European Cells and Materials Vol. 3. Suppl. 2, 2002 (pages 70-73) ISSN 1473-2262

EVALUATION OF FERRIFLUIDS CONTAINING PHOTOSENSITIZER

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INTRODUCTION: We have developed magnetic dextran-ferrite (DF) nanoparticles [1] and photogem (PG) [2] for tumor cell induction DF AC magnetic field hyperthermia (ACH) [3] and PG magneto- [4] and thermosensitization [5] in the dark (MTS). DF ferrifluids (DFFs), that had been prepared from DF, may be ideal magnetic carriers [1,3,6]. DF dissipates AC magnetic field energy and therefore causes hyperthermia in the area of their confinement [3]. PG in analogy to hematoporphyrin [4] may generate singlet oxygen or superoxide radicals and cause the destruction of tumor cells in the dark.

The inevitable technical problem of photodynamic therapy is the initiation of the absorbency of visible light by a tumor that has been injected with photo-sensitizing agent, because incident light at wavelengths between 600 and 1000 nm reacts with the photosensitizing agent only at shallow depth (0.1-1 cm) of tissue.

The purposes of this work were: to evaluate PG-containing dextran-ferrite ferrifluids for the combination of an ACH with MTS; to analyze the influence of AC magnetic field and hyperthermia on cell death and lysis in the presence of PG; to obtain further insights into the mechanisms of these processes.

METHODS AND MATERIALS: We have tested five water-based dextran-ferrite (DF) ferrifluids (DFFs): 12.0; 0.6; 0.2; 0.02 and 0.002%, that were prepared by a procedure modified from [6]. The sample of the initial DFF was lyophilized. DF specific saturation magnetization ($\acute{o}$) and DFF zeta-potential (ζ); specific power absorption rates (*SAR*); saturation magnetization (M_s), other physical and chemical characterizations and biological properties were determined as presented in [3,6]. The analytical fractionation of the samples of ferrimagnetic particles was performed by stepwise passage of their 1% aqueous sols through membrane filters (100 nm, 45 nm, 20 nm, XM300, XM100, XM50, UM20 – UM05u) at a nitrogen pressure of 0.1–0.3 atm on an Amicon TSF-10 thin-channel ultrafiltration system or in Model 12 and Model 202 cells, and using a column gel-filtration system. Gaussian/Nicomp and volume-weighted Gaussian distribution analysis of particles in the diluted DFFs and PG sols was performed by dynamic light scattering laser particle sizing system, Submicron Particle Sizer NICOMP™ 380/DLS (Particle Sizing Systems, Inc., Santa Barbara, Calif., USA).

PG was obtained by treatment of haemin with a 50% solution of hydrogen bromide in acetic acid followed by sequentially adding acetic acid, sodium acetate, and water. Hematoporphyrin diacetate, precipitated from solution, was filtered and treated with 0.1 M sodium hydroxide for 1 hour. PG was precipitated by acetic acid followed by filtering, washing with water, and drying in air. PG prepared as a deep-violet crystal powder represents a complex half-synthetic mixture of monomeric and oligomeric porphyrins. The solution of this substance in 0.5% sodium hydroxide and adjustment to pH 7.4 with 1 M hydrochloric acid, produced the PG sol.

For biotesting in vitro, two types of tumor cells were used: adherent human carcinoma ovarii (CaOv) and murine ascitic limpholeukosis P388 cells. The latter were obtained from tumor-bearing DBA_2 mice on the 7th day after intraperitoneal transplantation of 10^6 P388 cells. The survival of CaOv and P388 cells, as a result of exposure to increased temperature and a concentration of DF alone, PG alone, histidine (His) alone, DF in combination with PG (DF+PG), PG in combination with His (PG+His) was investigated. Magneto- and thermosensitization of tumor cells by PG in the dark and heating DFFs achieved simultaneously by an AC magnetic field or by a flow thermostat. For the CaOv and P388 cell survival study, the previously used experimental setup [3] was modified. An AC magnetic field 0.88 MHz, 9.3 kA/m, 0.15 kW was achieved inside a water-cooled copper induction coil of 4.5cm radius (20 turns with turn-to-turn distance of 0.9 cm). The tumor cells (concentration 10^6 cells/ml) alone and with reagents: DF, PG, His, PG+DF, PG+His were

