Research Article

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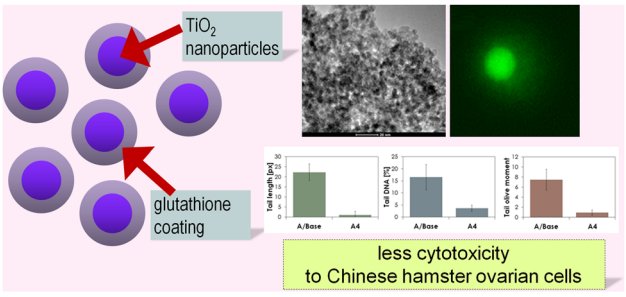
In vitro studies of titanium dioxide nanoparticles modified with glutathione as a potential drug delivery system

https://doi.org/10.1515/ntrev-2023-0126
received April 18, 2023; accepted September 7, 2023

Abstract: This article addresses issues related to the use of nanoparticles as drug delivery carriers, with a particular focus on titanium dioxide (TiO_2) nanoparticles. The aim of this study was to obtain TiO_2 nanoparticles modified with glutathione in order to inhibit the release of titanium ions and reduce the toxic effects of TiO_2 when used as a drug carrier. XRD analysis showed that some of the prepared samples had a crystalline structure, while others were amorphous. The size of crystallites was between 4.1 and 6.2 nm. The presence of glutathione in the structure of TiO_2 particles was confirmed through attenuated total reflectance-Fourier transform infrared analysis (1,385 and 1,516 cm^{-1} for δ_{N-H} and $\nu_{C=O}$ stretching bands in NH_3^+ and carboxylic groups). The size and stability of the nanoparticles assessed using the DLS technique revealed that the particles had a size range of 20–50 nm, and the electrokinetic potential of their suspensions was between −40.7 and −50.8 mV. The specific surface area, pore volume, and size were determined using nitrogen sorption. The measured specific surface area was equal to 230–390 m^2/g. The amount of titanium ions released from the modified carriers was determined. It was lower by even over 70% compared to the not-modified sample. Also, the study involved the synthesis and characterisation of modified TiO_2 nanoparticles loaded with tadalafil. It was found that the release of an active substance from the modified material was less, even 82% compared to the not-modified nanoparticles. Cytotoxic and mutagenic properties in relation to Chinese hamster ovary (CHO) cells were investigated. Titanium oxide nanoparticles modified with glutathione-enhanced CHO cell proliferation at over 60% compared to the reference material. Also, they had a less cytotoxic effect of over 37% compared to the reference material. The obtained materials show satisfactory purity and surface morphology, allowing the formation of carrier–drug interfaces. The results of *in vitro* studies let us claim that the prepared modified TiO_2 nanoparticles have a great potential for being applied as a drug carrier.

Keywords: titanium dioxide, nanocarrier, glutathione, tadalafil, drug delivery system



Graphical abstract

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1 Introduction

Non-specific distribution and uncontrolled release of active substances in conventional drug delivery systems have led to the development of nanocarrier-based drug delivery systems. They can deliver the drug to the target sites with a lower dosing frequency and in a spatially controlled manner [1]. It has been confirmed that nanoparticles can enhance the effectiveness of different drug delivery routes, such as topical administration [2] and others. Delivery of more drugs to the target site by nanoparticles should reduce the required doses of medicinal substances, which in turn will result in a reduction of toxic side effects [3]. There are several elements that need to be considered to ensure clinical potential for newly designed nanoparticle-based carriers. On the one hand, the design of such nanomaterials should take into account the following key issues such as sufficient biocompatibility and biodegradability, good stability under physiological conditions, and high loading capacity and low toxicity. On the other hand, in addition to the primary requirement of safety and therapeutic efficacy, industrial scale-up is also a prerequisite for this type of nanomaterial in clinical applications [4].

Titanium dioxide (TiO_2) nanoparticles have been confirmed as environmentally friendly antibacterial agents attributed to their potent oxidative capacity. The versatility of TiO_2 nanoparticles extends to a wide array of infectious microorganisms, encompassing various bacterial strains, endospores, fungi, algae, protozoa, viruses, microbial toxins, and prions [5]. The synthesis of TiO_2 nanoparticles through biological means has also been explored for their antibacterial applications [6]. The mechanism behind the effectiveness of TiO_2 nanoparticles involves the generation of reactive oxygen species (ROS) upon interaction with microbial cells [7]. This ROS production disrupts the integrity of the microbes' cell walls, particularly through the oxidation of phospholipids. Consequently, adhesion is compromised, and there is a shift in ionic balance. Upon entry into the cytosol, the nanoparticles hinder respiratory cytosolic enzymes and induce alterations in macromolecular structures, leading to a significant impact on cellular integrity and gene expression. Furthermore, these nanoparticles inhibit phosphate uptake and intercellular communication [8]. A visual representation of the impact of TiO_2 nanoparticles on bacterial cells is shown in Figure 1.

