine (DAC), doxorubicin	Breast cancer (MDA-MB-231 cells)
PLA-PEG-PLA	PC, cholesterol, DSPE-PEG	TGF-βreceptor-I inhibitor (SB505124), IL-2	Melanoma (B16-F10 cells)

TABLE 1. Combined Therapeutic Drug Delivery Agents.

Another effective chemotherapeutic agent, Paclitaxel has been delivered with different nano-delivery systems with various advantages over the standard-of-care therapy. One of properties to be highlighted in this case is the enhanced aqueous solubility of Paclitaxel when bioconjugated with water-soluble polymers, or encapsulated into lipid-based nanoparticles. Nowadays, there are several nanoparticles with size within a nanometer to several hundred nanometers in diameter, which favors the delivery of Paclitaxel into the tumor site with enhanced permeability and retention (EPR) effect. Alongside, due to their size they are not recognized by the reticuloendothelial system (RES) in healthy tissue reducing Paclitaxel side effects [18, 19].

Polymers	Lipids	Diagnostic & theranostic-Drugs	Cancer Model	References
PLGA	Lecithin, DSPE-PEG	Iron oxide nanoparticles, camptothecin	Breast cancer (MT2 cells)	20
PLGA	DPPC, DSPE-PEG	Gold nanocrystals and paclitaxel in polymer core; sorafenib and Cy7 NIR dye in lipid shell	Colon cancer (LS174 T cells)	21
PLGA	Lecithin, DSPE-PEG	Doxorubicin, Indocyanin-green NIR dye	Basal cell carcinoma (BCC cells)	22
PLGA	Soybean lecithin	Gold nanocrystals, quantum dots	N/A	23
BSA	PEG	Paclitaxel	Breast Cancer	24-27

TABLE 2. Diagnostic and theranostic Drug Delivery agents.

Paclitaxel-loaded cationic liposome formulations developed by MediGene (EndoTAG-1), had the potential to treat cancer. EndoTAG-1 consisted of N-(1-(2,3-dioleoyloxy)propyl)-N,N,Ntrimethyl-ammonium methylsulfate (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), which bound to negatively charged endothelial cells in tumor blood vessels. Trials in a prostate cancer mouse model, the mice treated with EndoTAG-1 had significantly reduced tumor volumes. [28].

2. ALBUMIN NANOPARTICLES

Human serum albumin (HSA) has a half-life of 19 days, and can bind reversibly hydrophobic drugs and then blood transport them, releasing the drugs at the cell surface [29]. Also, it is actively transported into tumors via a selective over-expression of a 60 kDa glycoprotein (gp60) receptor (albondin) [30]. Examples for HSA designed nanoparticles are those of diameter in the range of 100 nm and were prepared by the dropwise addition of acetone to an aqueous HSA solution at pH values between 7 and 9 and chemically crosslinked with glutaraldehyde [31]. A further improved method was carried out by using an aqueous desolvation process with ethanol as cosolvent [32, 33].

The highlight of albumin – based nanoparticle for the pharmaceutical industry was the conjugation of albumin with bound-Paclitaxel (PX NPs, Abraxane^{TM}) and was FDA approved in 2005 for the treatment of metastatic breast cancer. This was first applied to patients who have failed other chemotherapy

treatments or have relapsed. Seven years after (2012), the FDA approved Abraxane^{TM} to treat one of the most common lung cancers, the non-small cell lung cancer (NSCLC) [21].

The albumin-bound PX NPs have an average particle size of about 130 nm. Since this NP formulation completely eliminates Cremophor-EL and ethanol in Taxol, it is administered in a shorter period of 30 min with no need for premedication. For more details about Abraxane^{TM} product, please refer to the review articles [34-37].

Interestingly, targeted Abraxane formulations with two peptides of CREKA and LyP-1 (CGNKRTRGC), respectively, were developed: LyP-1-Abraxane demonstrated significantly improved antitumor efficacy compared to untargeted Abraxane in an MDAMB-435 xenograft mouse model [38]. In addition to human albumin, Zhao *et al*.; 2010, prepared PX-loaded bovine serum albumin (BSA) NPs using a desolvation method, and subsequently coated NPs by folic acid for targeting [39]. The folate-decorated NPs exhibited high stability and desired surface properties which specifically targeted to human prostate cancer PC3 cells. In another study, a novel octyl-modified bovine serum albumin (OSA) was synthesized to improve the lipophilicity of albumin and facilitate to form PX-loaded coreshell nanomicelles. The OSA NPs had smaller particle size, higher drug entrapment efficiency, and greater stability compared to unmodified NPs [40].

3. PREPARATION & CHARACTERIZATION OF ALBUMIN-BASED NPs

As aforementioned, albumin is the most abundant protein in the mammalian plasma and it serves as a carrier of hydrophobic biological and synthetic molecules such as anticancer drugs. Thus, preparation of albumin – based NPs should improve the drug delivery properties of this natural carrier. Micro and nanoparticles based on proteins have been prepared since many years. Serum albumins of different origin as well as gelatin were used as the starting material for the preparation of different particulate products.

Albumin-based nanoparticles have been designed for a variety of uses; among them, the ones that stand out the most are nanovehicles in a drug delivery system. Such choice relays in the fact that albumin is a protein with biocompatibility, is biodegradable and non – antigenic as well as malleable [41].

Three main methods have been described for their preparations: emulsion formation, desolvation, or coacervation. Extensive work has been done in the past concerning systematic studies of different experimental variables such as protein concentration, emulsification time and stirring power, heat stabilization temperature to obtain particles of different sizes [32, 42]. Preparation of NPs by desolvation process derived from the coacervation method of microencapsulation.

All these methods involve a partial or total protein denaturation as consequence of the presence of oils, surfactants, high concentration of alcohols and/or heat treatment. Therefore it is not expected that these methods will preserve the highly structured surface of native globular proteins onto the surface of NPs.

Among the different obtention process, the most common one is desolvation completed by chemical crosslinking. Globular proteins are macromolecules which are very sensitive to the microenvironment. Polar organic solvents, mainly ethanol, are able to precipitate globular proteins from a solution without loss of their native tridimensional structures. This property has been applied to design one of the most successful processes in the industrial purification of proteins [43]. This ethanol aggregation process of BSA, under sub precipitating conditions, was used to prepare protein NPs [14]. Examples of this technique are: albumin nanoparticles prepared with acetone as a desolvation agent (DA), prior dilution in urea [44]; nanoparticle prepared by ethanol desolvation method and glutaraldehyde as chemical crosslinker [45, 46]. There are some works that use another crosslinker in order to decrease the toxicity of the nanoparticle [47]. Nevertheless, some authors such as Nguyen *et al*., 2013, choose only the desolvation method with ethanol continuously or intermittently added to prepare the albumin NPs [48]. There are other authors who prepared BSA nanoparticle by also varying parameters such as temperature and pH during the process. Galisteo-Gonzalez *et al*., 2014 prepared BSA nanoparticles by adjusting temperature and pH during the desolvation with ethanol, prior to chemical crosslinking with glutaraldehyde [49]. Nab technology is also a commonly used technique to produce this type of nanoparticles [50].