placed in the center of the coil and exposed for 30 min to the AC magnetic field in the dark. To 6 test tubes (TTs) containing 2 ml of fresh peritoneal ascitic limpholeukosis P388 or CaOv cells (2×10^6/ml) were added respectively: 2 ml of 12% (w/v) DFF (net γ-Fe_2O_3 weight: 60 mg); 2 ml of 0.6% DFF; 2 ml of 0.2% DFF; 2 ml of 0.02% DFF; 2 ml of 0.002% DFF; and 2 ml of 0.9% saline to the sixth as a control. The TTs then were exposed to the AC magnetic field as described above, and the selected temperature in the range of 37 to 44°C was maintained for 30 minutes (Table 1). The cell temperature was measured during the AC magnetic field treatment using an alcohol thermometer. Alternatively, the cells were exposed to the AC magnetic field in the dark in the presence of PG alone, His alone, PG+DF, and PG+His. For the control, the cells were incubated at 37°C in the laboratory thermostat. To 6 isolated TTs were added 0.1 ml of fresh P388 or CaOv cells (2×10^6/ml) and 0.1 ml of reagents as listed above (Table 3-5); in the sixth (control TT, Tables 1,2) 0.1 ml 0.9 % saline was added. The volume of reaction mixture in the TTs, containing tumor cells and reagents, was 4 ml (Table 1) and 0.2 ml (Tables 2-6); the concentration in all the TTs was 10^6 cells/ml. The temperature of the reaction mixtures from 37 to 41°C was achieved using a flow thermostat (Tables 3 and 5); from 37 to 44°C (Table 1) and from 37 to 41°C (Tables 4 and 6) was achieved by the AC magnetic field. The survival of P388 and CaOv cells as a result of the exposure to concentrations of DF, PG, His, PG+DF and PG+His at 37 to 41°C during tumor cell PG magneto- and thermosensitization (MTS) achieved by AC magnetic field in the dark was fixed. After CaOv or P388 cells exposure to AC magnetic field, DFFs, PG, PG+DF, PG+His at various conditions, the survival of the cells was analyzed by a hemocytometer counting and by intraperitoneal injection of 0.1 ml analyzed compositions of P388 cells to DBA_2 mice. The interaction of DF with the cells was investigated, taking account of the recommendations in [7]. The results represent the mean ±SD from the four independent experiments.

RESULTS AND DISCUSSION: DF appeared as dark-brown leaflets and contained about 27 % of γ-Fe_2O_3, 71 % dextran and 2% H_2O; the value of σ was 18 $A{\cdot}m^2/kg$, LD_{50} 5 g/kg. 12% DFF appeared as a dark-brown sol, pH 7, ζ 15 mW, M_s 1.5 kA/m, SAR 240 W/g Fe. TEM data allowed evaluation of the DF particles size: the maxima of the microcrystal and microsphere diameters were 12 and 240 nm, respectively; in a good accordance with the results of analytical fractionation and dynamic light scattering analysis of the DF particle samples and Gaussian/Nicomp and volume-weighted Gaussian distribution analysis of the particle diameter in diluted DFFs that appeared as 2 peaks at 205 and 220 nm (Fig. 1).

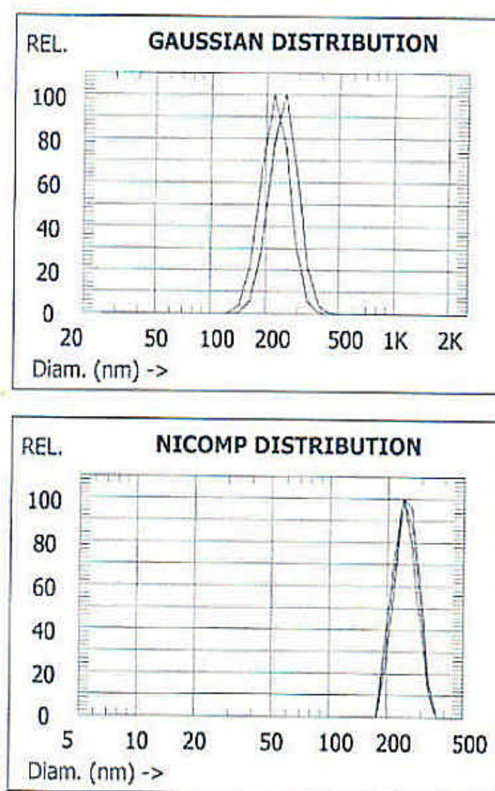

Fig. 1: Particle sizing analysis of DFF.

