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Application of Oil-in-Water Nanoemulsion Carrying Size-Defined Gold Nanoparticles Synthesized by Non-thermal Plasma for the Human Breast Cancer Cell Lines Migration and Apoptosis

Anna Dzimitrowicz¹ • Aleksandra Bielawska-Pohl² • Pawel Pohl¹ • Dorota Jermakowicz-Bartkowiak³ • Piotr Jamroz¹ • Magdalena Malik-Gajewska⁴ • Aleksandra Klimczak² • Piotr Cyganowski³

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Abstract

In this work, the gold nanoparticles (AuNPs) were synthesized using pulse-modulated radio-frequency atmospheric pressure glow discharge (pm-rf-APGD). By tailoring selected operating parameters of the pm-rf-APGD reaction-discharge system, the experimental conditions for the synthesis of raw-AuNPs with controlled optical and structural properties were found. The colloidal suspension of the size-controlled raw-AuNPs was mixed with an aqueous solution of gelatine and turmeric oil to produce an oil-in-water (O/W) nanoemulsion. AuNPs loaded into the nanoemulsion were characterized using ultraviolet-visible absorption spectrophotometry, dynamic light scattering, scanning electron microscopy supported by energy dispersive X-ray spectroscopy, and transmission electron microscopy equipped with selected area X-ray diffraction. Additionally, attenuated total reflectance Fourier-transform infrared spectroscopy was used to confirm the efficient functionalization of the AuNPs by nanoemulsion component. It was revealed that AuNPs were mostly spherical with an average size of 4.6 ± 1.0 nm and a face-centered cubic crystal system. The developed O/W nanoemulsion carrying AuNPs was applied towards the human breast cancer cell lines MCF7 and MDA-MB-231. It was found that it exhibited the cytotoxicity towards the breast cancer cells while were non-cytotoxic towards the non-tumour breast cells MCF10A. Moreover, it also inhibited the migration of the invasive cancer breast cells (line MDA-MB-231) and hence, could prevent the breast cancer metastasis.

Keywords Cold atmospheric pressure plasma · Pulse-modulated radio-frequency atmospheric pressure glow discharge · Nanomaterials · Cytotoxicity

Abbreviations

ATR FT-IR	Attenuated total reflectance Fourier-transform infrared spectroscopy
AuNPs	Gold nanoparticles
CAPP	Cold atmospheric pressure plasma

Anna Dzimitrowicz anna.dzimitrowicz@pwr.edu.pl

Extended author information available on the last page of the article

DAMP	4,6-Diamino-2-mercaptopyrymidyne
dc-APGD	Direct current atmospheric pressure glow discharge
D _H	Hydrodynamic diameter
DF	Degrees of freedom
DLS	Dynamic light scattering
DOE	Design of experiments
EDX	Energy dispersive X-ray spectroscopy
fcc	Face-centered cubic
FAAS	Flame atomic absorption spectrometry
FLA	Flowing liquid anode
FLC	Flowing liquid cathode
FLE	Flowing liquid electrode
GSH	Glutathione
LSPR	Localized surface plasmon resonance
MCF7	Human breast cancer cell lines
MCF10A	Non-tumour breast cells
MDA-MB-231	Human breast cancer cell lines
MS	Mean of squares
O/W	Oil-in-water
pm-rf-APGD	Pulse-modulated radio-frequency atmospheric pressure glow discharge
PSMA-1	Prostate-specific membrane antigen
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
RONS	Reactive oxygen and nitrogen species
RSM	Response surface methodology
SAED	Selected area electron diffraction
SEM	Scanning electron microscopy
SS	Sum of squares
TEM	Transmission electron microscopy
UV–Vis	Ultraviolet-visible

Introduction

Currently, a rapid development in the production and utilization of nanomaterials of certain properties has been observed. Among many nanomaterials designed for defined purposes, a special attention has been paid to the synthetic organic and inorganic nanoparticles (NPs) that are designed for the medical applications and can be produced in the laboratory settings. The organic NPs might be loaded into the polymeric micelles and liposomes, improving the therapeutic efficacy of the anticancer drugs [1]. Particularly, the polymeric micelles with the amphiphilic molecules are used in the delivery of the water-insoluble drugs [2]. The liposomes are of interest as well as they are highly biocompatible and biodegradable [3]. These novel selective drug-delivery systems are an important approach that has a great potential to overcome the problems associated with the systemic toxicity and poor bioavailability of the antineoplastic drugs [4].

Although the mentioned organic nanostructures draw a high attention, the inorganic gold nanoparticles (AuNPs) are involved in one of the most studied cancer treatment approaches [5]. Indeed, the AuNPs can provide many favourable properties to a drug, especially

including a longer elimination time, an increased drug-site contact time, and a reduced drug resistance [6, 7]. There are many methods used to produce the AuNPs in the laboratory settings, counting the chemical reduction [8], the sol–gel precipitation [9], and the green synthesis [10]. Unfortunately, all these methods usually involve the multi-step processes and complex chemical treatments that require a relatively high labour investment and generate costs. Moreover, the efficiency of the synthesis of the AuNPs with the above-mentioned methods can be limited, while the fabricated nanomaterials are still not ready-to-use and commonly need an additional purification before their specific biological or medical applications [10, 11].

A solution to the obvious disadvantages of the methods of the AuNPs synthesis cited above is the method based on cold atmospheric pressure plasma (CAPP), which has been proven to efficiently and simply fabricate them in a fast, single-step process [12–30]. In this novel method, the AuNPs' precursor solutions are treated by the different CAPP sources, resulting in the formation of the Au nanostructures of the certain morphology, which can additionally be tuneable by the appropriate changes in the operating parameters of these sources. Here, the AuNPs synthesis is associated with the action of the reactive oxygen and nitrogen species (RONS) as well as the hydrogen radicals (H·) and the hydrated electrons (e_{aq}^{-}) that are efficiently generated in the CAPP phase, the treated solutions, and the interfacial zone [12]. The method provides the AuNPs that have the desirable and reproducible physical dimensions, crystallinity, and spectral properties, and are ready-to-use without any initial pre-treatments. Nevertheless, all the previously reported CAPP sources were operated in non-flowing reaction-discharge systems [12–30], considerably limiting in this way the scale of the AuNPs production.

Bearing this in mind, our research group has developed and extensively examined the CAPP-based method for the noble metal nanostructures synthesis in the high-throughput, continuous-flow reaction-discharge systems [31-34]. In these systems, direct current atmospheric pressure glow discharge (dc-APGD) was operated between the surface of a flowing liquid anode (FLA) [32, 33] or a flowing liquid cathode (FLC) [31–34] and a nozzle Ar jet [31, 32, 34] or a sharpened pin-type electrode (with no discharge gas required, completely in the open-to-air atmosphere) [33]. By appropriately adjusting the working parameters of the dc-APGD systems, including the discharge current [31, 33], the AuNPs precursor solution flow rate [40, 42], and the flow rate of the jet-supporting gas [31], it was possible to find the operating parameters under which the size-defined AuNPs were reproducibly produced. In addition, the obtained AuNPs were stabilized by the addition of a natural biopolymer, i.e., gelatin [31, 32, 34], or a synthetic polymer, i.e., polyvinylpyrrolidone (PVP) [34] or polyvinyl alcohol (PVA) [34], to the precursor solutions or just by the electrostatical repulsions of the AuNPs [33]. To the best of our knowledge, this is a first work in which size-controlled raw-AuNPs have been synthesized with the aid of the pulse-modulated radio-frequency atmospheric pressure glow discharge (pm-rf-APGD) in the employed for that purpose highly-throughput reaction-discharge system under defined operating conditions. Because the polarity of the electrodes changes continuously, the obtained nanostructures might be monodisperse with the low size distribution.