The desolvation method with a chemical crosslinker is a high efficiency method, though the use of the agent to stabilize the protein aggregates adds an unwanted toxicity to the nanoparticle that should be best avoided. An option was suggested by Soto Espinoza *et al*., 2012, where the chemical agent was replaced by gamma irradiation induced crosslinking. In this specific method, the desolvation method with ethanol is followed by a gamma-irradiation step in order to prepare the nanoparticle: as a result the aggregate formed is solely protein in its composition [7, 14, 51].

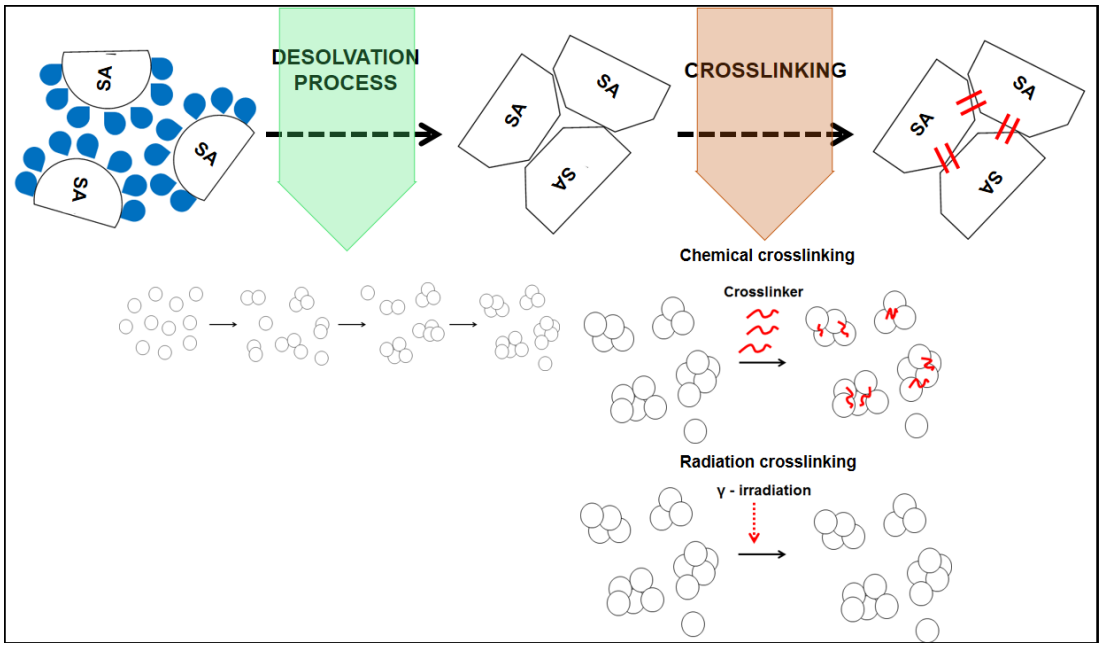

FIGURE 3: Process of BSA nanoparticle formation by the desolvation method and subsequent crosslinking.

Furthermore, it involves a conjugation of several effects where the most important are the dynamic aggregation of globular proteins by a cosolvent addition; and a crosslinking process induced by solvent radicals generated by the irradiation with gamma rays and accelerated electrons, according to Achilli, *et al*., 2015 [7]. Tailoring size of the protein NPs can be reached by changing the cosolvent proportion in the irradiated sample. The overall protein in the NPs retains a high percentage of the native structure according to the spectroscopic studies [14].

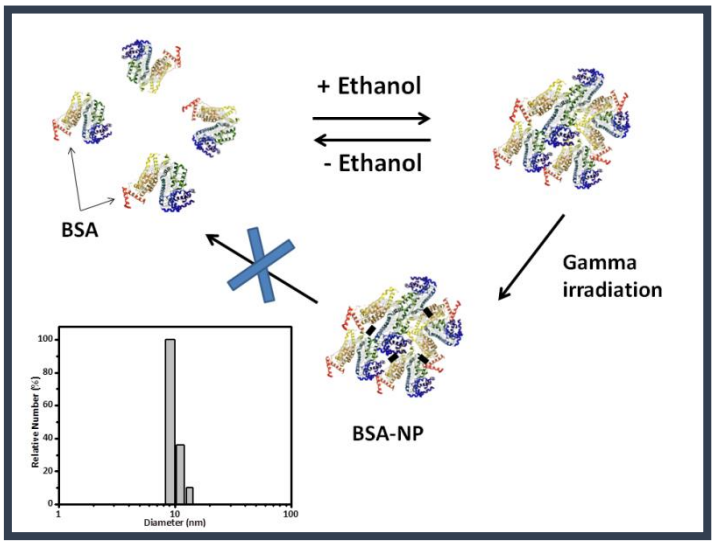

FIGURE 4. Scheme of γ- irradiation for NPs obtention and relative number of NP events with main peak between 8 to 10 nm; with author's permission [14].

4. NANOPARTICLE SIZE AND SURFACE CHARGE

In every technique mentioned, the particle size can be found in a range of diameters between 30 – 400 nm. The size can be modified by changing some parameters such as ethanol concentration [7]; protein concentration [44]; ionic strength concentration [45]; and pH value [45, 48] as well as temperature according to Galisteo-Gonzalez *et al*., 2014 [49]. Some of the parameters can alter in a deeper way the size of the nanoparticles than others during the desolvation process. The latter can be separated into different stages: super saturation, nucleation, condensation and coagulation [49]. Protein concentration is a parameter that highly affects the final output; as the concentration increases, so does the viscosity of the colloidal suspension. This causes a more intermittent protein transport between water and ethanol, which summing up with the aforementioned, causes the nanoparticles to be of a larger size due to lower nucleation. Also, having high protein concentration means having more of the smaller nuclei. But because of the high super saturation caused by the high protein concentration, agglomeration turns out to be a faster event resulting in large size NPs [49]. BSA NPs prepared with a 1 % solution of BSA were considerably smaller than those prepared with a 5 % BSA solution c.a. 61.53 nm and 211 nm at 34 °C [44]; 75 nm and 155 nm, approximately when prepared with 10 and 100 mg/ml BSA solution [49].

Another parameter that affects the size of the NP is the solution pH value. This was observed by Jun *et al*., 2011 where the size of the NP with increased pH (125 to 713 nm at pH 7.0 and 9.0, respectively) [45]. Contrary to this, Nguyen *et al*., 2013 observed a decrement in the diameter of the NP as the pH increased (300 to 100 nm at pH 6.0 and 9.0, respectively) [48]. The pH influence in the size of the NP relays on the charge acquired by the protein at a certain pH. When in pH higher than 5, the BSA will have a negative charge. As the pH value increases, so does the net charge of the protein causing the mobility to increase and, as a consequence, will not favor the coagulation into NPs, resulting in smaller ones [49]. Higher temperatures also cause an increase of the nanoparticle size as the super saturation degree is less favored: fewer nucleuses will be formed, but they will tend to have a larger size. Example of this can is observed in Shankar *et al*., 2016, where the BSA nanoparticle size varied from 128 – 49 nm, when the temperature decreased from 45 to 35 °C [44].

Ethanol concentration seems to have an effect on BSA nanoparticle size in Nguyen *et al*., 2013, when they measured the achieved nanoparticles at different stages of the preparation method [48]. Moreover, Soto Espinoza *et al*., 2012, found differences among the BSA nanoparticles; as the ethanol concentration increased from 10 to 40 % v/v, so did the diameter size of the nanoparticle [14].

