



Characterization and *in vitro* Evaluation of a New Levonorgestrel Silver Liposomes

MATILDA RADULESCU^{1#}, CRINELA UTESCU^{1#}, FLORIN BORCAN^{2*},
VICTOR DUMITRASCU¹, DAN NAVOLAN¹, IOANA ZINUCA PAVEL², DANA STOIAN¹

¹ Victor Babes University of Medicine and Pharmacy Timișoara, Faculty of Medicine, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

² Victor Babes University of Medicine and Pharmacy Timișoara, Faculty of Pharmacy, 2 Eftimie Murgu Sq., 300041, Timisoara, Romania

Abstract: *Levonorgestrel is the most widely used synthetic hormone due in particular to strong progestin and anti-ovulatory effects, without estrogenic effects, being also used in hormone supportive therapy and found in preclinical studies for the prevention / occurrence of malignant processes. In order to be able to eliminate the adverse effects but to benefit at the same time from the beneficial biological effects, the possibility of obtaining effective and safe biocompatible formulations is studied. In the present research silver nanoparticles loaded with lenorgestrel were obtained and were subsequently included in a liposomal transporter system. Physico-chemical characterizations showed good stability of the liposomal systems obtained and dimensions compatible with the biological environment. In vitro data conducted on breast cells (MCF 10A and MCF7) revealed a dose-dependent influence in terms of cell viability with no significant changes at low concentration of samples (0.1 μM), while higher concentration was associated with a decrease of the viable breast adenocarcinoma cells especially in the case of silver nanoparticles.*

Keywords: *levonorgestrel, silver nanoparticles, liposomes, breast cells*

1. Introduction

The use of synthetic hormones is necessary in different conditions, especially among women. The administration of combined oral contraceptives that contain a mixture from an estrogen derivative and a progesterone derivative is associated with the onset of venous thromboembolism due to increased serum estrogen levels in particular [1]. Newer generations of combined oral contraceptives present a higher risk of developing venous thromboembolism compared to the first generation [2]. In contrast, contraceptives based solely on progesterone derivatives do not appear to present such risks [3]. Levonorgestrel is extensively used as an active substance in certain contraceptive therapies and hormone replacement therapy. It is well known that steroid hormones are compounds that have repercussions on the endocrine gland, exerting influence on endocrine function even at extremely low concentrations [4].

To increase the stability and solubility of pharmaceutical substances for many years, different formulations are used by employing a series of macromolecules. Polyethers are polymers that have in their macromolecular structure the etheric group, some have FDA approval for use but in the pharmacological field they are useful only those that exert biodegradability. The most well-known and used such polymer is poly (ethylene glycol) (PEG) due to the advantages that make it ideal for pharmaceutical use: water solubility, non-ionic, non-toxic and biocompatible, already being approved by the FDA [5]. Liposomes were discovered decades ago but still capture the attention of researchers as being structurally distinct. They have a phospholipid structures with self-assembly role in lipid layers, which can incorporate lipophilic molecules and have an aqueous nucleus that can store hydrophilic molecules [6]. More recently, liposomes have been modified to extend the time of systemic circulation and retention of incorporated active substances. PEG molecules have proven to be

*email: fborcan@umft.ro # Authors with equal contribution



extremely useful in this case, with already approved forms which are used in cancer treatment: Doxil®, Lipodox®, Oncodox®PEG. [7]

The aim of the current study was to obtain levonorgestrel silver nanoparticles which were subsequently incorporated into liposomes in order to obtain a compatible and effective system correlated with the biological environment. The samples obtained were characterized by the usual physico-chemical methods and were afterwards tested *in vitro* for a preliminary evaluation of the behavior and safety.

2. Materials and methods

2.1. Materials

The reagents used in the current study were purchased from: Sigma Aldrich (Germany) - levonorgestrel (Lng) analytical standard, trisodium citrate, polyethylene glycol, lecithin, cholesterol; Merck (Germany) - silver nitrate, ethanol; Acros Organics (Belgium) - sodium lauryl sulfate. The specific materials utilized for the *in vitro* experiments (cell media, antibiotics, phosphate saline buffer, quantification reagents) were provided from American Type Culture Collection (ATCC, Germany), Sigma-Aldrich (Germany), Roche Diagnostics GmbH (Germany) and Thermo Fisher Scientific (USA).