According to the World Cancer Research Fund International, the breast cancer is the most prevalent cancer among women with approximately 2 million new cases reported in 2018 [35]. The high number of the breast cancer patients has led to a significant interest in the development of the novel, efficient, and faster therapies against it [5]. These therapies are supported by the recent progress in molecular biology [36, 37] and in pharmacology [38], as highlighted by many patents and research papers in the last 15 years [39–41]. Besides the application of the new therapies, decreasing the incidence of the breast cancer

and the number of the related deaths [5], there are many additional issues that must be overcome. It includes the improvement of the quality of live of the breast cancer patients by minimizing the side effects of the applied therapies [5, 42]. A special attention has lately been paid to nanotechnology, which seems nowadays to be one of the most promising research fields that may help to overcome the drawbacks related to the side effects of the convention cancer therapies and improve the quality of live as well [5]. The nanotechnology-based therapies are associated with the interaction of the cellular and molecular components with the nanomaterials that possess the certain properties [43].

To enhance the efficiency of the AuNPs in the cancer treatment and detection, several ligands, including lycopene [44], folate-decorated polymers [45], prostate-specific membrane antigen (PSMA-1) [46], as well as 4,6-diamino-2-mercaptopyrimidine (DAMP) and glutathione (GSH) [47], have successfully been attached to the surface of these nanostructures. However, *Curcuma longa* (turmeric or "Indian saffron"), a popular herbal raw material [48], is particularly interesting for that purpose. The turmeric rhizomes are widely applied in contemporary medicine because they contain the biologically active compounds such as p-cymene, 1,8-cineole, terpinolene, ar-turmeone, curlone, curcuphenol, turmerone, β -bisabolene, p-cymen-8-ol, and α -pinen [48]. Toden et al. [49] lately showed that the anti-inflammatory efficiency of turmeric towards the colitis could be improved by applying its essential oil [49]. Zhou et al. [50] and Mirzaei et al. [51] summarized that turmeric could affect the cellular and molecular pathways entangled in the cancer development.

Due to the biological activity of turmeric [48-51], it was decided in the present work to use turmeric essential oil (i) to functionalize the surface of the raw-AuNPs produced with pm-rf-APGD under defined operating conditions to enhance their cytotoxicity towards the human cancer cell lines and (ii) to prepare the homogenous oil-in-water (O/W) nanoemulsion carrying the produced raw-AuNPs. In the later case, it was motivated by the fact that the nanoemulsions are the most promising nanocarries used to improve the therapeutic efficacy of the anticancer drugs, commonly overcoming the problems associated with their systemic toxicity and poor bioavailability [52, 53]. According to the best of our knowledge, this is a first work in which size-defined raw-AuNPs have been immediately mixed with the gelatine and turmeric oil, resulting in the O/W nanoemulsion production. For that reason, the main aim of this studies was to synthesize the AuNPs with the aid of the pm-rf-APGD reaction-discharge system, working in the highly-throughput mode. The effect of the operating parameters of the pm-rf-APGD reaction-discharge system on the wavelength of the maximum (λ_{max}) of the localized surface plasmon resonance (LSPR) absorption band of the synthesized raw-AuNPs, as determined by ultraviolet-visible (UV-Vis) absorption spectrophotometry (UV–Vis), and their mean hydrodynamic diameter (D_H) , as determined by dynamic light scattering (DLS), was studied using the design of experiments (DOE) followed by the response surface methodology (RSM). The both responses of the examined system were next modeled in reference to the size-controlled production of the uncoated spherical Au nanostructures. The resultant colloidal suspensions of the AuNPs of a certain size were mixed with an aqueous solution of gelatine, being a natural biopolymer and emulsifier, and turmeric oil to obtain the O/W nanoemulsion carrying the AuNPs functionalized by nanoemulsion components (turmeric oil and aqueous solution of gelatine). The utilization of gelatine was related to the stabilization and protection from aggregation AuNPs produced with the aid of the pm-rf-APGD. The AuNPs loaded into the nanoemulsion were characterized to reveal their optical and structural properties as well as to confirm their functionalization. Finally, the developed O/W nanoemulsion carrying AuNPs was examined with respect to its migration and cytotoxic activity against the cancer cells, i.e., the human breast cancer cell lines MCF7 and MDA-MB-231, as well as a non-tumour breast cells (line MCF10A).

Experimental Procedures

Reagents and Solutions for the Raw-AuNPs Synthesis

Solid chloroauric acid tetrahydrate (HAuCl₄×4H₂O, Pol-Aura, Olsztyn, Poland) was used as the precursor for the synthesis of the uncoated Au nanostructures. To prepare the working solutions of the Au(III) at concentrations of 50, 125 and 200 μ g mL⁻¹, the appropriate amounts of HAuCl₄×4H₂O were dissolved in deionized water in the volumetric flasks and diluted using deionized water to the required volume.

Optimization of the Raw-AuNPs Synthesis by the Response Surface Methodology

Generally, to produce the raw-AuNPs by the CAPP-based synthesis method, the pm-rf-APGD based reaction-discharge system was used (Fig. 1) that was previously reported for the continuous-flow synthesis of the pectin-stabilized Ag nanostructures [54]. Here, a new set of the operating parameters that allows to obtain the entirely raw-AuNPs of the defined granulometric properties were selected and validated. Overall, in the high-throughput pm-rf-APGD-based reaction-discharge system, a AuNPs precursor solution (3), being the FLE of the discharge system, was pumped to a quartz chamber by a four channel peristaltic pump (Masterflex L/S, Cole-Parmer[®], Vernon Hill, II, USA) via a quartz-graphite tube (3, 7). At the interface between the surface of the FLE solution (3) and the pin-type tung-sten electrode of the OD=4.0 mm (1), pm-rf-APGD (6) was sustained and stably operated. Operating pm-rf-APGD under the defined experimental conditions (see below in Table 1), the treated solution was collected for the further experiments.



Fig. 1 The pm-rf-APGD-based reaction-discharge system used for the high-throughput continuous-flow production of the uncoated Au nanostructures. The description of the items in the scheme: (1) the pin-type tungsten electrode, (2) the product: the raw-AuNPs, (3) a AuNPs precursor solution (acting as the FLE), delivered to a quartz discharge chamber, (4, 5) the plugins of the pm-rf voltage, (6) pm-rf-APGD, (7) the quartz-graphite tube

Run order	Actual (and code	Response				
	$A (mL min^{-1})$	B (kHz)	C (%)	$D \ (\mu g \ m L^{-1})$	$\overline{\lambda_{max}^{a}\left(nm ight) }$	$D_{\rm H}^{\rm b}({\rm nm})$
1	5.0 (0)	0.7 (-1)	50 (0)	200 (+1)	544	2.1 ± 0.9
2	6.0 (+1)	1.5 (0)	50 (0)	50 (-1)	540	0.9 ± 0.5
3	6.0 (+1)	1.5 (0)	50 (0)	200 (+1)	549	3.4 ± 0.8
4	5.0 (0)	0.7 (-1)	50 (0)	50 (-1)	536	1.6 ± 0.6
5	4.0 (-1)	1.5 (0)	50 (0)	200 (+1)	545	1.8 ± 0.7
6	5.0 (0)	1.5 (0)	70 (+1)	50 (-1)	540	1.9 ± 0.7
7	5.0 (0)	0.7 (-1)	70 (+1)	125 (0)	542	1.5 ± 0.9
8	6.0 (+1)	1.5 (0)	70 (+1)	125 (0)	540	1.2 ± 0.5
9	4.0 (-1)	1.5 (0)	50 (0)	50 (-1)	537	3.1 ± 0.7
10 ^c	5.0 (0)	1.5 (0)	50 (0)	125 (0)	543	3.3 ± 0.6
11	5.0 (0)	1.5 (0)	30 (-1)	200 (+1)	546	2.7 ± 1.2
12	4.0 (-1)	1.5 (0)	70 (+1)	125 (0)	538	1.3 ± 0.6
13	5.0 (0)	2.3 (+1)	50 (0)	200 (+1)	543	0.9 ± 0.3
14	5.0 (0)	1.5 (0)	70 (+1)	200 (+1)	541	0.9 ± 0.4
15	5.0 (0)	1.5 (0)	30 (-1)	50 (-1)	534	1.5 ± 0.5
16	5.0 (0)	0.7 (-1)	30 (-1)	125 (0)	540	1.3 ± 0.6
17	5.0 (0)	2.3 (+1)	50 (0)	50 (-1)	533	1.1 ± 0.4
18	5.0 (0)	2.3 (+1)	30 (-1)	125 (0)	541	0.9 ± 0.4
19	4.0 (-1)	2.3 (+1)	50 (0)	125 (0)	537	1.9 ± 0.6
20	6.0 (+1)	2.3 (+1)	50 (0)	125 (0)	538	2.7 ± 0.5
21	4.0 (-1)	0.7 (-1)	50 (0)	125 (0)	539	3.0 ± 0.6
22 ^c	5.0 (0)	1.5 (0)	50 (0)	125 (0)	538	3.8 ± 0.8
23 ^c	5.0 (0)	1.5 (0)	50 (0)	125 (0)	540	3.2 ± 0.8
24	6.0 (+1)	1.5 (0)	30 (-1)	125 (0)	542	1.3 ± 0.5
25	6.0 (+1)	0.7 (-1)	50 (0)	125 (0)	539	1.2 ± 0.8
26	4.0 (-1)	1.5 (0)	30 (-1)	125 (0)	540	1.9 ± 0.8
27	5.0 (0)	2.3(+1)	70 (+1)	125 (0)	538	2.3 ± 0.8