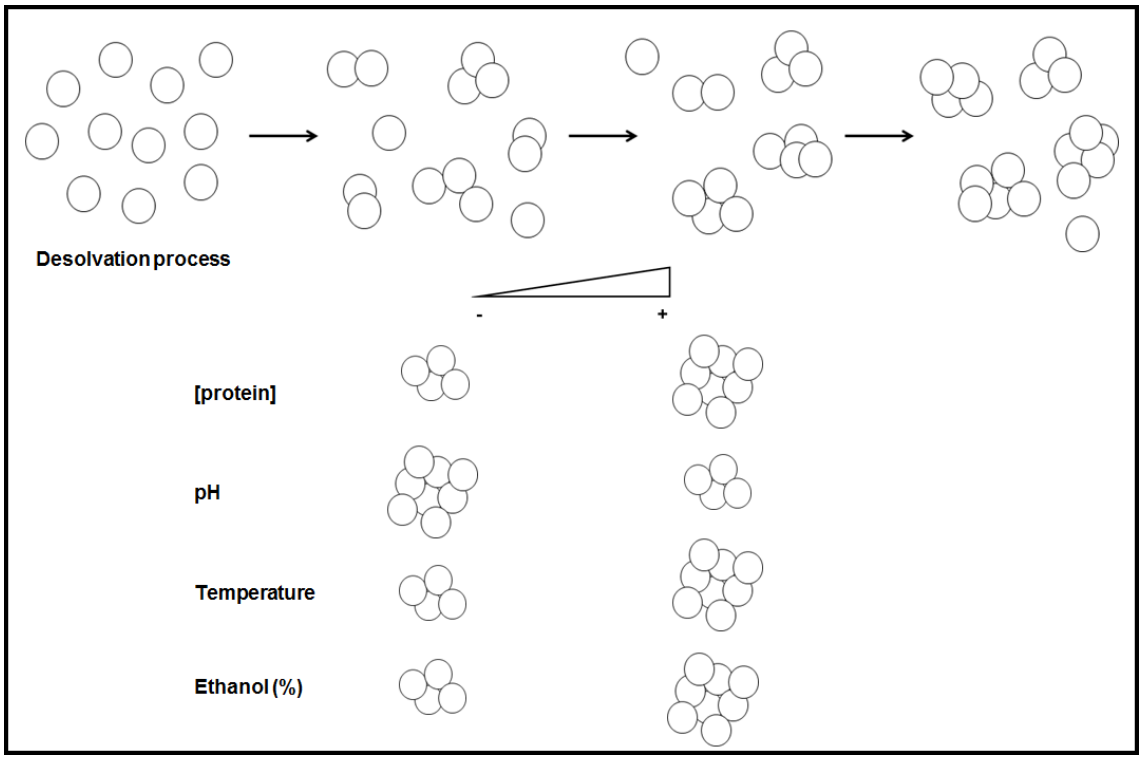

FIGURE 5: Four steps of the desolvation process is separated in. How the different factors influence on the process, resulting in different sizes.

Alongside the nanoparticle size, the Z potential value of each result within the preparation method must be tested. The result of this last experiment predicts the potential stability of the nanoparticle: if the module of the Z potential is large enough, then the stability of the sample will be high, otherwise possible flocculation of the nanoparticles is to be expected [52]. As it is, NP here described varies their Z potential value between – 27 and – 50 mV at pH 7.0. When in doubt of long term stability of the protein nanoparticle, lyophilization under specific conditions (with aid of trehalose as a cryoprotectant agent) could be applied [51, 53]. The Z potential seems to be affected by numerous parameters as described in several studies. For Jun *et al*., 2011, as the pH decreased from 9.0 to 7.0, the Z potential value increased; for Shankar *et al*., 2016, as the temperature increased so did ZP varying from -50 to -10 mV (1-4 % BSA) and 10 – 30 mV (5 % BSA) [44, 45].

5. NANOPARTICLE IMAGE MORPHOLOGY ANALYSIS

When testing the shape of every BSA nanoparticle aforementioned by microscopy techniques, the results were alike: all of them presented a spherical shape and smooth surface with defined edges. These experiments are mainly the most important ones while assessing the existence of the nanoparticle: in each case the nanoparticle yield of each method varied between 80 – 95 %, proving to be efficient methods. The variety of techniques results on the same spherical shape, denoting no difference in this aspect.

Despite this, consideration on the toxicity and function must be determined before deciding about a specific design of the nanovehicle [44, 47, 50].

6. NANOPARTICLE-CELL INTERACTION

Nanoparticles have been long designed for medical proposes. As it is, the BSA NP aforementioned was designed for the sole purpose of a nanovehicle in drug delivery system. The rigidity of the BSA NP's structure, should allow improving the BSA NP capacity to protect the drug from degradation or unspecific release. Because of the high number of albumin receptors present in tumor cells, BSA NP is very adequate to interact with these tissues. It is important to study first the behavior of each system in a situation that mimic the environment the nanoparticle will be after administration.

Accordingly, experiments to test the toxicity on cells in vitro is the first step taken by most authors, after testing the binding with the desired drug. Among the variety of cytotoxic experiments, the measurement for cell metabolic activity is the most popular. The choice of the cells to be used varies on the final aim of the study. Kim *et al*., 2016 managed to cobound Paclitaxel and curcumin in the BSA nanoparticle, resulting in different cell death percentage according to the concentration of the nanoparticle, ranging from 20 – 90 % cell death as concentration increased [50]. After a 48 h treatment with albumin NPs loaded with GNF-5837, Shargh *et al*., 2016 found enhanced cell mortality compared to the GNF – 5837 in free form. This work also showed no cell toxicity on behalf of the albumin NP alone [54]. Zhao *et al*., 2015 achieved a BSA nanoparticle by emulsification with cholesterol to bind Tacrolimus, a macrolide immunosuppressant [55]. By encapsulating this drug to the nanoparticle, a lower clearance was observed as well as, more favorable pharmacokinetics than Prograf®. Moreover, this system showed potentiality as a drug delivery system to reduce Tacolimus nephrotoxicity function [55].

Some of the authors aforementioned chose to use bovine serum albumin as a trial, before going for human albumin. This is done in light of lower costs and higher malleability. On their part, He *et al*., 2015, managed to achieve a human serum albumin nanoparticle to encapsulate Teniposide in order to enhance anti – tumor activity of the drug [56]. They observed a decrease in metastasis and increase in cellular uptake when the drug was bound to the nanoparticle, as well as, strong cell inhibition and less unspecific toxicity effect in the rest of the organism [56]. Other authors encapsulated Tacrolimus to combat rheumatoid arthritis in nab – technology prepared albumin NPs [55]. They observed not only highly inhibitory effect on the progression of the collagen – induced arthritis than the oral or intravenous administration of the drug, but also improved water solubility of hydrophobic drug together with a release profile suitable for injection.

As seen here, the albumin, specifically BSA has potentiality to act as a nanovehicle in a drug delivery system when forming a stable protein aggregate. The greatest use was given by Moreno–Aspitia *et al*., 2015 with the system Albumin-Paclitaxel (ABI – 007, Abraxane^{TM}) to fight against breast cancer, where the nanoparticle was prepared by the nab technology [57].