2.2. Synthesis and physico-chemical characterization

Silver nanoparticles (Ag_NPs) were obtained by chemical synthesis based on Turkevich's process with minor modification, as described in the literature: 1 mM silver nitrate aqueous solution was mixed with 4×10^{-1} mM trisodium citrate and 5×10^{-1} mM sodium lauryl sulfate solutions under continuous stirring until a yellow pale color specific for silver colloids appear [8,9]. In order to obtain PEGylated silver nanoparticles (Ag_NPs_PEG) PEG 400 was employed. Levonorgestrel (Lng) metallic colloids with a final concentration of 2×10^{-3} mM were synthesized with both silver nanoparticles and PEGylated silver nanoparticles by treating them with an ethanolic levonorgestrel solution. After purification, the resulting samples (Ag_NPs, Ag_NPs_PEG, Lng_Ag_NPs and Lng_Ag_NPs_PEG) were stored in the refrigerator in dark bottles.

The liposomes were obtained by thin-layer hydration method: lecithin and cholesterol were dissolved in chloroform and the mixture was stirred, then the solvent was removed and subsequently the formed film was hydrated with the colloidal silver solutions previously obtained (molar ratio drug:lecithin:cholesterol 0.15:12.5:1). After hydration, the solutions were subjected to ultrasound to obtain the desired size, lyophilized and kept at 4-6 °C until further use.

The physico-chemical characterization of the samples obtained was realized by usual methods: UV-Vis spectroscopy (Cary 60 UV-Vis and Secomam UviLine 9400), SEM-EDAX determination with EDAX detection on SEM, by utilizing an EDAX detector with FEI Quanta 250 microscope, TEM evaluation using a FEI Tecnai 12 Biotwin microscope and zeta analysis (Zetasizer Nano ZS system, Malvern Instruments).

2.3. *In vitro* studies

The experiments were conducted using two breast cell lines purchased from ATCC as frozen items: MCF 10A (ATCC® CRL-10317™) and MCF7 (ATCC® HTB-22™). The cells were grown and multiplied according to the manufacturer's specifications: MCF 10A with DMEM:F12 supplemented with EGF, insulin, hydrocortisone, FCS, and antibiotic mixture and MCF7 with EMEM supplemented with insulin and FBS. The cells were used in experiments only after reached a confluence of 85-90%.

The cell viability was assessed by MTT assay, as presented in the literature [8-10]: (1) cells were seeded in specific microplate (96 well) using the specific media for each cell type and allowed to attach; (2) after 24 h, the medium was replaced and the cells were treated with two concentrations of the samples (0.1 and 1 μM) for 24 h; (3) the MTT reagent was added and cells were incubated for 4 h; (4) samples were spectrophotometrically examined at 570 nm at a microplate reader (xMark Spectrophotometer - BioRad).

2.4. Statistical analysis

The GraphPad Prism 7 software was used for data processing and presentation and one-way ANOVA test was selected to establish the statistical differences followed by Tukey's post-test and p values were: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. control cells. The data are presented as the mean \pm standard deviation (SD).

3. Results and discussions

Silver nanoparticles loaded with levonorgestrel were obtained by chemical synthesis in order to better control their size. UV-Vis spectra were used during the syntheses to confirm the formation of silver nanoparticles in the form of colloidal solutions. The elemental composition of silver colloids by the EDAX analysis was conducted (Figure 1). The elements present in the unloaded silver nanoparticles samples were C, O, N, Na and Ag expressed in weight percent (Wt%) or atomic percent (At.%) (Table 1).