PG sol particle diameter distribution was in 3 peaks: peak 1 at 5 to 7 nm; peak 2 at 50 to 70 nm and peak 3 at 300 to 400 nm, and was in good accordance with PG gel-chromatography results. The obtained DFFs were resistant to gravitational forces, magnetic fields and liophylising. Determinations showed direct proportional decrease of $\grave{I}_s$ and heat production to decrease of DF concentration. Under the chosen conditions, the heating of a 0.9% NaCl solution was always below the detection limit. DFFs showed satisfactory heating to 2°C/mg Fe min. The experimental results are presented in Tables 1 and 4. No long-term toxicity or acute cell death was detected when cells were exposed to DFFs (up to 60 mg DF/ml) alone, or to AC magnetic field alone for periods of time up to 6 hours at +37°C. However, when P388 or CaOv cells were exposed to ACH at 41 to 44°C for 30 minutes in the presence of DFFs, the high hyperthermia effect was observed (Table 1).

Table 1. Influence of DF on CaOv cells during 30 min of AC magnetic field exposure.

Test tubes	Surviv. cells, %	Dead cells, %	DF, mg/ml	T, °C
1	0.0	100±6	60.0	43-44
2	4±0.8	96±5.8	6.0	42-43
3	48±3.4	52±3.6	1.00	41-42
4	91±5.5	9±1.4	0.10	39-40
5	95±5.8	5±1.0	0.01	37-38
6	96±5.8	4±0.8	0.00	37

Table 1 shows: the temperatures in TTs 1-6 were proportional to DF concentration. The cell death fractions were proportional to DF concentration: the cell survival fractions at 37 to 41°Ñ were high, at 42 to 43°Ñ were insignificant, and at 43-44°Ñ were absent.

Table 2. Influence of PG on CaOv cells during 30 min of AC magnetic field exposure at 37°Ñ.

T. T.	Surviv. cells, %	Dead cells,%	Cell lysis, %	PG (μg/ml)
1	0.0	0.0	100±6	325.00
2	9.5±1.5	60±4.0	30.5±2.5	32.50
3	54±3.7	28±2.5	18±1.9	3.25
4	87.3±5.5	5.7±1.0	7.0±1.3	0.65
5	91±5.5	5±1.0	4±0.8	0.06
6	95±5.8	4±0.8	1±0.1	0.00

Table 2 shows PG cytotoxicity obtained by magnetosensitization at 37°C for 30 minutes: the cell lysis and death rate fractions at the low PG concentrations were surprisingly high.

Table 3. Influence of PG on CaOv cells during 30 min exposure in the flow thermostat at 37°Ñ (TTs 1-3) and at 41°Ñ (TTs 4-6).

T. T.	Surviv. cells,%	Dead cells, %	Cell lysis, %	PG, (μg/ml)
1	3±0.6	6±1.1	91±5.5	325
2	29±2.5	50±3.5	21±2.0	32.5
3	66±4.3	16±1.8	18±2.4	3.25
4	10±1.5	55±3.8	35±2.8	32.5
5	38±2.9	39±3.0	23±2.2	3.25
6	82±5.1	14±1.7	4±0.8	0.32

Table 3 shows of PG cytotoxicity obtained as a result of CaOv cell thermosensitization at 41°C for a 30 minute period. The cell lysis and death fractions were proportional to PG concentration and increased with increasing temperature. The cell survival fractions at 37°Ñ, PG concentration 325 μg/ml, and at 41°Ñ, PG concentration 32.5 μg/ml, were insignificant.

Table 4. Influence of DF and PG on CaOv cells during 30 min of AC magnetic field exposure at 41 to 43°Ñ (TTs 1-3) and at 37°Ñ (TTs 4-6).

T. T.	Surv. cells (%)	Dead cells (%)	Cells lysis (%)	DF, mg/ml	PG, (μg/ml)
1	0	91±5.5	9±1.4	9.0	0.80
2	0	88±5.4	12±1.6	6.0	1.60
3	3±1	52±3.6	45±3.3	3.0	32.50
4	20±2	45±3.3	35±2.8	0.10	3.25
5	10±1	50±3.5	40±3.0	0.01	32.50
6	0.0	0.00	100±6	0.001	325.0

Table 4 shows PG+DF cytotoxicity that were obtained at 41 to 43°C and at 37°C for a 30 minute period; the cell lysis and death fractions were proportional to the concentrations of PG and DF. As the result of combination ACH with MTS at the moderate concentrations of PG (0.8-32.5 μg/ml) and the high concentrations of DF (3-9 mg/ml) the cell survival fraction was absent; at the moderate PG concentrations (3.25-32.5 μg/ml) and the low DF concentrations (0.001-0.1 mg/ml) the cell survival fractions were average; at the high PG concentration (325 μg/ml) and the low DF concentration the cell survival fraction was absent.

Table 5. Influence of PG on P388 cells during 30 min flow thermostat exposure at +41°Ñ (test tubes 1-3) and at +37°Ñ (test tubes 4-6).