7. NANOPARTICLES DECORATION WITH LIGANDS FOR ENHANCED SPECIFICITY

The evident advantage with SA NPs is the high quantity of functional groups exposed to the NP surface. According to Lee *et al*., 2009, no further modifications should be needed in order to enhance the NP functionality. For instance, sulfhydryl groups are sometimes attached in order to selectively target the chosen nanoparticle with thiol-selective reagents [58]. Not only that, but the BSA NP could be decorated with proteins such as T4 lysozyme, Her 2 and LRR domain of internalin by the cysteine residue while retaining its other characteristics, as done with some gold nanoparticles [59]. Cancer treatment has developed into two different ways; (i) the use of molecules to act as agents blocking protein expression pathways, or agents that are overexpressed in malignant cells, and (ii) the use of ligand which are compatible with an overexpressed receptor in a malignant cell [60]. The possibilities of ligands are such that they could decide the function of the NP; for imaging, drug delivery or any other use, providing a more amicable NP to work with than the synthetic manufactured [58].

The decoration with folic acid is normally done by activation of the ligand followed by incubation with the molecule to be decorated (Figure 6)

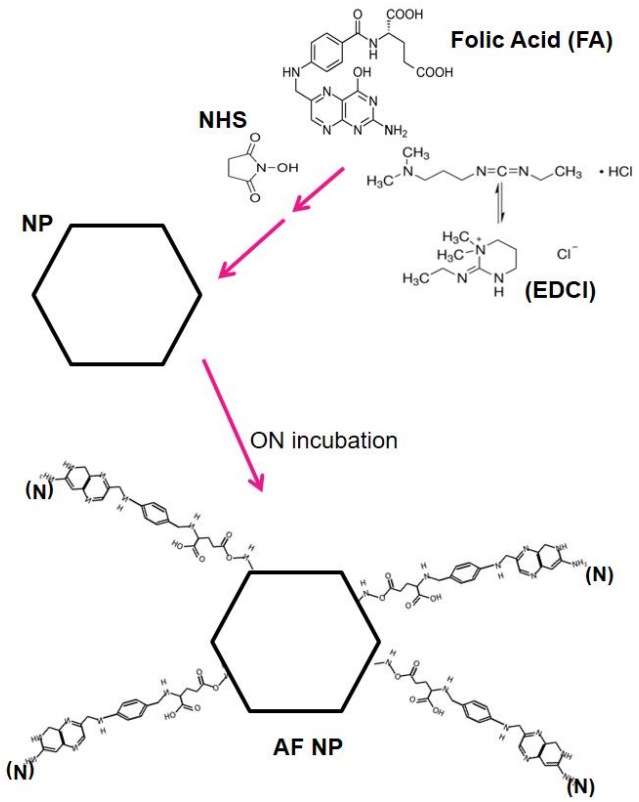

FIGURE 6: Scheme of how the Folic Acid is attached to the NP by previous activation with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and N-Hydroxysuccinimide (NHS). Briefly, folic acid is incubated in darkness, under constant stirring while a solution of EDIC is added dropwise together with a solution of NHS. After 4 hours incubation, the solution is added to the nanoparticle solution and kept overnight at room temperature and constant stirring. The process ends with the separation of the free folic acid to the one attached to the nanoparticle and quantification of the ligand adsorbed to the nanoparticle's surface [61].

There those who prefer to decorate the NP once formed. In our study we propose the decoration process following two protocols; decorated the already irradiated crosslinked BSA with FA (BSA NP), and to decorate BSA molecules with folic acid (FA) and then radiation crosslinking applied to the samples for the production of the BSA NP (Figure 7.1).

After each procedure the hydrodynamic diameter was measured on each sample by dynamic light scattering (Figure 7.2). Results showed an increase in the diameter of the NP as FA was adsorbed to the surface; this was a confirmation of the existence of a new kind of NP. While the increase in the diameter size BSA NP decorated after gamma irradiation (FA-BSA NPir) was not a marked one (BSA NP has a diameter size of 71.53 ± 6.66 nm and FA-BSA NPir has a diameter size of 102.90 ± 25.21), the diameter size in the BSA NP decorated before gamma irradiation (FA-BSA NP) increased considerably (540.20 ± 119.60). Not only it increased in size, but also its polydispersity did. BSA NP size population is

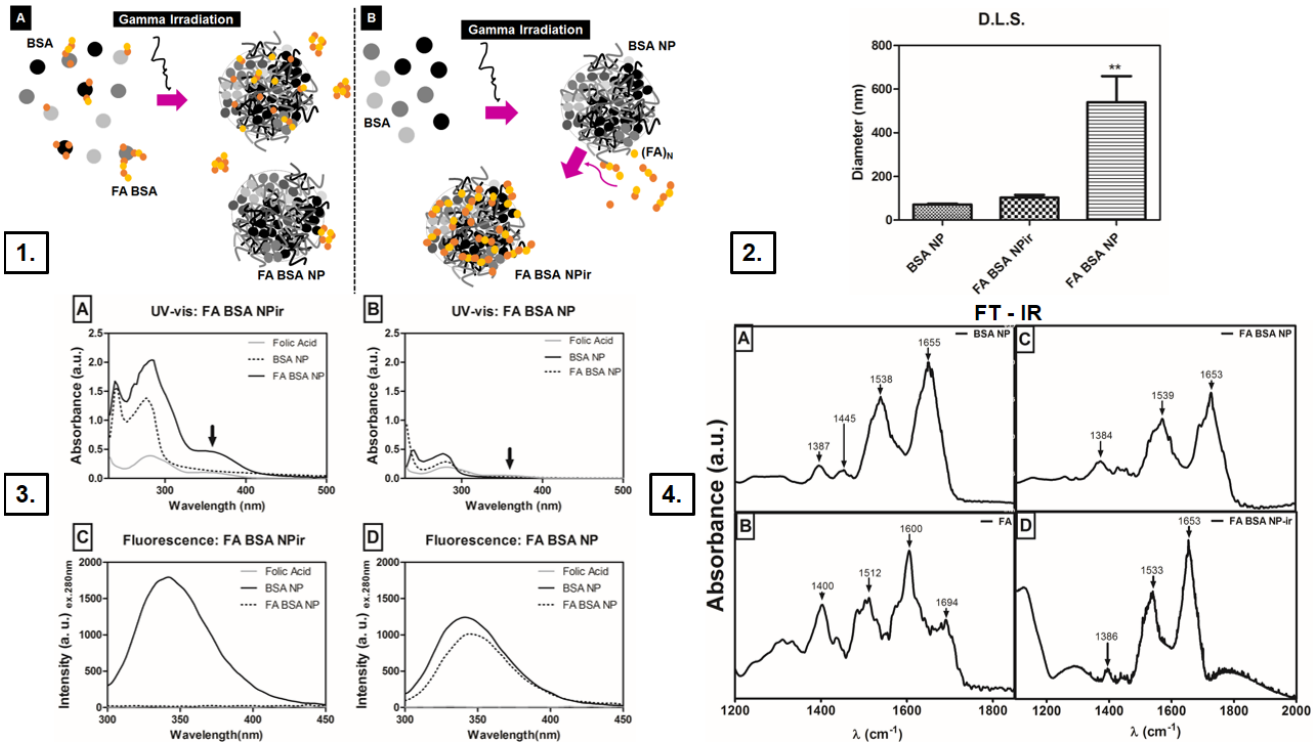

FIGURE 7: Biophysical characterization of the folic albumin – based nanoparticle (FA – BSA NP), prepared following both protocols. (1) Preparation diagram; (2) D.L.S. experiment with table of molecular ratio of [FA]/[BSA NP] of each method; (3) UV – vis and Fluorescence spectroscopy experiments on both FA–BSA NP;

rather monodispersed, whereas the decorated samples seemed to be composed by more than one size population. This may be due to the process it involves obtaining the FA-BSA NP. FA may not me adsorbed to every NP surface creating enhanced polydispersity among samples. Moreover, FA tends to form polymers during the activation step, which may then be the large molecules adsorbed to the NP surface increasing therefore the hydrodynamic diameter of the NP.

