ANTIPROLIFERATIVE EFFECT OF Fe_3O_4 / METHOTREXATE NANOPARTICLES ON METASTATIC PROSTATE CARCINOMA CELLS DU145

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Two aqueous dispersions of Fe_3O_4 / Methotrexate (nano-mtx) and Fe_3O_4 (nano) magnetic nanoparticles were synthesized by a modified Massart method. Dispersions were characterized by x-ray diffraction (XRD), thermogravimetry (TGA), dynamic light scattering (DLS) and fourier-transform infrared spectroscopy (FT-IR). The results confirm the obtaining of nanoparticles and the DLS analysis shows a 55.96 nm hydrodynamic diameter and a 42.2 mV potential for nano-mtx and 69.04 nm and 50.9 mV for nano. The analysis confirmed the magnetic nature of the nanoparticles and the metrotoxate nanostructure (nano- mtx) complex, the amount of methotrexate present in one mL of dispersion being 3.6 μg. The nano-mtx complex reduces the mitotic phases of the cell cycle and increases the percentage of apoptotic cells, suggesting inhibition of the development of metastases of independent androgenic prostate cells. The results are a first step in a series of cellular functional explorations that could improve the antitumor therapeutics through innovative solutions such as nano-structured complexes.

Keywords: Magnetic nanoparticles; Methotrexate; Human prostate.

1. Introduction

The use of magnetic nanoparticles in medicine has increased particularly lately due to the ease of synthesis and the size-dependent physical properties. In recent years, there has been growing interest in the development of core-shell nanoparticles based probes for tumor diagnosis and therapy[1,2,3]. Depending on the choice of the linker and the chemical structure of the shell, core-shell magnetic nanoparticles can be used in medicine as contrast agents in magnetic resonance

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imaging, the destruction of tumors by heating and targeting, tissue engineering, cell separation, medicines, embolotherapy[4,5,6,7,8,9]. However, the identification of certain targeting agents or drugs that can be used as shells for core-shell nanoparticles and which can be efficiently released within target cells remains a challenge and is the focus of current field studies. In some situations, the cell-chemotherapy agent impact does not guarantee cellular response and efficacy, requiring molecular targets to inhibit proliferation and essential metabolic changes[10,11,12]. Methotrexate (MTX) is a cytostatic antineoplastic agent acting as an antimetabolite of folic acid through a mechanism of competitive inhibition of its reductase, a mechanism by which it interferes with de novo synthesis of nucleic acids and with cellular replication in active proliferation tissues for the treatment of many cancers over time, including leukemia, breast cancer, head and neck cancer, lymphomas and carcinomas, prostate cancer. An active principle can be grafted onto the surface of the nanoparticles by the physical process of adsorption, ionic bonding or by covalent binding. In order to prolong the release of MTX at the target site, different groups of researchers graft different linkers on magnetic nanoparticles for covalent binding of MTX by creating an amide or ester linkage between the carboxyl group of MTX and the amino or free hydroxy groups of the linker. Studies of different groups of researchers by obtaining and testing various nanoparticles-linker-MTX complexes have demonstrated that the nanoparticles-linker-MTX complex has superior anti-tumor properties compared to MTX as such[13,14,15,16,17].

2. Materials and methods

Materials and method for synthesis of nano and nano-mtx

All used reagents (KOH, $FeCl_3$, $FeCl_2$, Methotrexate) were analytical grade (p.a.), and were purchased from Sigma-Aldrich. The ultrapure water used in all synthesis steps was obtained using a Millipore Elix 5 water purifier. A modified Massart synthesis was used for Fe_3O_4/Methotrexate and Fe_3O_4 core–shell synthesis [18]. The hydrated salts in stoichiometric Fe II and Fe III ratios (2 g $FeCl_3$, 1.25 g $FeCl_2$) were dissolved in 250 ml of ultrapure water (18,2 [MΩ]) and precipitated under basic condition (10 g KOH, 10 mg methotrexate, and 250 mL ultrapure water) for Fe_3O_4/Methotrexate and without methotrexate for Fe_3O_4, using an ultrasound bath. Several washing steps (with ultrapure water) involving soni-cation and magnetic separation were performed, in order to eliminate the KOH and methotrexate excess. Nanoparticles characterization involved FT-IR for the qualitative evaluation of the adsorption process of methotrexate, XRD analysis to establish the crystalline structure of the magnetic nanoparticles, DLS analysis to evaluate Zeta potential and the hydrodynamic diameter of the

Fe_3O_4/Methotrexate and Fe_3O_4, and TGA analysis for the quantitative evaluation of the adsorption process of methotrexate.

DLS analysis

Particle size measurements were performed by DLS with a Zetasizer Nano ZS (Malvern Instruments Ltd.,United Kingdom). The mean diameter (Z-average), and the polydispersity index (PdI) of the nanoparticles in aqueous dispersion were determined at an angle of 173º. All measurements were performed at 25ºC and data were given as an average of five individual measurements.