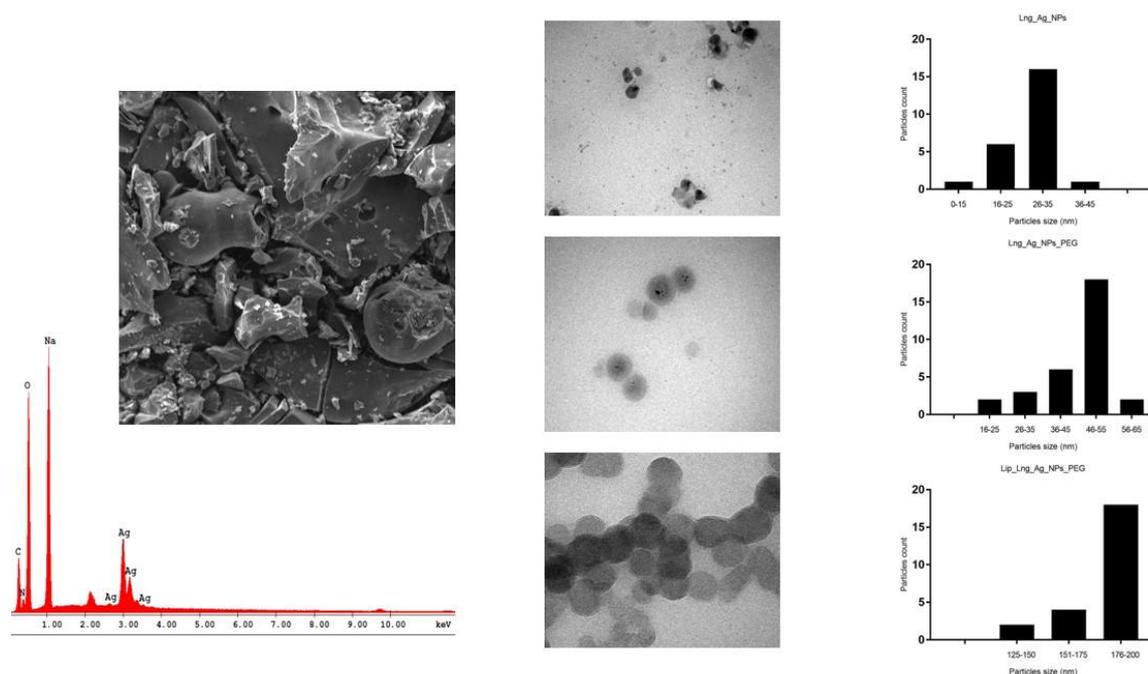


Figure 1. Energy dispersive X-ray spectra and SEM image of lyophilised AgNPs together with TEM images and particle size distributions of levonorgestrel silver nanoparticles, PEGylated particles and liposomal structure.

Table 1. Elemental composition of basic silver colloids

Element	Wt %	At %	K-Ratio	Z	A	F
C K	20.81	29.33	0.0609	1.0365	0.2819	1.0006
N K	5.40	6.52	0.0107	1.0288	0.1930	1.0013
O K	41.94	44.37	0.0889	1.0219	0.2073	1.0005
NaK	25.52	18.79	0.0565	0.9600	0.2306	1.0002
AgL	6.33	0.99	0.0639	0.7916	1.2751	1.0000
Total	100.00	100.00				

In order to assess the stability of the samples zeta potential was determined and the data obtained were satisfactory the values being negative and located in the range 39 mV-19 mV with the lowest values for liposomal systems (Table 2). Size of the samples obtained are biocompatible with the biological environment and are presented in the Table 2.

Table 2. Physico-chemical properties of silver nanoparticles, PEGylated silver nanoparticles, levonorgestrel metallic colloids and their liposomal formulations

Sample	Parameter		
	Particles size (nm)	PDI (%)	Zeta potential (mV)
Ag_NPs	27	0.210	-39
Lng_Ag_NPs	32	0.186	-27
Ag_NPs_PEG	52	0.241	-32
Lng_Ag_NPs_PEG	55	0.199	-38
Lip_Ag_NPs	138	0.188	-19
Lip_Ag_NPs_PEG	144	0.236	-22
Lip_Lng_Ag_NPs	157	0.192	-21
Lip_Lng_Ag_NPs_PEG	186	0.203	-22

Hormonal changes have an impact on the female mammary gland and decreasing hormone exposure decreases the risk of breast cancer, but there is also a theory that increased hormone exposure may increase the risk of disease. There are studies that have shown the mitogenic effects of estrogen and progesterone related to cancer initiation, but also effects that amplify the development of pre-existing tumor formations through the actions of estrogen metabolites. Some studies suggest that systemic progesterone treatment contributes to increased expression levels of reductive enzymes compared to oxidative ones, which results in a possible increase of estrogen hormones and thus increases the proliferation of breast epithelial cells. [11,12]