When the BSA molecules are decorated before irradiating, not all of them will end up with a linked FA molecule. Alongside, the FA-BSA solution is mixed with a BSA solution before the irradiation process; hence, the polydispersity is increased in the outcome of the whole process as the suspension could be composed by: NP with solely FA-BSA molecules in its composition, NP with solely BSA molecules or with NP with different percentages of FA-BSA and BSA molecules.

The number of FA molecules are adsorbed per NP can be estimated by a UV – vis experiment as follows: the concentration of FA was obtained by measuring the absorbance of the sample at 393 nm. The [FA]/ [NP] ratio was calculated by the NP concentration, knowing that the BSA NP molecular weight is 4.5 MDa (35 nm hydrodynamic radio)[51, 61] (Table 3).

Sample	[FA]/[NP]
BSA NP	0
FA BSA NPir	7.76 e5
FA BSA NP	1.37 e4

TABLE 3. Diagnostic and theranostic Drug Delivery agents.

The molecular weight of the NP serves as basis for the amount of BSA molecules conforming the NP, which give a total of 6000 NH exposed to the surface. As FA is present in a polymeric form in solution, considering that these polymers are of 10 units long, only 1/6 of the total amount of FA polymeric is expected to be adsorbed to the NP. Therefore, is not surprising the large quantities of the ligand associated with the NP.

The first thing we observed in the absorbance spectra of the FA-BSA NPir and FA-BSA NP that differentiate them from the absorbance profile of BSA NP, was the appearance of a shoulder at 300 nm that corresponds to the FA spectra (Figure 7.3 a and b). As samples were centrifuged to discard the excess of the FA, we assumed the shoulder represents the FA adsorbed to the surface of the BSA NP. It is important to highlight this result as it is a confirmation on the assumption over the existence of surface functional groups in the BSA NP.

While quantifying the amount of FA per BSA NP we observed more of the ligand present in the FA-BSA NPir than in the FA-BSA NP (Figure 7.3). Our hypothesis is that during the irradiation process some FA polymers might be broken as well as some FA molecules might be hidden inside the formed NP resulting in less FA molecules per BSA NP detected.

In order to elucidate if the structure of the BSA NP was affected by FA a fluorescence study was carried out. The emission spectra obtained showed that while FA-BSA NP only presented a slight decrease in the Trp emission of the BSA NP (Figure 7.3 c and b), FA-BSA NPir lost its capability to emit fluorescence signal (Figure 7.3 c). This meant that the BSA NP is heavily affected by the presence of FA molecules if they are added after the NP is formed; the FA polymers might act as an umbrella for the Trp, that means shading the primary binding site of the NP; not only preventing the Trp to emit fluorescence signal, but also preventing the Trp to interact with other substances. In other words, if the BSA NP is decorated with FA after the irradiation process, its function might be compromised. Contrary to this, FA-BSA NP showed a decrease in the fluorescence emission. Therefore, one might assume that the Trp might not be entirely covered or only a percentage out of the total of Trp are, but still the NP's function might not be entirely compromised (Figure 7.3d).

To a better understanding the interaction between the BSA NP and FA, a FT-IR spectroscopy experiment was carried out. For this particular analysis, we focused our attention into comparing the main signal maxima the BSA NP spectra showed and how this changed when the FA was present (Figure 7.4).

In both samples, FA-BSA NPir (Figure 7.4 c) and FA-BSA NP (Figure 7.4 d), when the FA was present, the α-helix signal experienced a frequency displacement from 1655 – 1653 cm^{-1}; this displacement to lower frequency waves mean less freedom of movement, assuming then a more compact structure as this maxima represents the global structure of the BSA NP. The β-sheet signal, another important maxima for the NP structure, shows a displacement to higher frequency waves in the FA-BSA NPir and to lower ones for FA-BSA NP; from 1538 cm^{-1} (BSA NP), to 1539 cm^{-1} (FA-BSA NPir) and 1533 cm^{-1} (FA-BSA NP). While in the BSA NP decorated after irradiation the FA did not do a major alteration in its β-sheet composition, in the BSA NP decorated before irradiation the FA molecules seem to have organized the β-sheet present in the NP in a more compact way.

With these findings we propose that the interaction with the FA is given by the –NH present in the mentioned structures. When added to the sample before irradiation, the FA molecules arrange themselves in a way that makes the β-sheet structure more compact, which may also mean that these molecules are also involved in the spatial arrangement of BSA molecules forming the NP. On the contrary, when added after the NP is formed, the FA molecules are only interacting with the –NH exposed to the surface, altering in a minor way the β-sheet structure arrangement but not the global structure of the NP (Figure 7.4).

Once we proved the existence of surface functional groups in the NP by the addition of FA molecules, we proceed to test its functionality as a drug delivery system carrier by studying its binding parameters as regards its interaction with the antitumoral drug Emodin (Emd). This was carried out with a fluorescence quenching experiment of the Trp fluorescence emission due to its interaction with Emd. As FA-BSA NPir did not have a fluorescence emission spectra (Figure 7.3 c), we calculate the binding constant; maxima loaded drug (B_{mx}) and disassociation constant (K_d) with an absorbance quenching spectra (Figure 8 a).

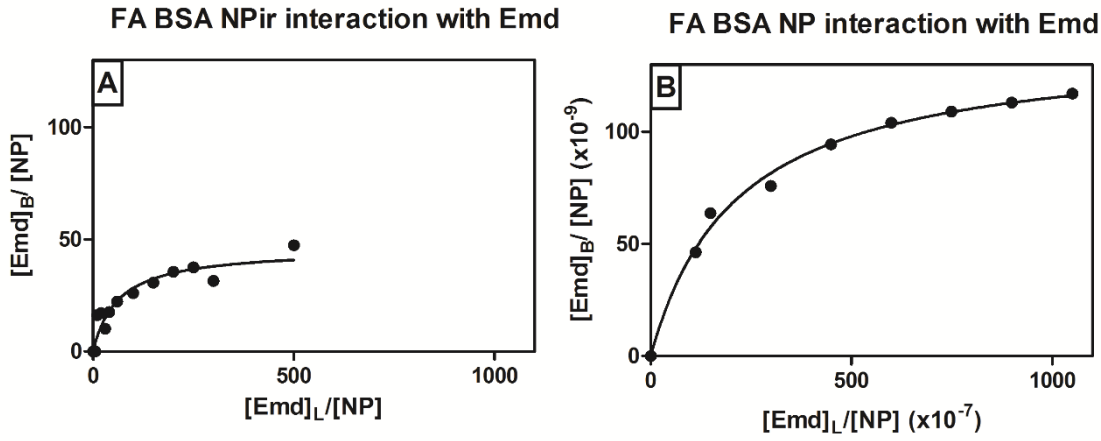

FIGURE 8: Fluorescence interaction experiment of both FA – BSA NP (a) irradiated NP with FA attached to it and (b) FA attached to BSA prior irradiation NP.

