TRABAJO FIN DE GRADO

FINAL DEGREE PROJECT

VEHICULIZATION OF DRUGS WITH ANTIPROLIFERATIVE ACTIVITY IN PROTEIN NANOPARTICLES AS AN ALTERNATIVE TO PEGYLATION TO INCREASE THEIR CIRCULATION TIME IN BLOOD

AUTHOR: Sheyla María Rodríguez Méndez

TUTOR: Dr. Edgar Pérez Herrero

CO – TUTOR: Dr. José Manuel Padrón Carrillo

COLLEGE OF PHARMACY – UNIVERSIDAD DE LA LAGUNA

La presente memoria de investigación ha sido realizada por Dña. Sheyla María Rodríguez Méndez durante el curso académico 2020-2021 en las instalaciones del Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) y bajo la dirección de los Dres. Edgar Pérez Herrero y José M. Padrón Carrillo.

La Laguna, a junio de 2021.

1. INDEX

2. ABSTRACT

Nanoparticles are colloidal vehiculization systems that are increasingly used by the scientific community to achieve the transport of drugs towards specific targets, improving their bioavailability, blood circulation and cell internalization. In particular, PEGylation of nanoparticles is a method in which nanoparticles are coated with polyethylene-glycol, achieving better efficiency in drug administration and increasing blood circulation time, but also reducing immunogenicity. Among the different alternatives to PEGylation, it is remarkable the use of albumin for the synthesis/coating of nanoparticles since, apart from improving the tumour accumulation of the transporters by means of the enhanced permeability retention effect, the incorporation of this protein results in the inhibition of the non–specific adsorption of plasma proteins, decreasing opsonization processes.

In this work, albumin-based nanoparticles have been synthesized by the desolvation method, using glutaraldehyde as crosslinker. These nanoparticles presented an average size of 131.55±2.62nm, a low polydispersity index ranged between 0.043-0.085 and a negative surface charge ranged between -30 and -48 mV. The particles were loaded with colchicine as a model cytotoxic drug that acts by binding to tubulin that prevent mitotic division, achieving encapsulation efficiencies between 50-60%.

Moreover, the colchicine-loaded nanoparticles were tested in HeLa and SW1573 cancer cells lines, performing clonogenic and antiproliferative assays. As conclusion, colchicine alone is more active than incorporated into nanoparticles, where it had a cytostatic activity. Thus, a concentration of 5 nM of colchicine is needed to inhibit growth by 50% and using nanoparticles a concentration of > 50 µM is required to achieve the same effect.

3. RESUMEN

Las nanopartículas, que son sistemas coloidales de vehiculización, están siendo cada vez más utilizadas por los científicos para lograr el transporte de fármacos hacia dianas específicas, mejorando su biodisponibilidad, circulación sanguínea e internalización celular. La PEGilación es un método en el que las nanopartículas se recubren con polietilenglicol, mejorando la eficiencia en la administración de fármacos, aumentando su tiempo de circulación y reduciendo su inmunogenicidad. Entre las alternativas a la PEGilación, destaca el uso de albúmina en la síntesis/recubrimiento de nanopartículas que, además de mejorar su acumulación en los tumores por el efecto-de-retención-y-permeabilidad-mejorada, permite la inhibición de la adsorción inespecífica de proteínas plasmáticas, lo que permite disminuir la opsonización.

En este trabajo se han sintetizado nanopartículas de albúmina por el método de desolvatación, utilizando glutaraldehido como agente reticulante. Éstas presentaron un tamaño medio de 131,55±2,62nm, un índice de polidispersión estrecho de 0,043-0,085 y una carga superficial negativa entre -30 y -48mV. Las partículas se cargaron con colchicina, utilizado como fármaco modelo al poseer actividad citotóxica debida a su unión a la tubulina que evita la división mitótica, logrando eficiencias de encapsulación del 50-60%.

Las nanopartículas cargadas con colchicina fueron evaluadas en líneas celulares cancerígenas (HeLa/SW1573), realizando ensayos clonogénicos/antiproliferativos que indicaron que la colchicina en solución es más activa que incluida en nanopartículas, donde mostraba actividad citostática. A pesar de que para inhibir el crecimiento celular en un 50% es necesario una concentración de 5 nM de una disolución de colchicina, en el caso de las nanopartículas cargadas, son necesarias concentraciones > 50 µM.

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

4. INTRODUCTION

Nanotechnology is a promising field increasingly used by the scientific community with many applications within different sectors, such as electronics, using quantum dots or carbon nanotubes to improve the conventional screens or using nanoscale materials to manufacture semiconductor nanowires, enviroment, allowing to design nanofilters for water treatment, textil sector, using nanoparticles to produce smart fabrics that are capable of remain intact, without spots or wrinkles or biomedicine, improving the diagnosis and treatment of many diaseases. In particular, in the area of biomedicine, nanotechnology provides new systems to transport molecules of interest towards specific targets and allowing to develop new formulations for vaccines, anticancer treatments or immunotherapy, improving their bioavailability, blood circulation and cell internalization, in such a way that it allows the development of new tools for the diagnosis and treatment of multiple diseases (Yu and Jin , 2016; Rusul et al, 2016; He et al, 2018; Bayda et al, 2019).

Nanotransporters are colloidal vehiculization nano-scale systems that, due to its size and properties, are perfect vehicles for introducing different molecules of interest into de body, being able to cross biological barriers, such as mucous membranes, increase its circulation time in blood and reach different organs, tissues or the therapeutic target to be treated (Mohanraj et al, 2006; Lühmann & Meinel 2016).

Nanocarriers can be classified mainly into polymer conjugates and particulate nanocarriers. Polymer conjugates are polymeric macromolecular structures linked to another agent by covalent bonds, resulting in polymer-drug or polymer-protein conjugates. These systems can provide more stability to the incorporated molecule, but not protection, and are not able to control the release of the molecule of interest. Instead, these properties can be achieved with particulate nanocarriers, where molecules are enclosed in the matrix of their structure, consisting mainly of lipids (liposomes) or polymers (micelles or nanoparticles) (Pérez-Herrero & Fernández-Medarde, 2015). Polymeric nanoparticles (NPs) provide higher loading capacity and a more controlled drug release than liposomes, in addition to a homogeneous size distribution accompanied by better

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

stability. Although they can be made by a wide range of materials, natural, synthetic or semi-synthetic, it is worth highlihting the use of polysaccharides, such as alginate or chitosan, polyamino acids or different proteins, like albumin (Pérez-Herrero & Fernández-Medarde, 2015).