FT-IR analysis

The FT-IR analysis were performed with a FT-IR Nicolet 6700 spectrometer by ATR measurement method. The spectra of the samples are the averages of 32 scans, in the range of 4000-400 cm^{-1}, with a resolution of 8 cm^{-1}.

XRD analysis

X-ray diffraction measurements were performed with a RigakuSmartLab diffractometer, using the following operating conditions:45 kV, 200 mA, CuK_αa radiation (1.54059Å), parallel beam configuration (2 theta/theta scan mode), from X to XX 2 degrees; the obtained diffractograms were interpreted using the Rigaku Data AnalysisSoftware PDXL 2, by comparison with ICDD database entries.

TGA analysis

Thermal analyzes (TG and DTG) were performed with a DuPont Instruments "Thermal Analyst 2000/2100" coupled with a "951 Thermogravimetric Analyzer" module, in inert gas atmosphere (nitrogen) with a flow rate of 50 mL / min and heating from room temperature (25 °C) to 750 °C with a heating rate of 10 °C / min.

2.2 Materials and method for assessing in-vitro effects

For in vitro testing was used **DU145** which is a hormone –independent standardized CELL LINE of human prostate epithelial cancer cells, having brain metastasis origin. Cells were cultured in monolayer in DMEM (Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham, code: D8437, Sigma-Aldrich) supplemented with 10% fetal bovine serum (code: F7524, Sigma-Aldrich) and 1% antibiotic/antimycotic.

Apoptosis induction – Flow cytometry monitoring of % of live cells / necrotic cells / early apoptosis / late apoptosis was done by double fluorescent

staining (FITC-A and PE) with **annexin V** (high affinity for phosphatidyl-serine translocated on the outer cellular membrane – the first event of apoptosis) and propidium iodide (bound to DNA when the cellular membrane is disrupted and allow its permeation). Reagents: ANNEXIN V-FITC APOPTOSIS DETECTION kit (BD PHARMINGEN), FACS Canto II flow cytometer and Diva 6 software.

Cell cycle progression

Flow cytometry measurements of DNA amount are possible due to the specific label of DNA with propidium iodide (PI) fluorochrome. The isolation and label of the nuclei in cell suspensions is done using Cycle TEST PLUS DNA Reagent (BD PHARMINGEN). Data were acquired with FACS CANTO II flow cytometer, and the cell cycle analysis is performed with FCS Express software – DNA cell cycle module.

3. Results and discussions

3.1 Results and discussions for synthesis and characterization of nano and nano-mtx

XRD, TGA and FT-IR analysis were performed on solid samples obtained by drying 100 ml of nano-mtx and nano dispersion in the oven at 50º for 48 hours, and DLS assays were performed on the dispersions obtained.

The confirmation of obtaining the core-shell magnetic nanoparticles from the xrd analysis(fig.1) is due to the presence of the peaks that correspond to the crystallographic phase of the magnetite.

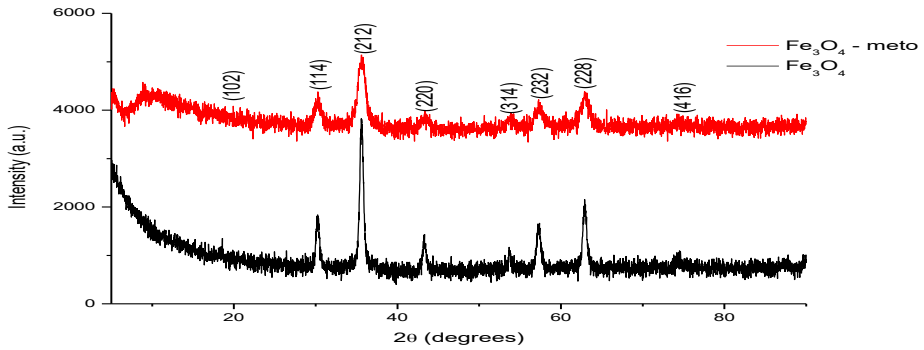

Fig.1: XRD spectra for nano and nano-mtx

Strong absorption bands at 524.49 cm^{-1} and 540.56 cm^{-1} present in the FT-IR spectra of the nano-mtx and nano-mtx samples correspond to the Fe-O bond in the magnetite, which confirms the obtaining of magnetic nanoparticles

(fig.2). The adsorption of mtx on the surface of magnetic nanoparticles is qualitatively confirmed by the valence vibration at 3412.94 cm^{-1} corresponding to the N-H bond in the primary and secondary amine bonds, the valence vibration at 1536.81 cm^{-1} corresponding to the C = O group of the amide bond and the valence vibration at 1437.6 cm^{-1} corresponding to the C = O groups of the carboxyl groups of the methotrexate (fig.2).