Parameters	NPirEmd	NPEmd
B_{mx}	47.71 ± 4.65	0.14 e-06 ± 0.004 e-06
K_d	61.55 ± 19.00	21.4 e-06 ± 2 e-06
R^2	0.90	0.99

TABLE 4: Binding parameters obtained from Figure 7 of the NP interaction with the therapeutic drug Emd.

Results showed a high K_d for FA-BSA NPir, meaning that the association constant would be a small one; the affinity for the drug is very low. In contrast, FA-BSA NP showed a low value for K_d; the affinity for the drug is high (Figure 8, Table 4). In previous studies, we reported the K_d for the interaction between Emd and BSA NP to be 1.43 e-04; FA-BSA NP showed a higher affinity for the drug as its K_d is lower than that reported by Siri *et al*., (2.14 e-05) [51]. It might mean that the reorganization into a more compact structure of the NP given by the FA decoration strengthens the affinity for the drug. This was also observed in previous studies, where lyophilization would enhance the affinity the BSA NP had for a theranostic drug (Merocianine 540) [51]. Referring to FA-BSA NPir, its K_d is increased compared to the one given by BSA NP, we assume that having the FA molecules added after the gamma irradiation process cause functionality loss. This might be by the FA polymerization and therefore blocking of the binding pocket entrance for other substances assumed while studying the fluorescence emission spectra (Figure 7.3 c). Hence, the BSA NP decorated with FA before irradiation does not lose the capability of drug loading (main function for this drug vehicle), but if the BSA NP is decorated with FA after irradiation, that function is lost. Therefore, we propose FA-BSA NP as a suitable vehicle to be tested as a drug delivery system, dismissing FA-BSA NPir as a suitable drug delivery system.

As FA-BSA NP was chosen to continue the study, a cytotoxicity test in MCF-7 cell line was carried out in order to study its potential effect on cell viability. After 4 hours of incubation no major cell metabolic activity alteration was observed for the cells treated with the bioconjugate; the decrease was that of the 10 % (Figure 8 a). It was slightly less toxic than that caused by Emd and BSA NPEmd treated cells, where the decrease on its metabolic activity was 20 %. Nevertheless, 24 hours after incubation a cytotoxic effect for the cells treated with the bioconjugated was observed (Figure 8 b). This decrease in the cell metabolic activity was similar to that generated in cells treated with Emd and BSA NPEmd. after 48 hours of treatment, cell metabolic activity for FA-BSA NPEmd treated cells was of around 50 %, this meant that the bioconjugated did not lose cytotoxic effect compared to the one caused by the BSA NPEmd in cells.

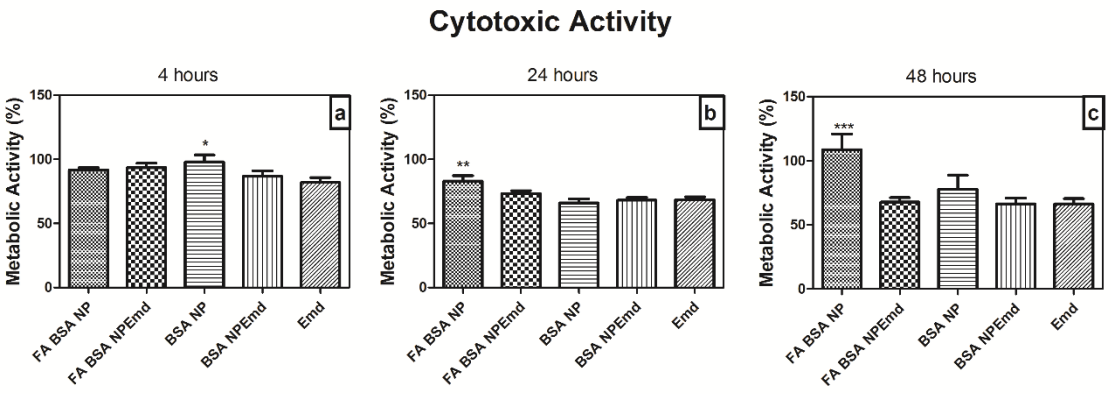

FIGURE 9: Cytotoxicity experiment using MCF – 7 cancer human breast cell lines with the chosen FA – BSA NP. The experiment was done in triplicates with an n = 8 in each repetition. Two way ANOVA $P<0.05$ (*). Results were analyzed against free drug (Emd).

Other studies showed BSA NP decorated with folate was designed for photodynamic therapy use. In this study it was shown a markedly improved phototoxicity in the mitochondria of cancerous cells, as well as highly improved tumor targeting and prolonged circulation time [62]. Especially, the designed nanoparticle showed preference for human cancer breast cells, known to have over expressed receptors for folic acid. Alam *et al*., 2015 designed a folate albumin nanoparticle in order to reduce the unwanted toxic effect Cisplatin had while being used in chemotherapy [63]. The NP in this work showed enhanced cellular uptake in cancerous cell which have over expressed receptors for this ligands. By histology experiments, they also observed a decreased in the unwanted toxicity of the drug while bound in the folic acid BSA NP [63]. Other group of authors managed to bind Gemcitabine to an albumin folate nanoparticle to fight against a wide range of solid tumors. Once again, the drug delivery system proved to be efficient for cells over expressing the targeted ligand, as well as, proving to have toxic effect on tumors [64].

8. SUMMARY AND FUTURE PERSPECTIVES

This review, focused on clinically used and in clinical trials NPs for cancer treatment, taking in account that nowadays, there is an ample varied number of nanoparticles being developed for cancer in different clinical and/or preclinical stages.

Therefore the future is promising based on that there is there is a great quantity of nanoparticles currently under characterization and in vitro and in vivo studies. It is well known that few of these will progress into preclinical studies and even less will reach the final clinical stage and approved. But, on the other hand, the great importance is that throughout the research world today, very many researchers are exploring the potential use of polymeric nanoparticles as carriers for a wide range of drugs for therapeutic applications. Technical methodologies and applications make this effort worthwhile and with great impact

in the near future. On top of this technical versatility collaborations and research networks ease the complete testing from design, in vitro, ex vivo and in vivo process. Also to take into considerations are newly discover polymeric materials. Because of the newly discovered and studied polymeric nanocarriers, their versatility and wide range of properties, they are increasingly being designed and tested as novel drug delivery systems. Particularly, this polymeric nanocarriers hold a great potentiality and promise in the near future to cover up areas of cancer therapy not envisioned and also if immune response is the primary response for controlled delivery of vaccines.

One of the most successful developments is the carrier mediated paclitaxel that has already shown significant efficacy in taxane resistant cancers, for prostate cancer. Other modifications include those with transferrin and folate receptors as targeted drug delivery molecules, presently under study. This branch of nanotechnology provides many exciting therapeutic approaches for targeted high concentration drug delivery to cancer cells, reducing damage of normal cells.

We have also highlighted the importance of FA-coated nanoparticles. The coupling of ligands on the nanoparticles need to be designed and studied for optimum circulation times, binding and tumor cell uptake. It is an important fact that targeting ligands lead to macrophage recognition and faster clearance compared to the non-targeted nanoparticles. In the future, nanoparticles design should introduce cleavable masking of the ligands till reaching the tumor cells. An initial study shows the enhance specificity of the NP giving by the attachment of FA in this present review. Experimental evidence in cancer nanotherapeutics are being implemented to solve several limitations of conventional drug delivery systems, like the efficiency of this FA coated NP which has been demonstrated and are on the horizon as the next generation of nanoparticles, facilitating personalized and tailored cancer treatment. More studies are required on the clearance mechanism, the metabolism and excretion of NPs and their components. NPs combined with therapeutic agents for cancer therapy have evolved rapidly during the last decades and it is expected that more will become in clinical practice.