One of the conventional methods to increase blood circulation time and reduce immunogenicity is pegylation, where NPs are coated with polyethylene glycol (PEG). The incorporation of PEG to the surface of the NPs allows to greatly reduce the binding with plasma proteins, decreasing opsonization and phagocytosis processes (Li and Huang, 2010; Karakoti et al, 2011; Suk et al, 2015).

Among the different alternatives to PEGylation, the use of albumin, a biodegradable and biocompatible protein with low immunogenicity, for the synthesis or coating of NPs stands out since, in addition to improving the tumor accumulation of transporters through the enhanced permeability retention (EPR) effect, the incorporation of this protein inhibits the non-specific adsorption of plasma proteins, reducing the opsonization processes after parenteral administration (Mariam J et al, 2016). In this regard, albumin NPs have the ability to bind to receptors that are overexpressed in cancer, like the Secreted Protein Acidic and Rich in Cysteine (SPARC) pathway, which ensures targeting without the use of other ligands, therefore, cell internalization is achieved by receptor-mediated endocytosis. When the binding of albumin with the receptor occurs, caveolin-1, an intracellular protein that causes invagination of the membrane, forms these vesicles that cause the albumin to be internalized in the tumor. All of this contributes to the accumulation of this type of transporters within the tumor (Spada et al, 2021). In addition, due to the physicochemical characteristics of albumin, that is the most abundant protein in plasma, its time in circulation is long being its half-life around 19 days.

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

Albumin-based nanoparticles can be prepared by different techniques, mainly emulsification or coacervation/desolvation methods. In the process of emulsification, NPs are formed by homogenizing an aqueous solution of albumin in an oily phase. Although this technique allows to achieve high encapsulation efficiencies, toxic active ingredients and high energy homogenization are required (Spada et al, 2021).
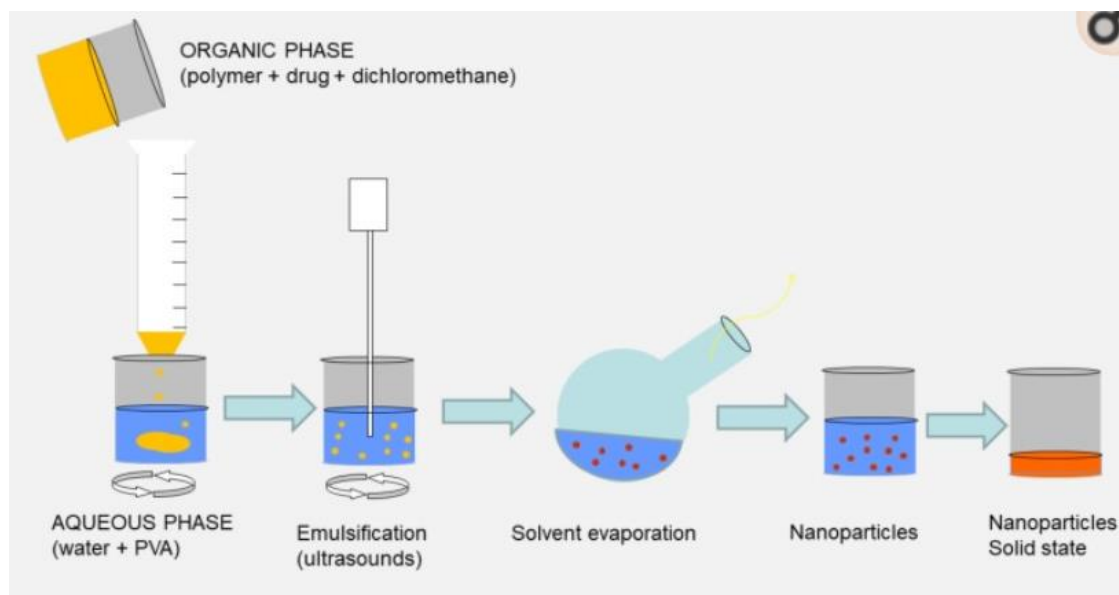

FIGURE 1. SCHEME OF THE PREPARATION OF PROTEIN NANOPARTICLES BY THE EMULSIFICATION METHOD.

Reproduced with permission from Umerska A, Gaucher C, Oyarzun-Ampuero F, Fries-Raeth I, Colin F, Villamizar-Sarmiento MG, Maincent P, Sapin-Minet A. Polymeric Nanoparticles for Increasing Oral Bioavailability of Curcumin. Antioxidants (Basel). 2018

With regard to the desolvation / coacervation method, a desolvating agent, like ethanol or acetone, is added by continuous dripping over an aqueous solution of albumin under continuous stirring, causing the albumin to desolvate, that is, the solubility of the protein in water is decreased, which lead to the spontaneous generation of NPs. Subsequently, a crosslinking agent is required, for example glutaraldehyde, to stabilize the NPs. It should be noted that this process does not require toxic organic solvents and is very simple and reproducible. (Karimi et al, 2016).
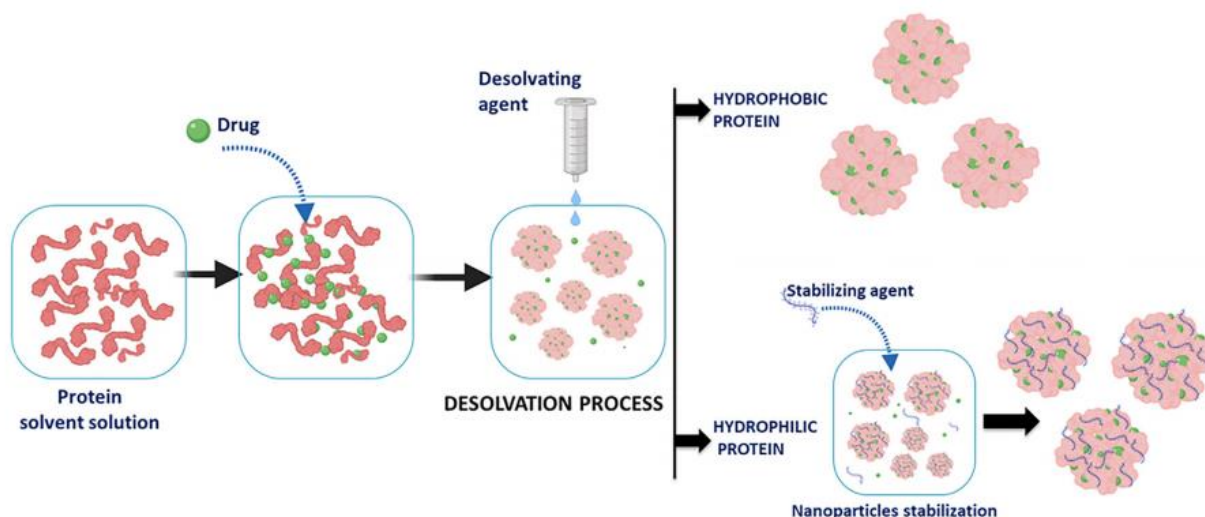
Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