T. T.	Surviv. cells, %	Dead cells, %	Cells lysis, %	PG (μg/ml)
1	9±1.4	54±3.7	37±2.9	32.5
2	39±3.0	36±2.8	25±2.3	3.25
3	70±4.5	18±1.9	12±1.6	0.325
4	29±2.5	40±3.0	31±2.5	32.50
5	88±5.4	8±1.2	4±0.8	3.25
6	91±5.5	5±1.0	4±0.8	0.325

Table 5 shows PG cytotoxicity that was obtained by the incubation of P388 cells at 41°Ñ and 37°Ñ for a 30 minutes period, the cell lysis and death fractions were proportional to the concentrations of PG and temperature. As a result of hyperthermia at 41°Ñ for 30 minutes with PG thermosensitization of P388 cells at concentrations of PG (0.325-32.5 μg/ml) the cell survival fractions were proportional to the concentrations of PG; at 37°Ñ and the same PG concentrations the cell survival fractions were three times as high.

Table 6. Influence of PG and His on P388 cells during 30 min of AC magnetic field exposure at 37°C (test tubes 1-3) and at 41°Ñ (test tubes 4-6).

T. T.	Surv. Cells, %	Dead cells, %	Cells lysis, %	PG, μg/ml	His, mg/ml
1	94±5.7	6±1.2	0.00	0.00	1.6
2	9±1.4	51±3.5	40±3.0	32.5	0.0
3	77±4.9	12±1.6	11±1.5	32.5	1.6
4	84±5.2	16±1.8	0.00	0.00	1.6
5	3±0.6	51±3.5	46±3.3	32.5	0.0
6	63±4.2	20±2	17±1.8	32.5	1.6

Table 6 shows that substantial inhibition of cell lysis and death by PG in the presence of 1.6 mg/ml His was observed. AC magnetic field cell damage enhancement by PG at 37°Ñ (TT 2) and simultaneous thermal- and AC magnetic field cell damage enhancements by PG at 41°Ñ (TT 5) was effectively suppressed by the addition of singlet oxygen scavenger, His (TTs 3,6).

We investigated the role of: DF, PG, His, PG+DF, PG+His alone; hyperthermia, AC magnetic field induction, DF AC magnetic field hyperthermia (ACH) alone; PG magneto- and thermo-sensitization in the dark (MTS) to increase the destruction of tumor cells. Two types of tumor cells: adherent human carcinoma ovarii (CaOv) and murine ascitic limpholeukosis P388 cells were incubated in the presence or absence of the enumerated reagents and physical factors. The cells were successively heated at 41 to 44°Ñ by AC magnetic field treatment with the 0.88 MHz, 9.3 kA/m, 0.15 kW induction coil. The combined effects of ACH and MTS were then examined and tested for statistical significance. Significant differences between cytotoxic effects produced by ACH, MTS and the combination of ACH with MTS were found. PG at nontoxic doses at 37°Ñ significantly enhanced magneto- and thermal tumor cell damage at 41°Ñ and above in a dose-dependent manner. Magneto- and thermal cell damage enhancement by PG was effectively suppressed by the addition of His, singlet oxygen and a superoxide scavenger. In the presence of PG+His non-toxic doses, the cell survival fractions were proportional to the temperature. Significant differences between cytotoxic effects produced by PG at 37°Ñ and 41°Ñ at the same concentrations of PG were found. Therefore the cytotoxicity of ACH should be attributed to the effects of heat itself. Combination of PG with DF have potential as a magneto- and thermosensitizer because of the following advantages: their dose-dependent enhancement of magneto- and thermal cell damage; lack of toxicity at physiological parameters, AC magnetic field (frequency, induction, strength, power and temperature), and at the non-toxic doses of PG+DF required for tumor cell magneto- and thermosensitization. Combination of ACH with MTS is the summary method. These data confirm the feasibility of using induction DF AC magnetic field hyperthermia in combination with tumor cell PG magneto- and thermosensitization. The advantage of this method is the much deeper penetration of the magnetic field into body tissues as compared to light. Further in vitro and in vivo investigations allow choosing of the PG+DF optimal doses and AC magnetic field range intensity and continuity.

CONCLUSIONS: Dissolution of dextran-ferrite in water results in formation of dextran-ferrite ferrifluids useful for the magnetically controlled combination of AC magnetic field induced hyperthermia with photogem magneto- and thermosensitization of tumor cells. The mechanism of ferrimagnetic heating most likely involves the magnetization relaxation loss process, and tumor cells photogem magneto- and thermosensitization most likely involves free-radical processes with a key role of superoxide radical.

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