LIST OF ABBREVIATIONS

Nanoparticle (NP)

Nanoparticles (NPs)

Serum Albumin (SA)

Polyethylene glycol (PEG)

Human serum albumin (HSA)

Bovine serum albumin (BSA)

Octyl-modified bovine serum albumin (OSA)

Folic acid (FA)

Emodin (Emd)

ACKNOWLEDGEMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Quilmes, Ministerio de Ciencia y Tecnología (MINCyT), Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Nuclear Atomic Energy Agency (IAEA) CRP codes F220064 and F23028. Dr.Roberto Candal and Lic. Juan Montes de Oca (INQUIMAE, FCEyN, UBA, Argentina) are gratefully acknowledged for DLS data obtention in preliminary experiments.

REFERENCES

[1] De Jong WH and Borm PJA. Drug delivery and nanoparticles: applications and hazards. *International J of Nanomed* 2008; *3*(2): 133.

[2] Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae- mediated endocytosis. *Biochem J* 2004; 377: 159–169.

[3] May RC, Machesky LM. Phagocytosis and the actin cytoskeleton. *J Cell Sci* 2001; 114(6): 1061–1077.

[4] Dvorak HF, Nagy JA, Dvorak JT and Dvorak A. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol* 1988*;* 133: 95-109.

[5] Nagy JA, Dvorak AM and Dvorak HF. Vascular Hyperpermeability, Angiogenesis, and Stroma Generation. *Cold Spring Harb Perspect Med* 2012; 2(2):a006544.

[6] Saptarshi S, Duschi A and Lopata AL. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. *Journal of Nanobiotech 2013;* 11:26.

[7] Achilli E, Casajus G, Siri M, *et al.* Preparation of protein nanoparticle by dynamic aggregation and ionizing-induced crosslinking. *Colloids Surf A Physicochem Eng Asp* 2015; 486: 161–171.

[8] Cho K, Wang XU, Nie S, Z. Chen, D.M. Shin DM. Therapeutic nanoparticles for drug delivery in cancer. *Clin Cancer Res* 2008; 14: 1310–1316.

[9] Shenoy DB, Amiji MM. Poly (ethylene oxide)-modified poly (e-caprolactone) nanoparticles for targeted delivery of tamoxifen in breast cancer. *Int J Pharm* 2005; 293(1): 261–270.

[10] Nobs L, Buchegger F, Gurny R, Allémann E. Poly(lactic acid) nanoparticles labeled with biologically active Neutravidin for active targeting. *Eur J Pharm Biopharm* 2004; 58(3): 483–490.

[11] Nobs L, Buchegger F, Gurny R, Allémann E. Current methods for attaching targeting ligands to liposomes and nanoparticles. *J Pharm Sci* 2004; 93(8): 1980–1992.

[12] Béduneau A, Saulnier P, and Benoit JP. Active targeting of brain tumors using nanocarriers. *Biomaterials* 2007; 28(33): 4947-4967.

[13] Liu Y, Hu Y and Huang L. Influence of polyethylene glycol density and surface lipid on pharmacokinetics and biodistribution of lipid-calcium-phosphate nanoparticles. *Biomaterials* 2014; 35(9): 3027–3034.

[14] Soto Espinoza SL, Sánchez ML, Risso V, Smolko EE and Grasselli M. Radiation synthesis of seroAlbumin nanoparticles. *Radiation Physics and Chemistry* 2012; 81(9): 1417-1421.

[15] Peer D, Karp JM, Hong S, *et al.* Nanocarriers as an emerging platform for cancer therapy. *Nature Nanotechnology* 2007; 2: 751-760.

[16] Pérez-Herrero E, Fernández-Medarde A. Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy. *Eur J of pharm and biopharm* 2015; 93: 52-79.

[17] Zhang L, Gu FX, Chan JM, *et al.* Nanoparticles in medicine: therapeutic applications and developments. *Clin Pharmacol Ther* 2008; 83(5): 761–769.

[18] Krishnamurthy S, Vaiyapuri R, Zhang L and Chan JM. Lipid-coated polymeric nanoparticles for cáncer drug delivery. *Biomater Sci* 2015; 3: 923-936.

[19] Ma P and Mumper RJ. Paclitaxel nano-delivery systems: a comprehensive review. *J of nanomed & nanotech* 2013; *4*(2): 1000164.

[20] Shah N, Chaudhari K, Dantuluri P, Murthy RS, Das S. Paclitaxel-loaded PLGA nanoparticles surface modified with transferrin and Pluronic® P85, an *in vitro* cell line and *in vivo* biodistribution studies on rat model. *J Drug Target* 2009; 17(7): 533–542.

[21] Green MR, Manikhas GM, Orlov S, *et al*. Abraxane®, a novel Cremophor®-free, albumin-bound particle from of paclitaxel for the treatment of advanced non-small-cell lung cancer. *Annals of Oncology* 2006; 17(8): 1263-1268.

[22] Zhang X, Jackson JK, Burt HM. Development of amphiphilic diblock copolymers as micellar carriers of Taxol. *International J of Pharm* 1996; 132(1): 195–206.

[23] Jin C, Bai L, Yang J, *et al*. Paclitaxel-loaded poly (D, L-lactide-co-glycolide) nanoparticles for radiotherapy in hypoxic human tumor cells *in vitro*. *Cancer Biology & Therapy* 2008; 7(6): 911–916.

[24] Desai N, Trieu V, Yang A, *et al*. Enhanced efficacy and safety of nanoparticle albumin-bound nab-docetaxel versus taxotere. *Cancer Research* 2006; 66(8 Supplement): 1277-1278.

[25] Desai N, Trieu V, Yao Z, *et al*. Increased antitumor activity, intratumor paclitaxel concentrations, and endothelial cell transport of Cremophor-free, albumin-bound paclitaxel, ABI-007, compared with cremophor-based paclitaxel. *Clinical Cancer Research* 2006; 12(4): 1317-24.

[26] Trieu V, Damascelli B, Soon-Shiong P and Desai N. SPARC expression in head and neck cancer correlates with tumor response to nanoparticle albumin-bound paclitaxel (nab-paclitaxel, ABI-007, Abraxane). *Cancer Research* 2006; 66(8 Supplement): 1050-1051.

[27] Trieu V, Hwang J and Desai N. Nanoparticle albumin-bound (nab) technology may enhance antitumor activity via targeting of SPARC protein. *New targets and delivery system for cancer diagnosis and treatment (SKCC)*, 2007; San Diego, Abstract 53.

[28] Bode C, Trojan L, Weiss C, *et al*. Paclitaxel encapsulated in cationic liposomes: a new option for neovascular targeting for the treatment of prostate cancer. *Oncology Rep* 2009; 22(2): 321–326.

[29] Paal K, Müller J, Hegedûs L. High affinity binding of paclitaxel to human serum albumin. *Eur J of Biochemistry* 2001; 268(7): 2187–2191.