FIGURE 2. SCHEME OF THE PREPARATION OF PROTEIN NANOPARTICLES BY DESOLVATION METHOD.

Reproduced with permission from Martínez-López AL, Pangua C, Reboredo C, Campión R, Morales-Gracia J, Irache JM. Protein-based nanoparticles for drug delivery purposes. Int J Pharm. 2020 May

Albumin nanoparticles have managed to encapsulate a diversity of drugs of diverse nature. In fact, Liu et al (2019) encapsulated dexamethasone in albumin-based nanoparticles to treatt lung injury. In addition, these nanoparticles were modified with the E – selectin binding peptide to direct the drug of more specific formula towards the vascular endothelial cells that are inflamed, although the accumulation rate after the study showed a lower accumulation with this peptide (52.2 %) than in the case of free drugs (88.2%) (Liu et al, 2019).However, in this final degree project, colchicine has been used as a low-cost model citotoxic drug since is capable of inhibiting tumor growth by actively binding to tubulin and blocking mitotic division.

5. OBJECTIVES

The main objective of this project is to synthesize and characterize colchicine-loaded albumin nanoparticles as an alternative to PEGylation of colchicine-loaded polymeric nanoparticles.

In particular, the specific objectives are:

1. Synthesis of colchicine-loaded albumin nanoparticles by the desolvation method, using glutaraldehyde as crosslinker.

2. Validation of an analytical method that allows determining the amount of encapsulated drug in the nanoparticles, that is, the encapsulation efficiency.

3. Characterization of the nanoparticles in terms of size, polydispersity and zeta potential (surface charge).

4. Biological evaluation of the synthesized nanoparticles on HeLa and SW1573 cell lines to determine their 50% growth inhibition (GI_{50}) and their capability to inhibit the formation of new colonies.

6. MATERIALS AND METHODS

Materials:

The products used to make the protein-based nanoparticles (NPs) were bovine serum albumin (BSA), colchicine and glutaraldehyde solution (Grade II – 25% in H_2O) (Sigma-Aldrich®) and absolute ethanol (Merck®). All the solutions were prepared using MilliQ water.

For cell lines experiments, the following products were used: medium RPMI – 1640 (HyClone Laboratories™), acetic acid (Prolabo®), methanol (Riedel – de Haën™) and Dulbecco's Phosphate Buffered Saline (PBS) and crystal violet (Sigma-Aldrich®).

Methods:

6.1 Synthesis of colchicine – loaded albumin nanoparticles

Colchicine-loaded albumin nanoparticles were prepared by the desolvation method, using glutaraldehyde as crosslinking agent. Briefly, 3 mL of the desolvating agent, ethanol, were added by means of a syringe pump (VARIOMAG® - MULTIPOINT) at a flow rate of 1 mL/min over 1mL of an albumin

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

solution of 10 mg/mL, which contains 3 mg of colchicine, under stirring, spontaneously generating a suspension of NPs.

After 15 min, and in order to stabilize the suspension, 13 µL of the crosslinking agent, glutaraldehyde (0,075% V/V), were added over the suspension of NPs under stirring (Kimura et al, 2018). The suspension of NPs was left under stirring overnight. The particles were isolated and purified by 3 cycles of centrifugation at 16 RCF for 25 min.
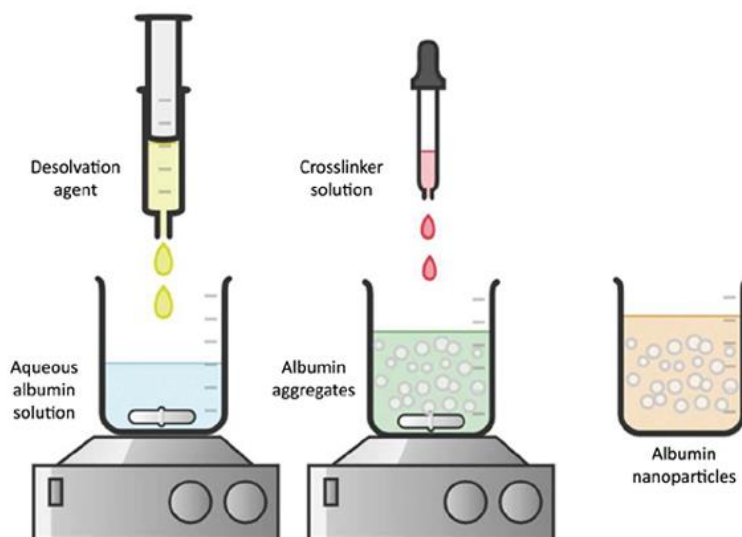

FIGURE 3. SCHEME OF THE PREPARATION OF ALBUMIN NANOPARTICLES BY DESOLVATION METHOD

Reproduced with permission from *Ozge Esim and Canan Hascicek, "Albumin-based Nanoparticles as Promising Drug Delivery Systems for Cancer Treatment", Current Pharmaceutical Analysis (2021) 17*

6.2 Nanoparticle characterization

Determination of particle size, polydispersion and surface charge:

The particle size and polidispersion (PDI) of the synthetized nanoparticles were measured using the Zetasizer NanoZS equipment (Malvern Instruments, UK) by the dynamic light scattering method (DLS), a non-destructive technique that is based on the fluctuation of the Brownian motion of the particles. A laser light beam passes through the sample and the analysis of their intensity fluctuations is correlated with the size of the particles, the smaller the size, the faster will be the movement, producing fluctuations of light more continuously (Manual ZetaSizer NanoZS, Malvern Instruments, UK).