Table 1 The Box–Behnken response surface design matrix with the actual and coded values of the studied operating parameters of the reaction-discharge system, the randomized run order, and the response of the system, i.e., the λ_{max} (in nm) of the LSPR absorption band of the resultant raw-AuNPs, and their D_H (in nm)

^aThe wavelength of the maximum (λ_{max}) of the localized surface plasmon resonance (LSPR) absorption band of the raw-AuNPs as determined by UV–Vis

^bThe average size corresponding to the hydrodynamic diameter (D_H) as determined by DLS

^cThe center point—the operating parameters A, B, C, and D were set at (0, 0, 0, 0). A—The flow rate of the FLE solution (in mL min⁻¹). B—The frequency of the pulse modulation of the rf current (in kHz). C—The duty cycle (in %). D—The AuNPs precursor concentration (in $\mu g m L^{-1}$)

To tune the size of the raw-AuNPs synthesized in the applied pm-rf-APGD-based reaction-discharge system (Fig. 1), the effect of the selected operating parameters of this system was studied and modeled with the aid of the Box-Behnken DOE followed by the RSM [31, 33]. The following operating parameters were considered: (1) the flow rate of the FLE solution (A, in mL min⁻¹); (2) the frequency of the pulse modulation of the rf current (B, in kHz); (3) the duty cycle (C, in %); (4) the AuNPs precursor concentration (D, in $\mu g m L^{-1}$). The response surface design in the Box–Behnken geometry included

twenty-seven randomized experimental treatments at three different levels (coded as "-1", "0" and "+1") of the studied operating parameters, counting three center points for which all the operating parameters were set at the central "0" level. The boundary levels "-1" and "+1" of the operating parameters were arbitrary selected based on the ranges within which the reaction-discharge system was stably operated, i.e., 4.0–6.0 mL min⁻¹ in the case of A, 0.7–2.3 kHz in the case of B, 30–70% in the case of C, and 50–200 µg mL⁻¹ in the case of D.

The responses of the system were: (1) the λ_{max} of the LSPR absorption band of the resultant raw-AuNPs, and (2) their average size corresponding to the D_H as measured by DLS (see section "*The characterization of the raw-AuNPs*" for more details). All the experimental treatments within the mentioned response surface design were carried out in one block (Table 1).

The values of the λ_{max} of the LSPR absorption band and the D_H of the raw-AuNPs were modeled using a full quadratic function that included the main and quadratic effects of the operating parameters as well as their two-way interactions. To reduce the dimensionality of the models, and to facilitate the interpretation of the effects of the operating parameters, the forward selection of terms algorithm with α equal to 0.15 was used to find the regression equations modeling the both responses, i.e., the λ_{max} and the D_H. The ANOVA test was applied to determine the accuracy of the models (one for the λ_{max} , the second one for the D_H) in fitting the experimental data. Here, the model summary statistics, including the *p*-values for the model and the terms included in it, and the R^2 and S values, were considered and used to assess the reliability of the model. The models were also tested using the lack-of-fit test that provided the evidence of the models adequacy in explaining the measured responses of the pm-rf-APGD-based reaction-discharge system. Finally, the residuals established for the both models, indicating the differences between the measured responses (λ_{max} and D_{H}) and their values predicted by the models, were analyzed to assess the quality of the models and their usefulness in optimizing the system by the RSM. This was realized by viewing the normal probability plots, the histograms of the distribution of the standardized residuals, and the scatter plots of the standardized residuals versus the fitted values and the run order. All of these graphs were useful in discovering any outliers and the non-normality in the distributions of the residuals, and making the final decision about the acceptance of the models.

Next, the models were applied to find the optimal experimental conditions of the pm-rf-APGD-based reaction-discharge system that enabled to produce the smallest and the largest AuNPs. The largest raw-AuNPs were recognized to be those with the highest red shift of the LSPR band in the UV–Vis spectra and the highest average size as determined by DLS, while the smallest raw-AuNPs had the highest blue shift of the LSPR band in the UV–Vis spectra and the lowest average size that could reliably be measured by the DLS instrument used, i.e., 0.8 nm.

The Characterization of the Raw-AuNPs

The optical properties of the raw-AuNPs were assessed using UV–Vis. The solutions containing AuNPs were introduced into a quartz cuvette and placed in a holder of a Jasco V730 spectrophotometer (MD, Easton, USA). The UV–Vis absorption spectra were recorded in the range from 300 to 800 nm immediately after the pm-rf-APGD treatment of the $AuCl_4^$ solutions. Deionized water was used as a reference sample. Dynamic Light Scattering (DLS) was used to determine the D_H of the raw-AuNPs and their size distribution by number. It was done using a Photocor Complex device (Photocor Instruments, Tallin, Estonia) equipped with a 657.04 nm/36 mW laser. The solutions containing the raw-AuNPs were introduced into round vials and submerged in decalin. The data were collected at a scattering angle of 90° at 21.8 °C, and then analyzed with the DynaLS software (Alango Ltd., Tirat Carmel, Israel). The size distribution by number was established as the mean value for 5 independent measurements.

Preparation of the Nanoemulsion Carrying the Raw-AuNPs

At first, 4 mL of a 1.5% (w/v) water solution of gelatin ($Mw = 80,0000 \text{ g mol}^{-1}$, Rousselot International, Dubuque, IA, USA) was prepared. Next, this solution was mixed with the colloidal suspension of the pm-rf-APGD-synthesized raw-AuNPs at a volume ratio 1:2. Simultaneously, turmeric oil (Hemani Live Natural, Dubai, UAE) was added to the resulting mixture (0.020 g of oil), to obtain nanoemulsion. The final concentrations of AuNPs, gelatin, and turmeric oil in the prepared mixture were 133 µg mL⁻¹, 0.5% (w/v), and 0.17% (w/v), respectively.

The Characterization of the AuNPs-Loaded Nanoemulsion

The optical properties of the AuNPs-loaded nanoemulsion were assessed using UV–Vis absorption spectrophotometry as described in the section "*The characterization of the raw-AuNPs*".

The structural properties of the AuNPs loaded into the nanoemulsion were investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) supported by energy dispersive X-ray scattering (EDX) and selected are electron diffraction (SAED), respectively. The SEM analysis was carried out on a Hitachi S-3400 N SEM instrument (Japan) equipped with a Thermo Scientific Ultra Dry EDAX system (USA). The TEM analysis was made with the aid of a Hitachi H-800 TEM instrument (Japan) equipped with 11 Mpix CCD Olympus Quemesa camera. The average size distribution based on the TEM analysis was carried out using the ImageJ software, by measuring 724 nanostructures.