In order to assess the cytotoxic impact of levonorgestrel silver nanoparticles and their liposomal systems on breast epithelial cells – MCF 10A, there were selected two concentrations 0.1 μ M and 1 μ M, respectively for 24 h. The data obtained revealed that at 0.1 μ M no significant influence on cell viability was noted for 24 h (data not shown). At a concentration of 1 μ M, there was a slight decrease in cell viability after treatment with Ag_NPs (viability ~93%), Lng_Ag_NPs (viability ~94%) and Lip_Ag_NPs (viability ~94%) (Fig. 2). The cytotoxic potential of the obtained samples on human adenocarcinoma breast cells has been conducted on MCF7 cells which were stimulated with the same concentrations used in non-cancer cells (MCF 10A) 0.1 μ M and 1 μ M, respectively. The viability was more or less statistically influenced by all the samples tested at the highest concentration as follows: ~90 % for Ag_NPs and Lip_Ag_NPs_PEG, ~91 % for Lip_Lng_Ag_NPs_PEG, ~92 % for Ag_NPs_PEG, ~93 % for Lip_Ag_NPs, ~94 % for Lip_Lng_Ag_NPs (Figure 3).

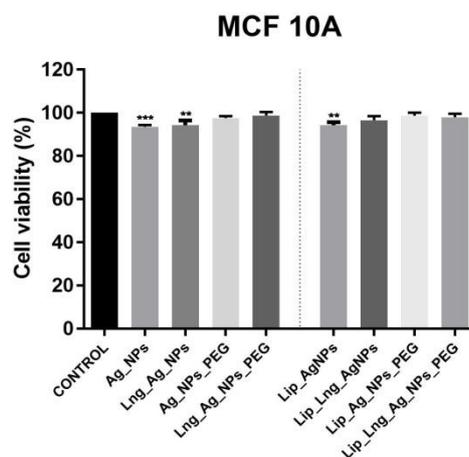


Figure 2. Cytotoxic-related impact of levonorgestrel silver nanoparticles and their liposomal systems on breast epithelial cells – MCF 10A related to control cells (untreated) based on MTT assay. All experiments were conducted in triplicate

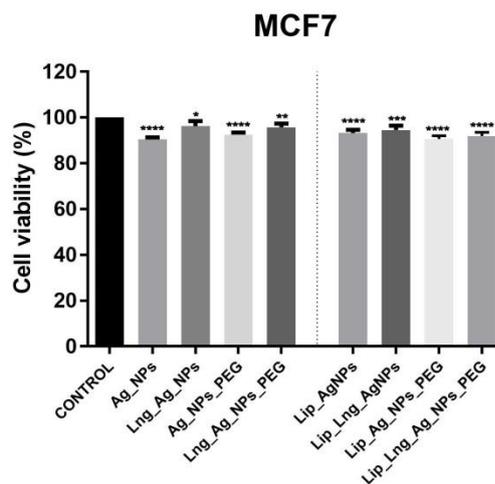


Figure 3. Cytotoxic-related impact of levonorgestrel silver nanoparticles and their liposomal systems on breast adenocarcinoma cells – MCF7 related to control cells (untreated) based on MTT assay. All experiments were conducted in triplicate

Currently, the action of pharmaceutical and medical products based on synthetic progestatives in the initiation and development of breast cancer is not sufficiently known due to studies using small samples, different types of progestins, frequency and use, environmental factors, genetic factors, etc. [13] Finding specific, biocompatible and no side effects formulations is necessary for effective and safe administration of patients' health. One of the most important parameters in the use of liposomes in the biomedical field is their size which manifests a direct influence on the half-life of the circulation in the release of active substances. The methods used to obtain liposomes involve the passive loading of the active substances. [14] Due to the complex mechanisms that occur in the biological environment, the association between the active substances, the metal nanoparticles and the carriers themselves is still a challenge for the specialists in these fields. The gold nanoparticles have been associated with liposomes but their release rate is still optimized despite the complexity of the methods of obtaining and optimizing the parameters. [15] In the study conducted by Yusuf and collaborators, liposomes loaded with silver nanoparticles (for use in anticancer therapies) suppressed the generation of reactive oxygen species, depleted GSH levels, damaged DNA and all of these could ultimately lead to cell death. [16]

4. Conclusions

The data from the present study provide that levonorgestrel silver nanoparticles and their liposomal systems meet the requirements for use in the biomedical field based on their stability and size. Preliminary testing of the obtained systems was performed on two mammary cell lines (non-tumoral – MCF 10A and tumoral – MCF7) to evaluate the activity at low concentrations taking into account that data from the literature often use concentrations that prove cytotoxicity on tumor cell lines but does not use comparisons with healthy lines. There was a slight reduction in the viability of the tumor cells, which varied statistically according to the components of the sample, those with levonorgestrel having a lower influence compared to those without these substance.

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