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

The surface charge or zeta potential was measured with the same instrument (Zetasizer NanoZS, Marlvern Instruments, UK), using the electrophoretic light scattering (ELS) technique. To do this, the sample was introduced into a cell with two electrodes to which an electric field is applied and the particles with a net charge, or what is the same, net zeta potential, migrate towards the electrode of the opposite charge at a certain speed, called mobility and this is correlated with the zeta potential value (Manual ZetaSizer NanoZS, Malvern Instruments, UK).
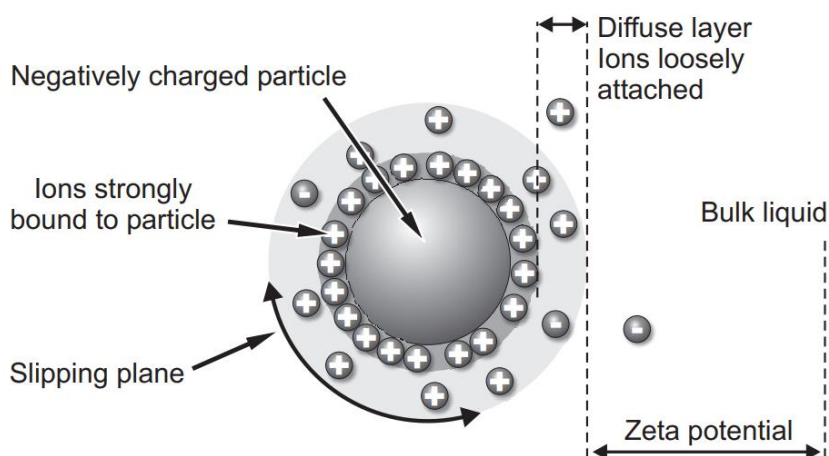

FIGURE 4. ZETA POTENTIAL SCHEME – REPRODUCED FROM ZETASIZER NANOZS MANUAL (MALVERN INSTRUMENTS, UK)

Determination of encapsulation efficiency:

In order to determine the amount of encapsulated drug in the synthesized nanoparticles (encapsulation efficiency - EE), a spectrophotometric method based on the measurement of the supernatant at 350 nm was developed.

The encapsulation efficiency was calculated using the following equation:

$$EE\ (\%) = \frac{(WT - WE)}{WT}\ x100$$

where WT is the total amount of drug (mg) that was added to the formulation and WE is the amount of free drug in the supernatant after centrifugation.

The spectrophotometric method was validated through the analysis of variance (ANOVA), a variability test and the F test, using Excel and RStudio software. To do that, 4 standard calibration curves composed of 7 reference

6.3 Biological activity

The biological tests were carried out in our group by PhD student Adrián Puerta under the supervision of Dr. José M. Padrón.

Cell and culture

HeLa and SW1573 were kindly provided by Prof. G. J. Peters (VUmc, Amsterdam). Cells were grown in RPMI 1640 medium containing 5% heat inactivated FCS, 2 mM L-glutamine, 100 U/mL of penicillin G and 0.1 mg/mL of streptomycin in a 37ºC, 5% CO_2 and 95% humidified air incubator. Cells were maintained in culture in 60 mm cell culture dishes with medium (10 mL) until 70-90% confluence was reached. Then, the medium was removed and cells were washed with sterile PBS without Ca and Mg (2 mL). After removal of PBS, a solution of trypsin/EDTA (1 mL) was added and the dishes were incubated for 2-3 minutes at 37ºC. Then, fresh medium (10 mL) was added to neutralize trypsin and collect the cells. Moxi Z was used to measure the concentration (cells/mL) of cell suspensions. Appropriate dilutions were made to inoculate the adequate amount of cells per well for nanoparticles exposure assays. Cell manipulation always took place in the culture chamber to keep sterilized conditions.

Antiproliferative test

Cell line suspensions of HeLa and SW1573 were seeded in 96 well plates (2500 cells/well). After 24 hours, nanoparticles containing Colchicine and Free Colchine dissolved in dimethyl sulfoxide (DMSO) were added. Compounds were tested in triplicates at different dilutions in the range of 0.01-100 µM. After 48 hours of incubation cells were precipitated with 25 µL ice-cold 50% (w/v) TCA and fixed for 60 min at 4ºC. Then, every well was rinsed with water and 25 µL of SRB solution (0.4% v/v in 1% of acetic acid) were added and stained for 15 min protected from light. SRB excess was removed and the wells were rinsed with 1% acetic acid. The remaining dye was diluted with 150 µL 10 mM Tris Base and

the optical density (OD) of each well was measured at 530 and 620 nm using BioTek absorbance microplate reader.

Clonogenic test

HeLa and SW1573 cells suspensions were seeded in a P6 well plate (500 cells/well). After 24 hours of incubation nanoparticles containing colchicine were added at 1 µM. DMSO was added as a control at the same concentration.

After 7 days of incubation, culture medium was removed and wells were rinsed with 1 mL of PBS for 5 min. Fixation was performed with 750 µL of methanol under stirring for 5 min. After removal of methanol, cells were rinsed again with PBS and 1% crystal violet was added for 10 min to stain the colonies. Cell colonies were counted using AutoCellSeg software, considering a colony as a group of 50 cells.

7. RESULTS AND DISCUSSION

7.1 Synthesis and characterization of colchicine – loaded albumin nanoparticles

In this work, six batches of albumin-based nanoparticles loaded with colchicine were synthetized in triplicate. The synthesis was based on the desolvation method, which has been widely used and optimized due to its simplicity and speed (Jun et al (2011); Yedomon et al., 2013).