Attenuated total reflectance Fourier-transform infrared spectroscopy (ATR FT-IR) was used to find out whether the AuNPs were functionalized by the nanoemulsion components. The corresponding spectra were acquired using a Vertex 70v FTIR spectrophotometer (Bruker, Bremen, Germany).

Evaluation of the Nanoemulsion Cytotoxicity Towards the Breast Cancer Cell Lines

Cell Culture Conditions and the Experimental Groups

The human cancer cell lines MCF7 (ATCC[®] HTB-22TM, non-invasive breast cancer cells) and MDA-MB-231 (ATCC[®] HTB-26TM, invasive breast cancer cells) were obtained from ATCC, and used to test the biological activity of the AuNPs-loaded nanoemulsion. As a control, the MCF10A cell line (ATCC[®] CRL-10317TM, non-transformed breast cells) was used. The cell lines were cultured at 37 °C in an incubator under the 5% CO₂ and 95% air atmosphere. The cells were maintained in the Opti-MEM

with GlutaMAX (Thermo Fisher Scientific Inc., USA) medium supplemented with a 2% fetal bovine serum (FBS, Gibco, UK), a 100 U mL⁻¹ penicillin solution and a 100 μ g mL⁻¹ streptomycin solution (Sigma-Aldrich, Steinheim, Germany). In the routine cell culture, the MCF7 cells were supplemented with insulin (at 10 μ g mL⁻¹), while the MCF10A cells—with EGF (at 20 μ g mL⁻¹), insulin (at 10 μ g mL⁻¹), ECGS (at 50 μ g mL⁻¹), and hydrocortisone (at 0.5 μ g mL⁻¹). The cells were routinely passaged using a 0.05% trypsin/0.02% EDTA (w/v) solution.

In order to evaluate the effect of the AuNPs on the cancer and non-transformed cell lines, the cells were treated with different agents, i.e., the nanoemulsion compounds (for groups II, III, IV and VII) or with a solution of the AuCl₄⁻ ions (group I) or the colloidal suspension of the raw-AuNPs (group V) and the AuNPs-loaded nanoemulsion (group VI) for 24 h. The concentration of Au in the groups I, V and VI was previously determined using flame atomic absorption spectrometry (FAAS) after the initial wet digestion of the respective samples in *aqua regia*. For all the investigated agents containing Au (groups I, V and VI), the IC₅₀ was assessed using the MTT tests. In all the subsequent MTT tests and the apoptosis assays, the concentration of Au in the examined agents was 25 μ g mL⁻¹, while the concentration of 10 μ g mL⁻¹ of Au was used for the scratch test. As a control, the culture medium with neither Au species nor supplements was used for 24 h (control group). The details of all the agents/compounds used in the in vitro tests are given in Table 2.

MTT Assay

The cells were seeded in the 96-well plates at a concentration of 2×10^5 cells/well in the dedicated culture medium. The next day, the cells were treated with 25 µg mL⁻¹ of Au (for groups I, V and VI) in the medium without serum (Opti-MEM). After 24 h, the cells were washed with PBS twice, followed by the addition of 100 µL of a MTT solution (0.4 mg mL⁻¹ of MTT) for the reaction for 3 h in the dark at 37 °C. Then, the MTT solution was removed, and 100 µL of DMSO was added to each well and incubated at 37 °C for 10 min to dissolve the purple crystals. The absorbance was measured at 570 nm with a Victor 2 multi-function microplate reader (Perkin Elmer, USA). The cell viability was calculated as the mean value of the absorbance acquired in triplicates in three independent experiments.

Name of the group	Treatment
Control	Cells treated with a culture medium alone
Group I	Cells treated with a solution of the $AuCl_4^-$ ions
Group II	Cells treated with the pm-rf-APGD-activated water
Group III	Cells treated with an aqueous solution of gelatin
Group IV	Cells treated with turmeric oil dispersed in water
Group V	Cells treated with the colloidal suspension of the raw-AuNPs
Group VI	Cells treated with the nanoemulsion carrying the AuNPs
Group VII	Cells treated with turmeric oil dispersed in an aqueous solution of gelatin

 Table 2
 The group of the agents/compounds used in the in vitro tests

Apoptosis Analysis

The apoptosis was measured by flow cytometry using an eBioscienceTM Annexin V Apoptosis Detection Kit APC (Invitrogen) to assess the number of the late apoptotic, early apoptotic, and necrotic cells by propidium iodide (PI) in all the groups compared to the control cells treated only with the culture medium alone. Briefly, the cells were cultured in the medium containing serum in the 48-well plates. After reaching the 80-90% confluence, the studied agents (see details in Table 4) at the Au concentration of 25 μ g mL⁻¹ were added to each well, and the cells were incubated for further 24 h. Annexin V and PI were then added to each sample according to the manufacturer's instructions. The early apoptotic cells (Annexin V positive, PI negative), late apoptotic cells (Annexin V positive, PI positive), necrotic cells (Annexin V negative, PI positive) and alive cells (Annexin V negative, PI negative) were detected using the FL4 $(\lambda_{em} = 660 \text{ nm})$ and FL2 $(\lambda_{em} = 535 \text{ nm})$ modes. The data were analyzed using a FACS-Calibur flow cytometer (Becton–Dickinson, USA). The percentage of the apoptotic cells was calculated using the Flowing Software 2 program. All the values were presented as the mean \pm SD values for three independent experiments, each consisting of the technical triplicates. The results were analyzed through the unpaired t-tests using the Graph-Pad Prism 5 software. The p-values for all the investigated groups were calculated compared to the control group (the cells treated with the medium alone).

Scratch Test

The human cell lines were seeded in the 48-well plates with each well containing 2×10^5 cells in the Opti-MEM medium. After the cell adhesion, the studied agents (see details in Table 4) were added to each well, and the cells were incubated for further 24 h. The final concentration of Au was reduced to 10 µg mL⁻¹ in order to investigate migratory effect of alive cells. Next, the cells were washed twice in PBS and a scratch area was formed by scratching with a 10 µL tip and the residual cells were washed twice with PBS. Then, the photographs were taken by an optical microscope at 0 h and after the incubation for 24 h, which were subjected to the analysis by using the ImageJ software. The cell migration area on the both sides was calculated based on the difference between the whole area and the middle scratch area (the relative scratch area). The migration rate over the 24-h period was calculated by dividing the cell migration area after 24 h of the treatment by that at 0 h. The results were analyzed through the unpaired t-tests using the GraphPad Prism 5 software. The *p*-values for all the investigated groups were calculated compared to the control group (the cells treated with the medium alone).

Results and Discussion

Response Surface Regression Models for the λ_{max} of the LSPR Absorption Band of the Synthesized Raw-AuNPs and Their D_H as Measured by DLS

Response surface regression models were developed for both the (1) optical and (2) granulometric properties of the resultant raw-AuNPs. These parameters were collected either by determining the λ_{max} of the LSPR absorption band (as determined by UV–Vis absorption spectrophotometry) or by the D_H of the raw-AuNPs (as determined by DLS).