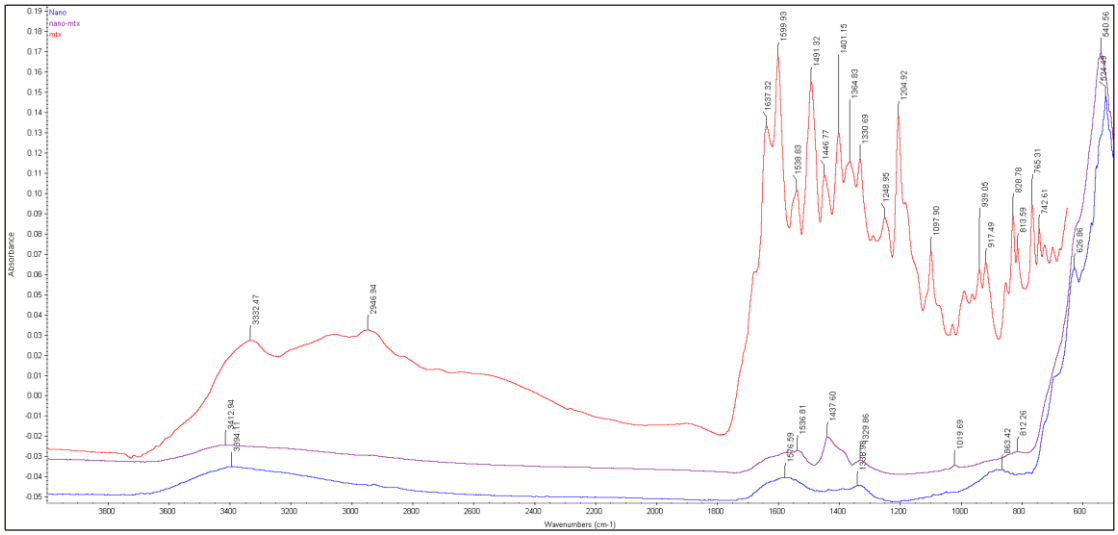

Fig.2: FT-IR spectra for nano, nano-mtx and mtx

The amount of methotrexate adsorbed on the surface of magnetic nanoparticles is 0.3608 mg / 100 ml nano-mtx dispersion determined by thermogravimetry (fig.3).

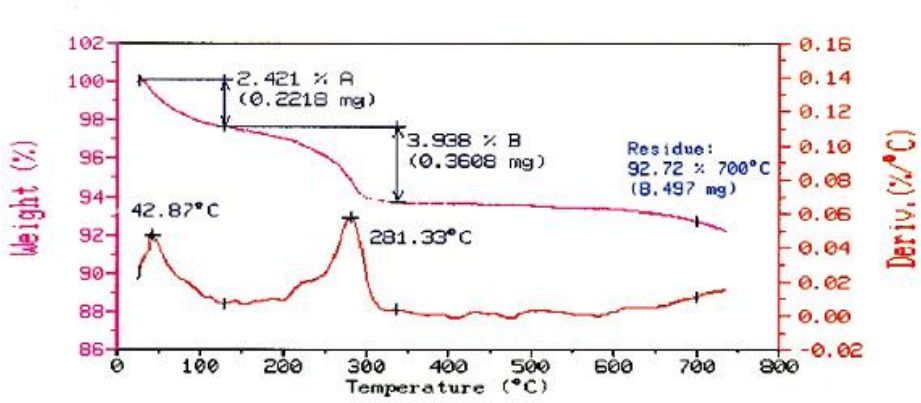

Fig.3: TGA curve for nano-mtx

The stability of the dispersions obtained is confirmed by DLS analysis with a potential of 42.2 mV for nano-m.tx and 50.9 mV for nano, values which confirm excellent stability for both dispersions (table 1). Polydispersity index values correlated with the hydrodynamic diameters of dispersions obtained from DLS analysis confirm that the nano-mtx and nano dispersions are monodisperse (table 1).

Table 1:

DLS results for nano and nano-mtx

Synthesized materials	Dimension(nm)	Potential zeta(mV)	Polydispersity index (Pdl)
Nano-mtx	55,96	42,2	0,119
Nano	69,04	50,9	0,114

3.2 Results and discussions for in-vitro testing

To increase survival of men with metastatic prostatic cancer, a modality that can effectively eliminate androgen independent cancer cells is needed, a possible solution being the therapy with two types of agents, one having antiproliferative activity affecting the small number of dividing androgen independent cells, and the other able to increase the low rate of cell death for the androgen independent prostatic cancer cells[19]. Androgen-independent prostate cancer cells are remarkably resistant to therapeutic agents that work by triggering apoptosis or decreasing the proliferation rate. The individual prostate cancer cells express multiple antiapoptotic mechanisms and it's not possible to enhance cellular sensitivity to therapeutics by disabling just one target in a pathway. The development of novel therapeutic strategies may have a significant improvement by the use of targeted nano-structures, with higher bioavailability[20,21].