As can be seen in *Table 1*, the particle size is ranged between 128.4 ± 3.44 and 135.6 ± 6.18 nm, being the average size, 131.55 ± 2.62 nm, adequate for intravenous administration, since for this type of administration a size between 100-200 nm is required to fit the size of capillaries, avoiding the formation of thrombi due to the possible aggregation of the NPs (Oropesa-Nuñez and Ulises-Javier, 2012). With regard to the polydispersity index (PDI), which indicates the distribution of sizes that exists in the sample, i.e. if the sample is homogeneous or not (Danaei et al, 2018), the values are ranged between 0.043 ± 0.011 and 0.085 ± 0.010, being all the values below 0.1, which means that samples are homogeneous in size. The highly negative values of the surface charge (zeta

potential), ranged between -30.1 ± 1.30 and -47.5 ± 1.84 mV, demonstrate the great stability of the nanoparticles in solution since zeta potential indicates the probably of forming aggregates through electrostatic interactions between the NPs. It should be noted that values above ± 20 mV are indicative of the absence of aggregation. It is worth mentioning that the surface of the nanoparticles is negative because of the albumin itself that has a negative electrical charge at physiological pH (isoelectric point, pI: 4.5-5.0).

TABLE 1. SUMMARY OF PHYSICAL-CHEMICAL CHARACTERISTICS OF THE SYNTHETIZED ALBUMIN-BASED NANOPARTICLES

Batch	PDI	Size (nm)	Z Potential (mV)	Encapsulation efficiency (%)
1	0.043 ± 0.011	132.3 ± 2.44	-47.5 ± 1.84	55.767 ± 1.99%
2	0.066 ± 0.016	128.4 ± 3.44	-31.7 ± 1.82	53.181 ± 1.61%
3	0.043 ± 0.015	132.7 ± 3.82	-43.6 ± 1.99	52.071 ± 0.21%
4	0.045 ± 0.031	129.2 ± 5.34	-30.1 ± 1.30	57.661 ± 8.93%
5	0.085 ± 0.010	135.6 ± 6.18	-38.3 ± 0.40	52.277 ± 0.90%
6	0.082 ± 0.020	131.6 ± 3.74	-37.4 ± 1.40	50.804 ± 1.15%

Finally, the values obtained for the encapsulation efficiency, ranged between 50.80 ± 1.15 and 57,66 ± 8.93 % were within the range found in literature for other cytotoxic drugs, although no data were found in reference to colchicine It must be taken into account that the different structure and characteristics of drugs can interfere in the incorporation of these molecules within the particles. In particular, colchicine has a net zero charge, which makes its encapsulation very difficult. For example, sulfasalazine were encapsulated in BSA nanoparticles by the desolvation method, obtaining a PDI <0.1, but with a particle size between 200 and 400 nm and an encapsulation efficiency of 28% (Olaitan et al, 2019). On the other hand, Chen et al. (2020), also using albumin NPs, but cross-linked with hyaluronan, were loaded with paclitaxel, obtaining a PDI of 0.051 ± 0.021, a particle size of 331.4 ± 5.29 nm and an encapsulation efficiency of 74.5% ± 3.0%.

7.2 Validation of the analytical method for colchicine quantification

As described in the "Materials and Methods" section, in order to determine the encapsulation efficiency, i.e. the loading of colchicine in the synthesized albumin-based nanoparticles, a spectrophotometric method based on the measurement of the supernatant at 350 nm (Omar et al, 2019) was developed and validated, as an alternative to the widely used high-performance liquid chromatography (HPLC) method (Hadad GM et al, 2013). Figure 5 shows the standard calibration curve that was obtained from data related to 4 calibration curves composed of 7 reference samples in triplicate with a concentration range between 1 – 50 µg/mL.
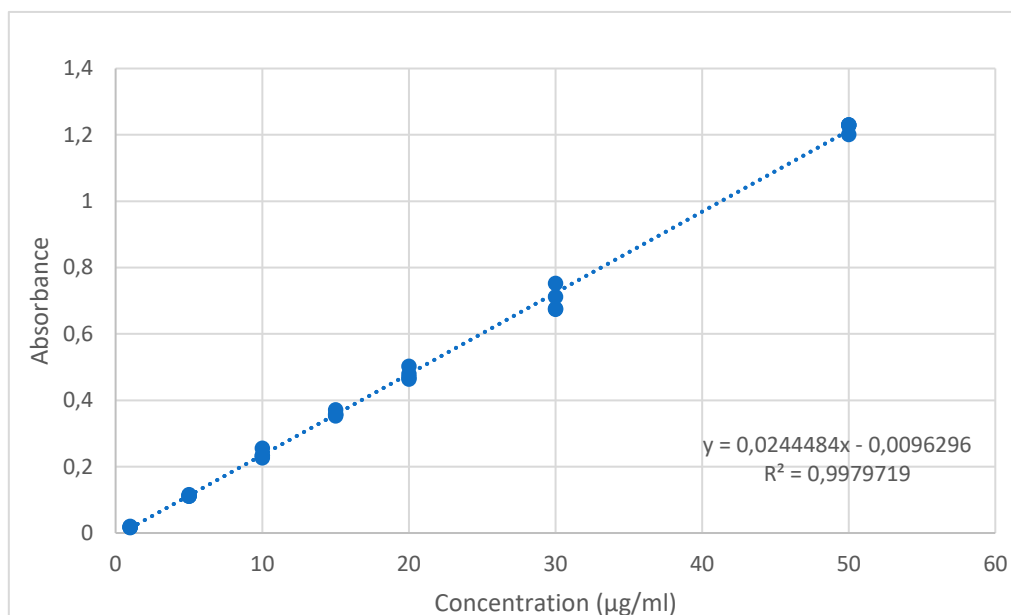

FIGURE 5. COLCHICINE CALIBRATION CURVE AT 350 NM.