The quality of the both responses acquired according to the Box-Behnken surface response design was verified. The mean values of the λ_{max} of the LSPR absorption band of the raw-AuNPs and their D_H were viewed versus the run order. The mean values of the both responses versus their standard deviations were also considered. In the both cases, no trends were found in the resultant scatter plots. In addition, the differences in the both responses between the treatments were higher than the variability of these responses within the treatments. This particularly pointed out that the both responses acquired for the studied reaction-discharge system, i.e., the λ_{max} of the LSPR absorption band of the synthesized raw-AuNPs and their D_{H} , were varied due to the changes in the operating parameters. Therefore, the both responses were not transformed to stabilize their variance observed during the operation of the studied reaction-discharge system [31, 33]. Next, the experimental data for the λ_{max} of the LSPR absorption band of the synthesized raw-AuNPs and their D_H (the means for 3 repetitions) were fitted with the full quadratic regression models. The number of the possible terms in the response surface regression equations was reduced by using the forward-selection-of-terms algorithm. Accordingly, the developed response surface regression model for the λ_{max} was given by the following equation (Eq. 1):

$$\lambda_{\max}(\text{in nm}) = 514.2 + 1.00\text{A} + 6.93\text{B} + 2.12 \times 10^{-1}\text{C} + 1.45 \times 10^{-1}\text{D} - 3.00\text{B}^2$$

$$-1.83 \times 10^{-3}\text{CD}.$$
(1)

In the case of the size of the synthesized raw-AuNPs, corresponding to the $D_{\rm H}$, the response surface regression model was given by the following equation (Eq. 2):

$$D_{\rm H}({\rm in\ nm}) = -9.65 + 2.22 \times 10^{-1}{\rm B} + 3.41 \times 10^{-1}{\rm C} - 1.05 \times 10^{-2}{\rm D} - 5.24 \times 10^{-1}{\rm A}^2$$

-1.00B²-2.96 × 10⁻³C²-1.33 × 10⁻⁴D² + 8.05 × 10⁻¹{\rm AB} + 1.29 × 10⁻²{\rm AD}
-3.77 × 10⁻⁴CD. (2)

The reliability of the both response surface regression models was tested with the aid of the analysis-of-variance (ANOVA) test and the lack-of-fit test. The results of these tests are given in Tables 3 and 4, respectively, for the λ_{max} and the D_H.

In the case of the response surface regression model for the λ_{max} of the LSPR absorption band of the raw-AuNPs synthesized using the continuous-flow reaction-discharge system proposed in the present work, the linear terms A (p=0.000), B (p=0.058), and C (p=0.110), in addition to the non-linear terms, i.e., B² (p=0.019) and CD (p=0.005), were statistically significant because the p values for them were lower than $\alpha = 0.15$. The statistically insignificant term D (p=0.511) was however included in the equation Eq. 1 of the model because of the hierarchy of the terms. The R-squared (R^2) value for this model, showing its goodness-of-fit, was 81.6% and indicated that there was a quite high closeness of the experimental data with the data fitted by the model; almost 82% of the total variance in the acquired experimental data was explained with the developed response surface regression equation Eq. 1. The adjusted (R^2 adjusted) and predicted (R^2 predicted) R-squared values were also high, i.e., 76.0 and 67.0%, respectively, showing that the mentioned model had a high power for explaining the measured data and predicting the completely new data. The p value calculated for the lack-of-fit test (p=0.884) was much higher than $\alpha = 0.15$ taken, hence, the model was certainly statistically significant. The standard error of the regression (S) was also relatively low, while the inspection of the residuals (see Fig. 2a) let to the conclusion that the goodness-of-fit of the response surface regression

Source of data	DF	Adjusted SS	Adjusted MS	F value ^a	p value ^b
Model	6	263.18	43.86	14.75	0.000
Linear	4	213.67	53.42	17.96	0.000
А	1	12.00	12.00	4.03	0.058
В	1	8.33	8.33	2.80	0.110
C	1	1.33	1.33	0.45	0.511
D	1	192.00	192.00	64.56	0.000
Square	1	19.27	19.27	6.48	0.019
B ²	1	19.27	19.27	6.48	0.019
2-Way interactions	1	30.25	30.25	10.17	0.005
CD	1	30.25	30.25	10.17	0.005
Error	20	59.48	2.97		
Lack-of-fit	18	46.82	2.60	0.41	0.884
Pure error	2	12.67	6.33		
Total	26	322.67			

Table 3 The ANOVA test table for the response surface regression model of the λ_{max} of the LSPR absorption band of the uncoated AuNPs synthesized by using the reaction-discharge system with pm-rf-APGD generated in contact with the FLE solution containing the AuNPs precursor

p values < 0.15 are given in italics

^aThe value of the *F* test for the comparison of the model variance with the residual variance

^bThe forward-selection-of-terms algorithm was used, α to enter equal to 0.15. LSPR—The localized surface plasmon resonance. pm-rf-APGD—Pulse-modulated radio-frequency atmospheric pressure glow discharge. FLE—The flowing liquid electrode. DF—Degrees of freedom in the source. SS—The sum of squares due to the source. MS—The mean of squares due to the source. A—The flow rate of the FLE solution (in mL min⁻¹). B—The frequency of the pulse modulation of the rf current (in kHz). C—The duty cycle (in %). D—The AuNPs precursor concentration (in µg mL⁻¹)

model to the measured values of the λ_{max} was high, confirming the correctness and appropriateness of the developed model for the optimization of the system. Accordingly, the distribution of the standardized residuals had no signs of the non-normality, the standardized residuals were randomly placed on the both sides of the "0" axis, falling rather a symmetric pattern with a more or less constant spread throughout the range of the fitted values or the run order.

In the case of the response surface regression model for the average D_H of the raw-AuNPs, the following non-linear terms were statistically significant in the response surface regression equation Eq. 2: A^2 (p=0.040), B^2 (p=0.001), C^2 (p=0.000), D^2 (p=0.006), and AB (p=0.030), AD (p=0.003), as well as CD (p=0.053). All the linear terms, i.e., A, B, C, and D, were statistically insignificant ($p > \alpha = 0.15$), but they were kept in the regression equation because of the hierarchy of the terms. The goodness-of-fit of the established response surface regression model, as represented by the R² value of 79.6%, was also high. The R² adjusted and predicted values (64.6 and 36.3%, respectively) were fair but not as high as in the case of the R² statistics for the model of the λ_{max} . Nevertheless, the *p*-value (p=0.247) for the lack-of-fit test was higher than $\alpha=0.15$, while the S value was 0.5; hence, there was no reason to reject the model. The inspection of the residuals also confirmed that the developed response surface regression model for the D_H fitted the experimental data well. The normal probability plot, the histogram of the frequency distribution of the standardized residuals and the scatter-plots of the standardized residuals

Source of data	DF	Adjusted SS	Adjusted MS	F value ^a	p value ^b
Model	11	16.95	1.54	5.31	0.002
Linear	4	0.76	0.19	0.66	0.630
А	1	0.44	0.44	1.50	0.239
В	1	0.07	0.07	0.23	0.635
С	1	0.02	0.02	0.09	0.773
D	1	0.24	0.24	0.82	0.381
Square	4	9.49	2.37	8.19	0.001
A ²	1	1.46	1.46	5.04	0.040
B^2	1	4.55	4.55	15.69	0.001
C^2	1	7.48	7.48	25.81	0.000
D^2	1	3.00	3.00	10.34	0.006
2-Way interactions	3	6.69	2.23	7.69	0.002
AB	1	1.66	1.66	5.73	0.030
AD	1	3.75	3.75	12.92	0.003
CD	1	1.28	1.28	4.41	0.053
Error	15	4.35	0.29		
Lack-of-fit	13	4.16	0.32	3.46	0.247
Pure error	2	0.18	0.09		
Total	26	21.30			

Table 4 The ANOVA test table for the response surface regression model of the average D_H of the raw-AuNPs synthesized by using the reaction-discharge system with pm-rf-APGD generated in contact with the FLE solution containing the AuNPs precursor

p values < 0.15 are given in italics

^aThe value of the F test for the comparison of the model variance with the residual variance

^bThe forward selection of terms algorithm was used, α to enter equal to 0.15. D_H—The hydrodynamic particle diameter. pm-rf-APGD—Pulse-modulated radio-frequency atmospheric pressure glow discharge. FLE—The flowing liquid electrode. DF—Degrees of freedom in the source. SS—The sum of squares due to the source. MS—The mean of squares due to the source. A—The flow rate of the FLE solution (in mL min⁻¹). B—The frequency of the pulse modulation of the rf current (in kHz). C—The duty cycle (in %). D—The AuNPs precursor concentration (in μ g mL⁻¹)

versus the fitted values and the run order did not exhibit any unusual structures or patterns (see Fig. 2b). This confirmed the correctness of the model and its usefulness for the optimization of the experimental conditions of the reaction-discharge system for the synthesis of the size-defined surfactant free AuNPs.