[30] Vogel SM, Minshall RD, Pilipovic M, Tiruppathi C, Malik AB. Albumin uptake and transcytosis in endothelial cells *in vivo* induced by albumin-binding protein. *American J of Physiology-Lung Cellular and Molecular Physiology* 2001; 281(6): 1512-1522.

[31] Lin W, Coombes A, Davies M, Davis S and Illum L. Preparation of sub-100 nm human serum albumin nanospheres using a pH-coacervation method. *J of Drug Targ* 1993: 1(3): 237-243.

[32] Müller BG, Leuenberger H and Kissel T. Albumin nanospheres as carrier for passive drug targeting: an optimized manufacturing technique. *Pharmaceutical Research* 1996; 13(1): 32-37.

[33] Langer K, Balthasar S, Vogel V, *et al*. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. *International J of Pharm* 2003; 257(1): 169-180.

[34] Petrelli F, Borgonovo K, Barni S. Targeted delivery for breast cancer therapy: the history of nanoparticle-albumin-bound paclitaxel. *Expert opinion on Pharmacother* 2010; 11(8): 1413–1432.

[35] Hawkins MJ, Soon-Shiong P, Desai N. Protein nanoparticles as drug carriers in clinical medicine. *Adv drug delivery Rev* 2008; 60(8): 876–885.

[36] Kratz F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. *J of Controlled Release* 2008; 132(3): 171–183.

[37] Stinchcombe TE. Nanoparticle albumin-bound paclitaxel: a novel Cremphor-EL®-free formulation of paclitaxel. *Nanomed* 2007; 2(4): 415–423.

[38] Karmali PP, Kotamraju VR, Kastantin M, *et al*. Targeting of albumin-embedded paclitaxel nanoparticles to tumors. *Nanomedicine: Nanoth Biol and Medicine* 2009; 5(1): 73–82.

[39] Zhao D, Zhao X, Zu Y, *et al*. Preparation, characterization, and *in vitro* targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. *Int J Nanomedicine* 2010; 5(1): 669–677.

[40] Gong J, Huo M, Zhou J, *et al*. Synthesis, characterization, drug-loading capacity and safety of novel octyl modified serum albumin micelles. *Int. J. Pharmaceutics* 2009; 376(1): 161–168.

[41] Elzoghby AO, Samy WM, Elgindy NA. Albumin-based nanoparticles as potencial controlled release drug delivery systems. *J of Controlled Release* 2012; 157(2): 168-182.

[42] Gallo JM, Hung CT and Perrier DG. Analysis of albumin microsphere preparation. *International J of Pharm* 1984; 22(1): 63-74.

[43] Cohn EJ, Hughes WL and Weare JH. Preparation and properties of Serum and Plasma Proteins. XIII. Crystallization of Serum Albumin from Ethanol-Water Mixtures 1a, b. *J American Chem Soc* 1947; 69(7): 1753-1761.

[44] Shankar KR, Ameta RK, Singh M. Preparation of BSA nanoparticles using aqueous urea at T=308.15, 313.15 and 318.15 K as a function of temperature. *J of Molecular Liquids* 2016; 216: 808-813.

[45] Jun JY, Nguyen HH, Chun HS, Kang BC and Ko S. Preparation of size-controlled bovine serum albumin (BSA) nanoparticles by a modified desolvation method. *Food Chemistry* 2011; 127(4): 1892-1898.

[46] Yedomon B, Fessi H, Charcosset C. Preparation of Bovine Serum Albumin (BSA) nanoparticles by desolvation using a membrane contactor: A new tool for large scale production. *European J of Pharmaceutics and Biopharm* 2013; 85(3): 398-405.

[47] Cui W, Wang A, Zhao J, *et al*. Layer by layer assembly of albumin nanoparticles with selective recognition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Journal of Colloid and Interface Science* 2016; 465: 11–17.

[48] Nguyen HH, Ryu J, Che JH, *et al*. Robust size control of bovine serum albumin (BSA) nanoparticles by intermittent addition of a desolvating agent and the particle formation mechanism. *Food Chemistry* 2013; 141: 695–701.

[49] Galisteo-González F and Molina-Bolívar JA. Systematic study on the preparation of BSA nanoparticles. *Colloids and Surfaces B: Biointerfaces* 2014; 123: 286-292.

[50] Kim B., Lee C., *et al*. Paclitaxel and curcumin co-bound albumin nanoparticles having antitumor potential to pancreatic cancer. *Asian Journal of Pharmaceutical Sciences* 2016; 11: 708–714.

[51] Siri M, Grasselli M, Alonso, SDV. Albumin-based nanoparticle trehalose lyophilisation stress-down to preserve structure/function and enhanced binding. *J of pharm and biomedical analysis* 2016, *126*: 66-74.

[52] Salis A, Bostrom M, Medda L, *et al*. Measurements and Theoretical Interpretation of Points of Zero Charge/Potential of BSA Protein. *Langmuir* 2011; 27: 11597–11604.

[53] Costantino HR, Firouzabadian L, Wu C, *et al*. Protein spray freeze drying. 2. Effect of formulation variables on particle size and stability. *J of pharm sci* 2002; *91*(2): 388-395.

[54] Shargh VH, Hondermarck H, Liang M. Albumin hybrid nanoparticles loaded with tyrosine kinase A inhibitor GNF-5837 for targeted inhibition of breast cancer cell growth and invasion. *International J of Pharm* 2016; *515*(1): 527-534.

[55] Zhao L, Zhou Y, Gao Y, *et al*. Bovine serum albumin nanoparticles for delivery of tacrolimus to reduce its kidney uptake and functional nephrotoxicity. *International J of pharm* 2015; *483*(1): 180-187.

[56] He X, Xiang N, Zhang J, *et al*. Encapsulation of teniposide into albumin nanoparticles with greatly lowered toxicity and enhanced antitumor activity. *International J of pharm* 2015; *487*(1): 250-259.

[57] Moreno-Aspitia A and Perez EA. Nanoparticle albumin-bound paclitaxel (ABI-007): a newer taxane alternative in breast cancer. *Future Medicine* 2005; 755-762.

[58] Lee JE, Lee N, Kim J, *et al*. Uniform Mesoporous Dye-Doped Silica Nanoparticles Decorated with Multiple Magnetite Nanocrystals for Simultaneous Enhanced Magnetic Resonance Imaging, Fluorescence Imaging, and Drug Delivery. *J Am Chem Soc* 2010; 132 (2): 552–557.

[59] Blum AS, Soto CM, Wilson CD, *et al*. Cowpea mosaic virus as a Scaffold for 3-D patterning of Gold Nanoparticles. *Nano Letters* 2004; 4(5): 867–870.

[60] Xia W and Low PS. Folate-Targeted Therapies for Cancer. *J Med Chem* 2009; 53(19): 6811–6824.

[61] Du C, Deng D, Shan L, *et al*. A pH-sensitive doxorubicin prodrug based on folate-conjugated BSA for tumor-targeted drug delivery. *Biomaterials* 2013; 34(12): 3087-3097.

[62] Battogtokh G and Ko YT. Mitochondrial-targeted photosensitizer-loaded folate-albumin nanoparticle for photodynamic therapy of cancer. *Nanomedicine: Nanotechnology, Biology, and Medicine* 2016.

[63] Alam N, Dubey RD, Kumar A, *et al*. Reduced toxicological manifestations of cisplatin following encapsulation in folate grafted albumin nanoparticles. *Life Sciences* 2015; 142: 76–85.