The data collected in Tables 2 and 3 which show the results of the analysis of variance (ANOVA) of the linear regression model, confirm the validation of the proposed analytical method to quantify colchicine in terms of regression (b1 ≠ 0) and linearity. Null hypothesis of Table 3 lead to affirm that the intercept is cero and the recalculated slope is 0.02442, so the function that relates absorbance with concentration is:

$$Absorbance = 0.02442 \, x \, Concentration$$

TABLE 2. VARIATION ORIGIN

Variation origin	Sum of squares	Degrees of freedom	Middle squares
TOTAL	4.07141619	27	-
REGRESSION	4.063158762	1	4.063158762
RESIDUAL	0.008257428	26	0.000317593
DL	0.002113206	5	0.000422641
PURE ERROR	0.006144222	21	0.000292582

TABLE 3. SUMMARY OF ANOVA STATISTICAL DATA

Hypothesis tested	Calculated statistic	Tabulated statistic	Decision
$H0: \beta_0 = 0$ $H1: \beta_0 \neq 0$	1.829475612	2.055529439	b0=0 and recalculate b1.
$H0: \beta_1 = 0$ $H1: \beta_1 \neq 0$	12793.58717	4.225201273	The null hypothesis is rejected; b1≠0
H0: Linearity deviation = 0 H1: Linearity deviation ≠ 0	1.444522172	2.68478073	The hypothesis is acceptedthere is no linearity deviation

Using R-project and RStudio® software, a variability test was carried out to determine the variability between assays, considering the following statistical model:

$$y_{i,j}=(b_0+\alpha_{0,i})+(b_1+ \alpha_{1,i})x_{i,j} + e_{i,j}$$

where i is the number of the assay and j the concentration within the assay, b_0 and b_1 are the estimates of the intercept and the slope corresponding to the assay 1, $\alpha_{0,i}$ and $\alpha_{1,i}$ refer to the difference between the intercept or the slope, of the assay i with respect to the first one, and $e_{i,j}$ is the residue and .

The null hypothesis of the model (H0: $\boldsymbol{\alpha_{0,i}} = 0$ and H0: $\boldsymbol{\alpha_{1,i}} = 0$) were accepted with a significance level of 0.05 ($p < 0.05$), which means that the 4 assays can be interpreted as only one, i.e. there is no variability between assays. Thus, the statistical model could be expressed as:

$$y_{i,j}=b_0+b_1x_{i,j} + e_{i,j}$$

where b_0 is the estimated intercept that is statistically cero ($b_0 = 0$) and b_1 is the slope that is statistically different to cero ($b_1 = 0.02442$). Note that the parameters of the calibration curve obtained with this test (b_0 and b_1) are the same as those obtained previously with ANOVA.

Additionally, the variability between assays was confirmed by an F test, using the R-project and RStudio® software.

The precision of the analytical method was determined as coefficient of variation:

$$C.V. = \frac{s/b_1}{\bar{x}} x100 = 2.89\ \%$$

where s is the residual standard error, b_1 is the slope of the calibration curve and $\bar{x}$ is the midpoint of the concentration range in the calibration curve. Note that the value obtained for the coefficient of variation (2.89 %) is below 5 %, so the analytical method can be considered as precise.

Finally, to determine the accuracy for the analytical method, 5 solutions of colchicine of 20 µg / ml were prepared and measured. The average value (µ) was compared to the real value (T) and the accuracy (µ - T) was expressed as the following confidence interval:

$$-4.5167 \le (\mu - T) \le -4.5179$$

The accuracy range obtained is not satisfactory. This can be due to several factors: operator or instrument failure. It is suspected that the errors may come from the balance and the micropipettes. In fact, a calibration of the pipettes was performed, finding that they always failed to the left, that is, they added less volume than referred. Therefore, this lack of accuracy may be justified by the addition of a smaller volume by the pipettes at the same weight, so the solution will be more concentrated than expected.

7.3 Biological activity

As a model to study the antiproliferative activity, we used the human solid tumor cell lines HeLa (cervix) and SW1573 (lung). We selected two general methods to study cell viability and cell survival, the so-called antiproliferative assay and the clonogenic assay, respectively.

Antiproliferative assay

The results are shown in *Table 3.* As negative control, we used the nanoparticles alone –without colchicine– (NP0), which did not induce cell growth inhibition. From the three sets of nanoparticles loaded with colchicine, only NP1 induced growth inhibition in the tested range and in HeLa cells. The GI_{50} of the colchicine alone is much lower than that of the charged nanoparticles. Therefore, colchicine alone ($GI_{50} < 5$ nM) is more effective than encapsulated into albumin-nanoparticles ($GI_{50} > 50$ µM). We speculate that the release of colchicine from the nanoparticles is very low and can explain the obtained results.

TABLE 3. ANTIPROLIFERATIVE ACTIVITY (GI_{50}) AGAINST SOLID TUMOR CELLS

Sample	GI_{50} (µM)	
	HeLa	SW1573
NP0	>100	>100
NP1	58.96 ± 31.98	>100
NP2	>100	>100
NP3	>100	>100
Colchicine	<0.001	0.00445 ± 0.00079

GI_{50} values are mean of two independent experiments.

The GI_{50} of the colchicine solution is much lower than that of the charged nanoparticles as we can see on *Table 3*. Therefore, colchicine alone ($GI_{50} < 5$ nM) is more effective than encapsulated into albumin-nanoparticles ($GI_{50} > 50$ µM).

Clonogenic assay

Based on the previous results, only sample NP1 was tested in the clonogenic assay. The number of colonies formed after 7 days of treatment is

given in *Table 4*. Similarly to the abovementioned tests, the nanoparticles did not prevent colony formation when compared to colchicine (*Figure 6*).

TABLE 4. EFFECT OF NP ON COLONY FORMATION.

Sample	Number of colonies	
	HeLa	SW1573
Control	135.0	378.5
NP1	130.0	393.5
Colchicine	0	0