The Effect of the Operating Parameters on the λ_{max} of the LSPR Absorption Band of the Synthesized Raw-AuNPs and Their D_H

The both responses of the studied pm-rf-APGD-based reaction-discharge system were related to the size of the uncoated AuNPs. The λ_{max} of the LSPR absorption band, as evaluated by UV–Vis absorption spectrophotometry, was indirectly associated with the size of the AuNPs, as based on the Mie theory [12], while the D_H was directly measured by DLS. Considering the response surface regression models, the effect of the operating parameters A, B, C, and D on the λ_{max} of the LSPR absorption band of the raw-AuNPs and their D_H



Fig. 2 The distribution of the residuals in the developed response surface regression models (the normal probability plot, the histogram of the frequency distribution of the standardized residuals, the scatter-plots of the standardized residuals versus the fitted value and the run order) for the λ_{max} of the LSPR absorption band of the synthesized uncoated AuNPs (**a**) and their D_H (**b**)

(see Fig. 3) was acknowledged similar; however, for the λ_{max} this effect was primarily linear, whereas the squared terms were predominant in the model representing the D_H . In the both response surface regression models, by increasing the flow rate of the FLE solution (the operating parameter A) and the concentration of the AuNPs precursor (the operating parameter D), the raw-AuNPs with a larger size could be synthesized, as manifested by a "red shift" of the λ_{max} of their LSPR absorption band and their higher D_H values. This was



Fig.3 The graphical representation of the effect of the operating parameters on the responses of the reaction-discharge system with pm-rf-APGD, i.e., the λ_{max} of the LSPR absorption band of the synthesized raw-AuNPs and their D_H. **A** The flow rate of the FLE solution. **B** The frequency of the pulse modulation of the rf current. **C** The duty cycle. **D** The AuNPs precursor concentration

certainly coincident with the increase of the concentration of the $AuCl_4^-$ ions available for the reduction by the appropriate plasma-liquid interactions-derived reducing agents, e.g., H_2O_2 [13–15]. In such conditions, according to the Fike–Watzky two-step mechanism of the nucleation and growth of the Au nanostructures [16], at the higher concentrations of the $AuCl_4^-$ ions, the growth of the nucleated AuNPs seeds was likely faster because the reduction of the precursor ions could also take place on the surface of the AuNPs that adsorbed these ions. As a result, the larger raw-AuNPs were produced rather than the new nucleation seeds or the small AuNPs were formed in the solution due to the above-mentioned surfaceassisted reduction of the existing nucleated seeds and/or the small AuNPs [13–16].

The increase of the frequency of the pulse modulation of the rf current (the operating parameter B) gave rise to the gradual elevation of the λ_{max} of the LSPR absorption band of the raw-AuNPs and their D_H up to a certain level, i.e., 1.6 kHz. A further growth of the frequency of the pulse modulation of the rf current above this level was responsible for decreasing the both responses. In the case of the duty cycle of the pulse (the operating parameter C), its increase from 30 to 70% resulted in shifting the λ_{max} of the LSPR absorption band toward the shorter wavelengths (the "blue shift") and lowering the size of the synthesized raw-AuNPs as expressed by the $D_{\rm H}$ measured in these conditions. Such behavior of the studied reaction-discharge system with pm-rf-APGD occurred because the both operating parameters B and C affected the discharge power associated with the pulse width in the subsequent on-and-off cycles and the duration of the period with the active discharge, advancing the plasma-liquid interactions. The higher discharge power resulted in increasing the production rate of the raw-AuNPs, i.e., the higher formation of the new nucleation seeds rather than the growth of the small AuNPs [12, 14, 17]. However, this was not the only one benefit related to the usage of pm-rf-APGD. The fast interchanges of the polarity of the FLE solution from negative to positive within the pulses were certainly convenient for etching the raw-AuNPs and stabilizing their colloidal structure,

preventing from the aggregation of the nuclei and agglomeration of the nanostructures. This undoubtedly distinguishes the studied pm-rf-APGD-based reaction-discharge system from other reported so far in the literature [12–30]. All the cited works propose non-flow-through reaction-discharge systems with dc-driven APGDs generated in contact with the positively polarized solutions, being the example of the plasma cathode and the solution anode, where the surface of the solutions is irradiated with the electrons (e_g^-) coming from the discharge column. Although rf-APGD operated in the atmosphere of Ar in a non-flow-through system was previously applied for the synthesis of the DNA stabilized AuNPs, the gas pressure had to be reduced in this case just to 40.0 kPa because the plasma-treated solutions were overheated and evaporated [23, 24].

The novelty of the reaction-discharge system proposed in the present study was that pmrf-APGD was sustained at the atmospheric pressure (approximately 101.3 kPa) and completely operated in the ambient air (no additional gas was needed). The previously reported problem with the overheating of the plasma-treated solutions was solved by using the flowthrough system (the surface of the solution was continuously replenished) and the duty cycle operation that related to the certain discharge activity "on" and "off" times within the rf current pulse period.

Summarizing, aside from its flow-through character and possibility for the continuousflow production of the raw-AuNPs, the uniqueness of the developed reaction-discharge system lied in two additional phenomena that concurrently occurred due to the application of pm-rf-driven APGD. Accordingly, the pH of the FLE solutions lowered because the surface of the solutions, containing the AuNPs precursor, was bombarded with the $H_2O_{\sigma}^+$ ions when it was negatively polarized within the pulse (see the following reaction: $3H_2O + H_2O_g^+ = 2H_3O_{ag}^+ + 2OH^{\bullet} + e_{ag}^-$ [13, 15]). In the presence of the formed H_3O^+ ions and the Cl⁻ ions, released from the AuCl₄⁻ ions during their reduction [14], the resulting raw-AuNPs were etched to make their colloidal structure lower-sized and more monodispersed. Concurrently, when the surface of the FLE solutions was bombarded by the e_{σ}^{-} during its positive polarization within the pulse, the very large amounts of the charge were transferred to the solution [12–14]. The $e_{\overline{g}}$ that did not participate in the production of H_2O_2 molecules, i.e., $e_{\overline{g}} + H_2O = OH^{\bullet} + H^{-}$, $2OH^{\bullet} = H_2O_2$, likely interacted with the raw-AuNPs, which could have captured them, and contributed to the formation of a negative charge on their surface [13]. Hence, the electrostatic stabilization of the colloidal structure of the synthesized surfactant free AuNPs was provided in these conditions by the Coulomb repulsions in the solution [13].

Size-Controlled Synthesis of the Raw-AuNPs

On the basis of the developed response surface response regression models for the λ_{max} and the D_{H} , the operating parameters of the reaction-discharge system with pmrf-APGD were selected to provide the electrostatically stable raw-AuNPs with the largest and the smallest size. In the case of the largest raw-AuNPs, it was established that the following parameters should be applied for the synthesis: the flow rate of the FLE solution (A)—6.0 mL min⁻¹, the frequency of the pulse modulation of the rf discharge current (B)—1.6 kHz, the duty cycle (C)—40%, and the AuNPs precursor concentration (D)—200 µg mL⁻¹ (see Fig. 3). According to the both developed models, the synthesized in these experimental conditions AuNPs would be characterized by the λ_{max} of their LSPR absorption band of 547±1 nm and have the D_H of 3.2±0.4 nm. To verify the accuracy of the both models and the correctness of the selection of the optimal conditions for the synthesis of the largest raw-AuNPs, the examined reaction-discharge system was run under the selected operating parameters. The resultant pm-rf-APGD-treated solutions, containing the synthesized raw-AuNPs, were collected and analyzed by UV–Vis absorption spectrophotometry and DLS. In five independently repeated experiments, it was established that the raw-AuNPs produced in these conditions exhibited the λ_{max} of the LSPR absorption band at 552±1 nm and had the D_H of 3.2±0.4 nm as displayed in Fig. 4a.