Based on these ideas, and according our previous studies[22,23], we design the experiments on DU-145, an androgen independent, metastatic prostate cancer cell line, trying to find improved therapeutical agents with a antiproliferative effect and enhance the apoptosis.

The cells were adhered 48h, then treated 48h with the test substances: NANO, MTX-NANO and MTX (methotrexate 9 μM). Results, compared with the solvent control and cellular control, are presented in Fig.4 and table 2 and 3.

Table 2:

Prostate carcinoma cell cycle sequentiation modulated by Nano, MTX-NANO and MTX

	DU 145 cells – prostate carcinoma cell cycle				
	G0/G1	**%S**	**%G2/M**	**%S+%G2/M**	**% of (S+G2/M) decrease**
Cellular control	50,62	37,43	11,95	49,38	100,00

Solvent Control	52,61	35,88	11,51	47,39	100,00
Nano 5%	46,45	41,79	11,76	53,55	3,36
Nano 2,5%	46,69	39,47	13,58	53,05	4,26
Solvent control 5%	44,59	46,64	8,77	55,41	100,00
Mtx-Nano 5%	49,24	42,45	8,32	50,77	8,37
Mtx-Nano 2,5%	48,17	36,95	14,88	51,83	6,46
MTX 9µM	56,82	41,18	2,13	43,31	12,20

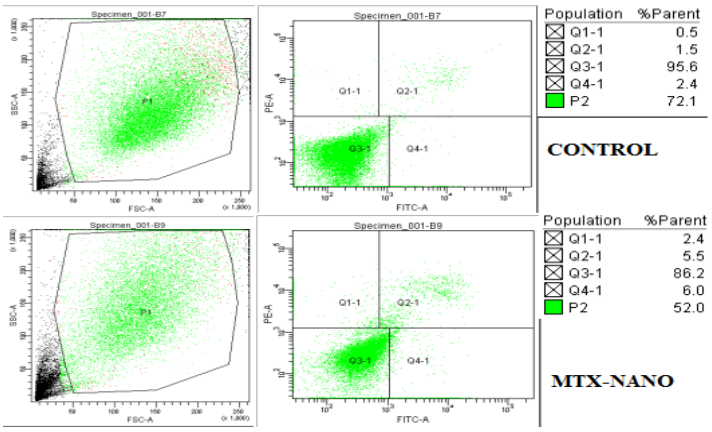

Fig. 4: Apoptosis induction by MTX-NANO compound

Table 3:

Prostate carcinoma apoptosis modulated by Nano, MTX-NANO and MTX

	DU 145 cells – prostate carcinoma apoptosis				
	Q3 % (% live cells)	**Q4 %(% early apoptosis)**	**Q2 % (late apoptosis)**	**Q1 % (%Necrosis)**	**% total apoptosis (early+late)**
Cellular control	93,7	2,8	2,6	0,9	5,4
Solvent Control	93,4	3,1	2,6	0,9	5,7
Nano 5%	96,7	1,6	1,1	0,5	2,7
Nano 2,5%	95,8	1,9	1,7	0,5	3,6
Solvent control 5%	95,6	2,4	1,5	0,5	3,9
Mtx-Nano 5%	91	4,1	3,3	1,6	7,4
Mtx-Nano 2,5%	86,3	5,9	5,4	2,5	11,3
MTX 9uM	94,8	2,6	2,1	0,6	4,7

The chemotherapeutic agent Methotrexate alone reduces the cellular percent that undergo the G2/M check point of the cell cycle, inhibiting the proliferation of DU145 cells. The Nano structure by itself doesn't affect the cell

cycle sequentiation nor the apoptotic status of the prostate metastasis. MTX-NANO proves to be the only one agent that both inhibits the DU145 proliferation and induces apoptosis, including the cellular membrane permeabilisation (late apoptosis

4. Conclusions

Following the synthesis, nano and nano mtx dispersions with high water stability were obtained. In order to evaluate the anticancer effect of mtx for in-vitro tests, mtx as such and magnetic nanoparticles without mtx (nano) were used as control.

The MTX-NANO compound reduces the cell cycle mithotic phases and rise the percent of apoptotic cells, suggesting a promising role in inhibition of androgen-independent prostate cells development. These results are only the first step for the identification of new therapeutic modalities in advanced prostate cancer targeted therapy, considering the folowing aspects: apoptotic response for the uncontrolled growth behavior, drug sensitivity, nanostructures impact on modulation of molecular mechanisms underlying cancer progression.

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