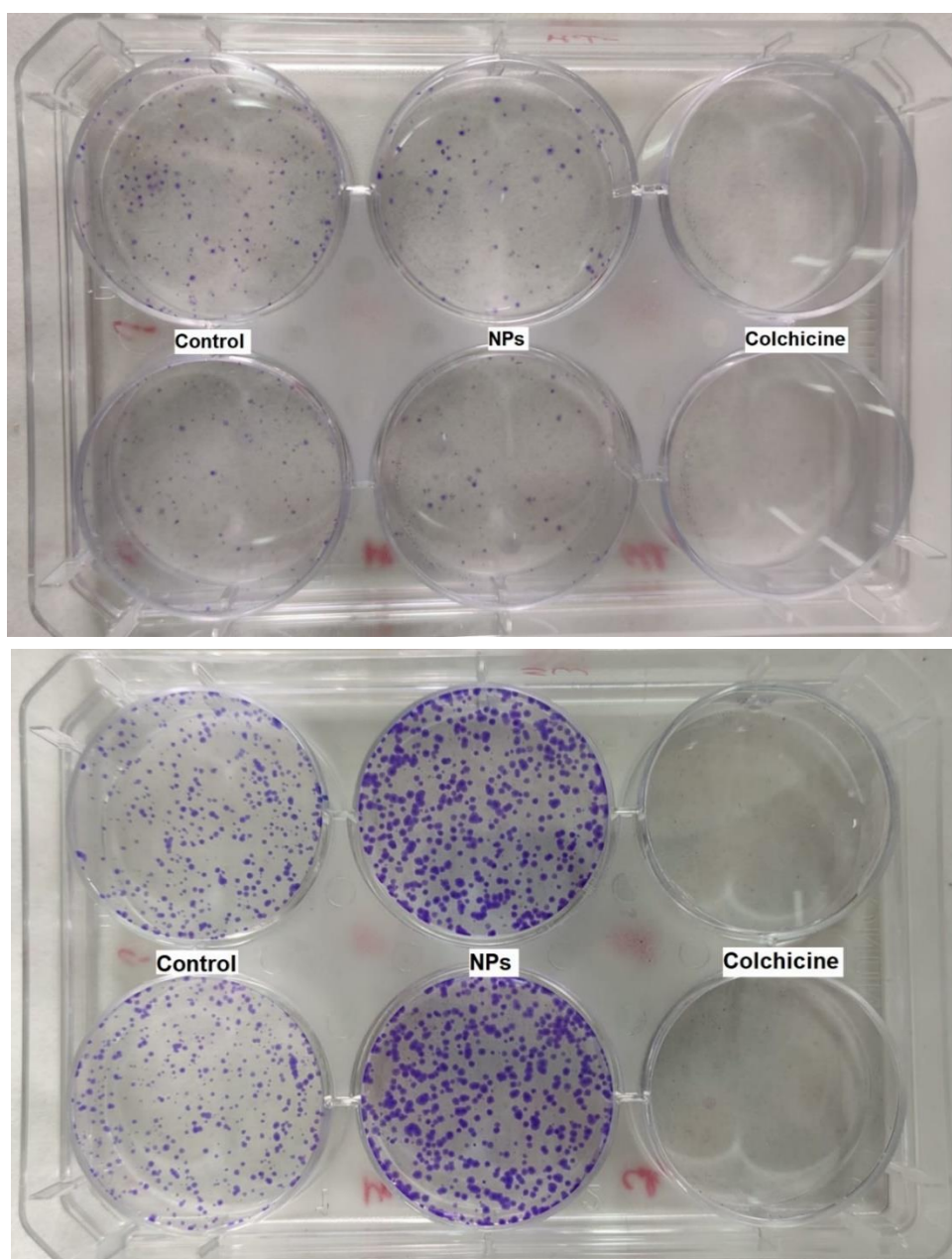**FIGURE 6. EFFECT OF NANOPARTICLES LOADED WITH COLCHICINE ON COLONY FORMATION OF HELA (TOP) AND SW1573 (BOTTOM) CANCER CELLS.**

Facultad de Farmacia
UNIVERSIDAD LA LAGUNA

BioLab

Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

8. CONCLUSIONS

1. Colchicine-loaded albumin nanoparticles were synthesized. The particles showed an adequate average particle size of 131.55 ± 2.62 nm, a narrow size distribution with a PDI below 0.1, a highly negative surface charge ranged between -30.1 ± 1.30 and -47.5 ± 1.84 mV and an encapsulation efficiency ranged between 50.80 ± 1.15 and 57,66 ± 8.93 % that were within the range found in literature for other cytotoxic drugs.

2. It has been possible to produce albumin protein nanoparticles loaded with colchicine with a PDI between 0.043 – 0.085; a size between 128 – 135nm; a potential between –30 and –48mV and an encapsulation efficiency between 50 – 60%.

3. Nanoparticles were tested against the human solid tumor cell lines HeLa and SW1573. The results indicate that colchicine loaded into albumin nanoparticles were less active ($GI_{50} > 50$ µM) than in solution ($GI_{50} < 5$ nM).

9. REFERENCES

- Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The History of Nanoscience and Nanotechnology: From Chemical-Physical Applications to Nanomedicine. Molecules. 2019 Dec 27;25(1):112. doi: 10.3390/molecules25010112. PMID: 31892180.

- Chen X, Wang M, Hu Y, Gong T, Zhang ZR, Yu R, Fu Y. Low-dose paclitaxel via hyaluronan-functionalized bovine serum albumin nanoparticulate assembly for metastatic melanoma treatment. J Mater Chem B. 2020 Mar 11;8(10):2139-2147. doi: 10.1039/c9tb02780g.

- Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, Khorasani S, Mozafari MR. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. Pharmaceutics. 2018 May 18;10(2):57. doi: 10.3390/pharmaceutics10020057.

- Duan Y, Liu W, Tian L, Mao Y, Song C. Targeting Tubulin-colchicine Site for Cancer Therapy: Inhibitors, Antibody- Drug Conjugates and Degradation Agents. Curr Top Med Chem. 2019;19(15):1289-1304. doi: 10.2174/1568026619666190618130008.

- Espinoza, S. L. S., SÁNchez, M. L., Risso, V., Smolko, E. E., & Grasselli, M. (2012). Radiation Synthesis Of Seroalbumin Nanoparticles. Radiation Physics And Chemistry, 81(9), 1417-1421.

- Galisteo-González F, Molina-Bolívar JA. Systematic study on the preparation of BSA nanoparticles. Colloids Surf B Biointerfaces. 2014 Nov 1;123:286-92. doi: 10.1016/j.colsurfb.2014.09.028. Epub 2014 Sep 22.

- Hadad GM, Badr JM, El-Nahriry K, Hassanean HA. Validated HPLC and HPTLC methods for simultaneous determination of colchicine and khellin in pharmaceutical formulations. J Chromatogr Sci. 2013 Mar;51(3):258-65. doi: 10.1093/chromsci/bms135. Epub 2012 Aug 21.

- He X, Deng H, Hwang HM. The current application of nanotechnology in food and agriculture. J Food Drug Anal. 2019 Jan;27(1):1-21. doi: 10.1016/j.jfda.2018.12.002. Epub 2018 Dec 24.