In a similar way, it was ascertained that the following operating parameters would provide the raw-AuNPs exhibiting the λ_{max} of their LSPR absorption band at 532 ± 1 nm and the D_H of 0.8 ± 0.5 nm: A—4.0 mL min⁻¹, B—2.2 kHz, C—35%, and D—50 µg mL⁻¹. Again, the studied reaction-discharge system was used to synthesize the raw-AuNPs under these conditions, and the collected plasma-treated solutions were subjected to the analysis by UV–Vis absorption spectrophotometry and DLS. The synthesized nanomaterial had the λ_{max} of the LSPR absorption band at 535 ± 4 nm and the D_H of 0.8 ± 0.6 nm (Fig. 4b). The both measures confirmed the usefulness of the RSM approach to reliably find the operating parameters for the size-controlled synthesis of raw-AuNPs with the aid of the studied pm-rf-APGD-based reaction-discharge system. The proposed methodology was also found to be useful for the continuous-flow



Fig. 4 The average size distribution and the UV–Vis absorption spectra recorded for \mathbf{a} the largest and \mathbf{b} the smallest raw-AuNPs synthesized by using the studied reaction-discharge system with pm-rf-APGD generated in contact with the FLE solution

synthesis of the pectin-stabilized AgNPs, exhibiting the cytotoxicity towards the human melanoma cancer cells [54].

The Optical and Structural Properties of the AuNPs Colloidal Suspension Loaded into Nanoemulsion

The Au nanostructures colloidal suspension obtained under the operating parameters for the production of the largest nanostructures were immediately loaded into an O/W nanoemulsion consisting of turmeric oil and a gelatin aqueous solution. The raw-AuNPs contained in the mixture were not purified before their loading into the nanoemulsion. The purification process could lead to their aggregation and sedimentation, hence, it was avoided. The optical and structural properties of the AuNPs loaded into the O/W nanoemulsion are presented in Fig. 5.

The λ_{max} of the LSPR absorption band of the AuNPs loaded into the nanoemulsion was located at 566 nm. As compared to the determined λ_{max} of 552 nm for the largest raw-AuNPs, this was red-shifted, suggesting a slight decrease in the average size of the Au nanostructure loaded into the nanoemulsion (see Fig. 5a). This could be the effect of gelatin and turmeric oil, which -OH functional groups could stabilize the AuNPs carried by the emulsion formed as reported in Ref. [55] for the Au and Ag nanostructures. To reveal the preliminary structural properties of the AuNPs loaded into the O/W nanoemulsion, the SEM was employed. As can be seen from Fig. 5b, the differentially shaped AuNPs were present in the nanoemulsion. Additionally, the EDX spectroscopy was used to find the elemental composition of the resulted nanoemulsion carrying the Au nanostructures. The EDX spectrum, corresponding to the point labeled as (1) in the SEM photomicrography (see Fig. 5b), confirmed the presence of the metallic Au. Additionally, C, O, and N, were detected due to the chemical structure of gelatin and turmeric oil. An imperceptible band (barely visible in Fig. 5b), attributed to the presence of Cl suggested that the conversion of the AuNPs precursor due to the plasma-liquid interactions was almost complete.

To verify the structural properties of the AuNPs loaded into the nanoemulsion, TEM supported by the SAED was also used. As displayed in Fig. 6a, the AuNPs of spherical, and triangular, were found in the nanoemulsion. Based on the all captured photomicrographs their size ranged from 0.5 to 237 nm; however, the majority of the AuNPs did not exceed 10 nm as shown in the particle size distribution in Fig. 6b. In fact, the mean



Fig. 5 a The UV–Vis absorption spectrum of the AuNPs-loaded nanoemulsion and b the SEM/EDX photomicrographs thereof



Fig. 6 a The TEM photomicrographs, b the average size of the AuNPs determined based on TEM and c the SAED pattern recorded for the AuNPs-loaded nanoemulsion

diameter calculated based on 724 nanostructures was 4.6 ± 1.0 nm. This well corresponded to the size of the raw-AuNPs determined using DLS (3.2 ± 0.4 nm). The SAED pattern displayed in Fig. 6c allowed to define the d-spacings of the analyzed nanostructures. Their values confirmed that the nanoemulsion indeed contained the face-centered cubic (FCC) crystalline AuNPs [56].

The Surface Functionalization of the AuNPs

In the next step, an attempt was made to confirm that the loading of the raw-AuNPs into the nanoemulsion had led to the functionalization of their surface by turmeric oil and gelatin. Accordingly, the ATR FT-IR spectra of all the separate components, i.e., gelatin, turmeric oil, raw-AuNPs, and all of the components combined together to form the nanoemulsion were recorded as shown in Fig. 7.

The spectrum of gelatin normally shows a series of the protein vibrations, mainly related to the amide absorptions, generating the bands called Amide A, I, II, and III [57, 58]. Indeed, the intense IR absorptions of the ν N–H (and ν O–H from H₂O) stretching vibrations, seen as a broad band at 3313 cm⁻¹ (Amide A band), were identified in the spectrum of a gelatin solution. The ν C=O group stretching vibration, observed as a strong band at 1644 cm⁻¹, was associated with the Amide I band (Fig. 7a). Finally, the medium band at 1534 cm⁻¹ was likely due to the ν N–H bending vibration and with a small contribution of the ν C–N stretching vibration recognized as the Amide II band (Fig. 7a) [59, 60].

The spectrum of turmeric oil contained a weak band at 1744 cm⁻¹ that was attributed to the ν C=O stretching vibrations of the ester groups occurring in natural oil (Fig. 7b). This



Fig. 7 The ATR FT-IR spectra recorded for the a gelatin, b turmeric oil, c raw-AuNPs, d AuNPs-loaded nanoemulsion

band was also observed in the spectrum of the AuNPs-loaded nanoemulsion, confirming the presence of turmeric oil in the final formulation. Besides, the medium broad band at 3316 cm⁻¹, assigned to the ν O–H stretching vibrations from water and turmeric oil, was also observed (Fig. 7b). Other absorption bands in this spectrum were also characteristic for oils; however, they were shifted as a resulting of the dispersion of turmeric oil in water as reported before [61].

The spectrum of the AuNPs-loaded nanoemulsion displayed some characteristic bands in the fingerprint region coming from gelatin and turmeric oil. However, these bands were shifted to the lower wavenumbers, which likely indicated the formation of the weak hydrogen bonds between the emulsion components and the AuNPs. This primarily included the shift of the strong peaks at 1644 cm⁻¹ (Fig. 7a) and 1658 cm⁻¹ (Fig. 7b) (ν C=O) in the spectra of gelatin and turmeric oil, respectively, to 1634 cm⁻¹ (Fig. 7d) in the spectrum of the nanoemulsion. Furthermore, some small wavenumber differences were found for the Amide II bands in gelatin (~3 cm⁻¹) (Fig. 7a vs. d) and the ν C=C stretching vibrations in turmeric oil (~5 cm⁻¹) (Fig. 7b vs. d). These differences suggested that the AuNPs were likely capped by turmeric oil and gelatin. Moreover, as the ATR FT-IR spectra suggested the presence of the weak hydrogen bonds between the AuNPs and turmeric oil and gelatin, it was concluded that the nanoemulsion not only capped the AuNPs but might also functionalized their surface.