- Ibrahim RK, Hayyan M, AlSaadi MA, Hayyan A, Ibrahim S. Environmental application of nanotechnology: air, soil, and water. Environ Sci Pollut Res Int. 2016 Jul;23(14):13754-88. doi: 10.1007/s11356-016-6457-z. Epub 2016 Apr 14.

- Karakoti AS, Das S, Thevuthasan S, Seal S. PEGylated inorganic nanoparticles. Angew Chem Int Ed Engl. 2011 Feb 25;50(9):1980-94. doi: 10.1002/anie.201002969. Epub 2011 Jan 27. PMID: 21275011.

- Karimi M, Bahrami S, Ravari SB, Zangabad PS, Mirshekari H, Bozorgomid M, Shahreza S, Sori M, Hamblin MR. Albumin nanostructures as advanced drug delivery systems. Expert Opin Drug Deliv. 2016 Nov;13(11):1609-1623. doi: 10.1080/17425247.2016.1193149. Epub 2016 Jun 3.

- Kimura K, Yamasaki K, Nakamura H, Haratake M, Taguchi K, Otagiri M. Preparation and in Vitro Analysis of Human Serum Albumin Nanoparticles Loaded with Anthracycline Derivatives. Chem Pharm Bull (Tokyo). 2018;66(4):382-390. doi: 10.1248/cpb.c17-00838.

- Langer, K., Balthasar, S., Vogel, V., Dinauer, N., Von Briesen, H., & Schubert, D. (2003). Optimization of the preparation process for human serum albumin (ha) nanoparticles. International Journal of Pharmaceutics, 257(1), 169-180.

- Li SD, Huang L. Stealth nanoparticles: high density but sheddable PEG is a key for tumor targeting. J Control Release. 2010 Aug 3;145(3):178-81. doi: 10.1016/j.jconrel.2010.03.016. Epub 2010 Mar 23.

- Liu Y, Yang B, Zhao X, Xi M, Yin Z. E-Selectin-Binding Peptide-Modified Bovine Serum Albumin Nanoparticles for the Treatment of Acute Lung Injury. AAPS PharmSciTech. 2019 Jul 30;20(7):270. doi: 10.1208/s12249-019-1403-2.

- Lühmann T, Meinel L. Nanotransporters for drug delivery. Curr Opin Biotechnol. 2016 Jun;39:35-40. doi: 10.1016/j.copbio.2015.12.013. Epub 2016 Jan 11.

- Manual ZetaSizer NanoZS, Malvern Instruments, UK.

- Mariam J, Sivakami S, Dongre PM. Albumin corona on nanoparticles - a strategic approach in drug delivery. Drug Deliv. 2016 Oct;23(8):2668-2676. doi: 10.3109/10717544.2015.1048488. Epub 2015 Jun 9.

- Martínez-López AL, Pangua C, Reboredo C, Campión R, Morales-Gracia J, Irache JM. Protein-based nanoparticles for drug delivery purposes. Int J Pharm. 2020 May 15;581:119289. doi: 10.1016/j.ijpharm.2020.119289. Epub 2020 Mar 31.

- Mohanraj, V. J., & Chen, Y. (2006). Nanoparticles – A Revies. Tropical Journal of Pharmaceutical Research, 5(1), 561 – 573.

- Olaitan, V.; Chaw, C. S. Desolvation Conditions for Production of Sulfasalazine Based Albumin Nanoparticles: Physical Properties. Pharm. Front 2019, 1, e190006.

- Pérez-Herrero E, Fernández-Medarde A. Advanced targeted therapies in cancer: Drug nanocarriers, the future of chemotherapy. Eur J Pharm Biopharm. 2015 Jun;93:52-79. doi: 10.1016/j.ejpb.2015.03.018. Epub 2015 Mar 23.

- Pérez-Herrero, E. R-Project Template – Pharmaceutical Technology Practices, 2019.

- R. Oropesa – Nuñez, J. Ulises-Javier. Las nanopartículas como portadores de fármacos: características y perspectivas, 2012.

- Reis, C. P., Neufeld, R. J., Ribeiro, A. J., & Veiga, F. (2006). Nanoencapsulation I. Methods For Preparation Of Drug-Loaded Polymeric Nanoparticles. Nanomedicine: Nanotechnology, Biology And Medicine, 2(1), 8-21.

- S. Omar, F. Firuze, M. Maan Rajab and Noreldin S. Y Abdolla. Spectrophotometric method for the determination of Colchicine in pure and pharmaceutical forms, 2019.

- Spada A, Emami J, Tuszynski JA, Lavasanifar A. The Uniqueness of Albumin as a Carrier in Nanodrug Delivery. Mol Pharm. 2021 May 3;18(5):1862-1894. doi: 10.1021/acs.molpharmaceut.1c00046. Epub 2021 Mar 31.

- Suk JS, Xu Q, Kim N, Hanes J, Ensign LM. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. Adv Drug Deliv Rev. 2016 Apr 1;99(Pt A):28-51. doi: 10.1016/j.addr.2015.09.012. Epub 2015 Oct 9.

- Umerska A, Gaucher C, Oyarzun-Ampuero F, Fries-Raeth I, Colin F, Villamizar-Sarmiento MG, Maincent P, Sapin-Minet A. Polymeric Nanoparticles for Increasing Oral Bioavailability of Curcumin. Antioxidants (Basel). 2018 Mar 24;7(4):46. doi: 10.3390/antiox7040046.

- Y., Nguyen, H. H., Chun, H. S., Kang, B. C., & Ko, S. (2011). Preparation Of Size-Controlled Bovine Serum Albumin (Bsa) Nanoparticles By A Modified Desolvation Method. Food Chemistry, 127(4), 1892-1898

- 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. Eur J Pharm Biopharm. 2013 Nov;85(3 Pt A):398-405. doi: 10.1016/j.ejpb.2013.06.014. Epub 2013 Jun 27.

- Yu X, Jin C. Application of albumin-based nanoparticles in the management of cancer. J Mater Sci Mater Med. 2016 Jan;27(1):4. doi: 10.1007/s10856-015-5618-9. Epub 2015 Nov 26.