Biological Effect of the Nanoemulsion Carrying the Au Nanostructures

Colorimetric MTT Assay

The cytotoxic effect of the AuNPs-loaded nanoemulsion (group VI) towards the human breast cancer and non-cancerous cell lines was examined using 3-(4,5-Dimethyl-2-thiazolvl)-2.5-diphenvl-2H-tetrazolium bromide (MTT). The AuNPs induced the dosedependent toxicity to the cancer cells. For the non-tumor MCF10A cell line, the half maximal inhibitory concentration (IC₅₀) was >40 μ g mL⁻¹ of Au. In contrast, for the noninvasive MCF7 cell line the IC₅₀ was around 30 μ g mL⁻¹ of Au, while the IC₅₀ towards the invasive MDA-MB-231 cell line was 25 μ g mL⁻¹ of Au (data not shown). Based on these results, all the cytotoxicity tests were done on the cells treated with 25 μ g mL⁻¹ of Au in all three groups where Au was present (groups I, V and VI). A strong inhibition of the proliferation of the invasive MDA-MB-231 breast cancer cells treated with the AuNPsloaded nanoemulsion (group VI) was observed (Fig. 8). In contrast, the proliferation of the non-invasive MCF7 breast cancer cell line was reduced after the treatment of the cells with an AuCl₄⁻ solution (group I), however, the proliferation inhibition was not statistically significant. The control non-tumor cell line MCF10A was not sensitive to all the examined groups containing Au. Additionally, for all other groups without Au (groups II, III, IV and VII, see details in Table 2) there were no difference in the absorbance between the investigated groups and the control in all the cell lines. These results showed that AuNPs-loaded nanoemulsion (group VI) could inhibit the proliferation of the invasive breast cancer cells and did not moderate the non-invasive cancer cells as well as the non-transformed breast cells. Similar results were reported by other researchers, who observed the inhibition of the breast cancer cells treated with the AuNPs [62, 63]. However, in our study it was observed that the proliferation activity of the human cancer invasive cells was selectively inhibited



Fig.8 The proliferation measured as the absorbance at 570 nm of the human non-transformed breast cell line (MCF10A), non-invasive cancer cell line (MCF7), and invasive cancer cell line (MDA-MB-231) treated with different agents (see the description of the groups given in Table 2). The data are given as the mean \pm SD values for three analyses done in triplicate. The statistical comparison was performed using the unpaired *t* test and compared to the untreated controls **p < 0.01

as compared with the proliferation activity of the non-invasive and non-transformed cells after the treatment with the AuNPs-loaded nanoemulsion (group VI for MDA-MB-231 vs. MCF7 and MCF10A cell lines).

Apoptosis Test

As shown in Fig. 9, the reduction of the viable cells (62% for MCF7 and 55% for MDA-MB-231; p < 0.05 and p < 0.01; respectively) was detected following their treatment with the AuNPs-loaded nanoemulsion (group VI, 25 µg mL⁻¹ of Au) and compared to the control cell population treated only with the culture medium. The majority of the tumor cells underwent the necrosis within 24 h. Similar results were observed when the invasive tumor cells MDA-MB-231 were treated with the AuCl₄⁻ ions (group I). No statistically significant apoptosis was detected when the non-transformed breast cells MCF10A were treated with the $AuCl_4^-$ ions (group I, 25 µg mL⁻¹ of Au) and the AuNPs-loaded nanoemulsion (group VI, 25 μ g mL⁻¹ of Au). The same was for the breast cancer cells treated with pm-rf-APGD-activated water (group II), a water solution of gelatin (group III), and turmeric oil dispersed in water (group IV). No statistically significant effect on the apoptosis rate compared to the control sample was observed and the apoptosis rate of 10-20% for the different cell lines was established (see Fig. 9). It was previously reported that combo nanoemulsion carrying the Au nanostructures in size 4.7 ± 1.1 nm and 3.3 ± 0.6 nm successfully induced the apoptosis in the colon cancer cell line [44]. Our study documented that the developed AuNPs-loaded nanoemulsion, carrying AuNPs in similar size 3.2 ± 0.4 nm, significantly induces the apoptosis of the breast cancer cells compared to the control conditions where the Au nanostructures are not present, confirming its unique anti-cancer activity.

Scratch Test for the Migration Activity

Next, the migration activity of the cells treated with all the studied agents was examined. As shown by the other researchers [63], the highest migratory activity is displayed by the MDA-MB-231 cell line, while the non-invasive MCF7 and non-tumor MCF10A cell lines migrate slower. As shown in Fig. 10, only the invasive MDA-MB-231 cell line displayed the impaired cell migration, i.e., the relative scratch closure (RSC) of 18%, when treated



Fig. 9 The percentage of the apoptotic cells. The human breast cell line (MCF10A) and the human cancer cell lines (MCF7 and MDA-MB-231) were treated for 24 h with different agents (see description of the groups given in Table 2). The percentages of the alive cells, early apoptotic cells, late apoptotic cells and necrotic cells were calculated as the mean \pm SD values of three independent experiments, each performed with triplicate wells for each treatment group. The statistical comparison was performed using the unpaired t-test and compared to the untreated controls *p < 0.05, **p < 0.01



Fig. 10 The scratch assays with the human breast cell line (MCF10A) and the human cancer cell lines (MCF7 and MDA-MB-231) performed to monitor the migratory activity of the cancer cells. All the cell types were grown to confluence and treated with different agents (see description of the group presented in the Table 4) for 24 h. Subsequently, the monolayers were scratched, and the migration rate was measured after 24 h by quantifying the relative scratch closure (RSC %). The data are presented as the mean \pm SD values for three analyses done in triplicate. The statistical comparison was performed using the unpaired *t* test and compared to the untreated controls *p < 0.05

with the AuNPs-loaded nanoemulsion (group VI, 10 μ g mL⁻¹ of Au). Concurrently, the RSC values for the MCF10A and MCF7 cell lines were merely 12 and 8%. Our results confirmed the previous observations and indicated that the AuNPs-loaded nanoemulsion could successfully be used to inhibit the migration of the invasive cancer cell lines.

Conclusions

In the current work, the pm-rf-APGD-based high-throughput reaction-discharge system was employed for the synthesis of the size-defined, uncoated AuNPs. Based on the established statistical models, the operating parameters of the system that resulted in the fabrication of the largest raw-AuNPs were selected and their appropriateness was validated in the independent experiments. Loading the pm-rf-APGD-produced size-controlled raw-AuNPs into the nanoemulsion, consisting of turmeric oil and a gelatin aqueous solution, the aggregation and sedimentation of the AuNPs was limited. As compared to the raw-AuNPs, the developed AuNPs-loaded nanoemulsion was found to effectively inhibit the proliferation of the invasive breast cancer cell lines MDA-MB-231 as well as to significantly induce the apoptosis thereof. Hence, the results presented in this work make an important impact on the development of the new anti-breast cancer strategies.

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Affiliations

Anna Dzimitrowicz¹ · Aleksandra Bielawska-Pohl² · Pawel Pohl¹ · Dorota Jermakowicz-Bartkowiak³ · Piotr Jamroz¹ · Magdalena Malik-Gajewska⁴ · Aleksandra Klimczak² · Piotr Cyganowski³

- ¹ Department of Analytical Chemistry and Chemical Metallurgy, Faculty of Chemistry, Wroclaw University of Science and Technology, Wybrzeze St. Wyspianskiego 27, 50-370 Wroclaw, Poland
- ² Laboratory of Biology of Stem and Neoplastic Cells, Hirszfeld Institute of Immunology and Experimental Therapy Polish Academy of Science, R. Weigla 12, 53-114 Wroclaw, Poland
- ³ Department of Polymer and Carbonaceous Materials, Faculty of Chemistry, Wroclaw University of Science and Technology, Wybrzeze St. Wyspianskiego 27, 50-370 Wroclaw, Poland
- ⁴ Department of Inorganic and Structural Chemistry, Faculty of Chemistry, Wroclaw University of Science and Technology, Wybrzeze St. Wyspianskiego 27, 50-370 Wroclaw, Poland