TiO_2 found an application also as a photocatalyst. The anatase phase demonstrates superior efficiency as a photocatalyst compared to the other phases of TiO_2. Singh and Soni employed the hydrothermal method to synthesise mixed-phase TiO_2 nanoflowers, utilising them as photocatalysts for water remediation applications. Their findings

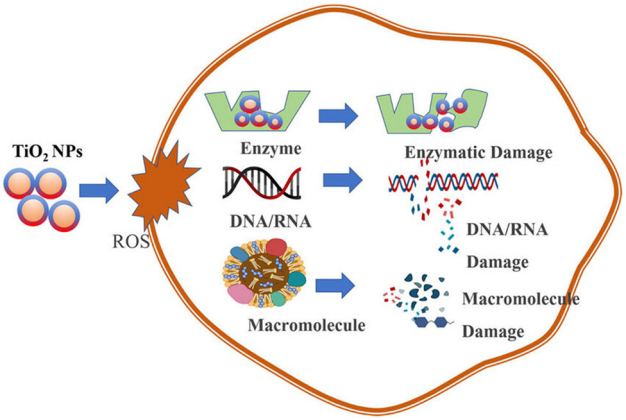

Figure 1: Antibacterial activity of titanium oxide nanoparticles, reprinted with permission from the study of Sagadevan *et al.* [9].

indicate that mixed-phase TiO_2 nanoflowers exhibit remarkable photocatalytic activity due to the formation of heterojunction interfaces between the rutile and anatase phases of TiO_2 [10].

In contrast to their bulk counterparts, nanostructured TiO_2 exhibits significantly enhanced photocatalytic efficiency owing to its effective active sites and high surface-to-volume ratio. This characteristic promotes robust molecular interactions. TiO_2, possessing a wide band gap of 3.2 eV, readily absorbs ultraviolet light. Following this absorption, charge separation occurs, leading to the generation of photo-induced electrons in the conduction band and corresponding holes in the valence band. However, the transient nature of these photo-generated carriers often results in rapid recombination, leading to a decrease in overall photocatalytic efficiency. To tackle these challenges, diverse research groups have explored various approaches. These include methods like metal doping [11], non-metal incorporation [12], and, more recently, surface modification through the integration of noble metal nanoparticles [13]. Metal nanoparticles introduced to TiO_2 serve as electron sinks, capturing electrons from TiO_2. Moreover, they contribute to generating additionally charged carriers through their localised electric fields or surface plasmon resonance [14,15].

Titanium(IV) oxide nanoparticles, being chemically stable, environmentally friendly, and non-cytotoxic, are considered intelligent drug delivery systems to pathogenic sites. Their small nanometric size makes them suitable for targeted therapy, *e.g.* cancer. In such systems, active substances deposited on carriers based on nanomaterials reach sick cells, bypassing healthy cells and tissues. This solution increases the effectiveness of therapy and reduces the possibility of negative side effects. The analysis of TiO_2 nanoparticles for use in drug delivery systems is based on observing the loading efficiency or the effect of pH on the release of the

active substance from the carrier surface. Additionally, uptake by neoplastic cells and cytotoxicity in target cells are assessed [16]. The titanium(IV) oxide nanoparticles can deliver anti-cancer drugs such as paclitaxel, doxorubicin (DOX), or temozolomide on their surface. Moreover, the carrier itself, thanks to its properties, increases its anti-cancer effect [4].

The use of nanometric TiO_2 in biosystems may have several difficulties due to their poor dispersibility and stability in water and biological fluids. In order to improve their colloidal properties, it is necessary to study their surface properties and stability. Coating nanoparticles of titanium(IV) oxide with polymeric materials to eliminate aggregation and sedimentation is becoming popular. This treatment also reduces toxicity and increases biocompatibility. Polyethylene glycol (PEG) is successfully used as a modifier. It has a hydrophilic character. This enables the modification of the nanoparticle surface in order to eliminate the aforementioned agglomeration and make the carrier resistant to protein adsorption [16]. PEG forms a specific, polymeric, thin layer on the nanoparticle's surface. The *in vivo* studies so far show that the TiO_2-PEG nanoparticle conglomerate can be successfully used as one of the cancer treatment tools. The next step to confirm its effectiveness is the analysis of the effects of nanoparticle therapy as drug carriers along with other treatment methods, such as photodynamic therapy. One of the ways to increase the selectivity of TiO_2 nanoparticles is to combine them with folic acid, which allows high selectivity for certain types of cancer. Like antibodies, folic acid increases the affinity of molecules to pathological tissues, increasing their accumulation at the target site [17].

The toxic effects associated with titanium(IV) oxide nanoparticles in humans are mainly long-term effects resulting from chronic exposure *via* various routes (respiratory, digestive, and dermal). Human exposure to TiO_2 *via* various consumer products in Western countries has been estimated to be 2.8–21.4 mg per person per day [18]. These values may increase by about 10–100 times for some risk groups who are exposed to inhalation of large amounts of these particles in the workplace (bleaching of paper, production of paints, *etc.*), or who consume large amounts of products coated with these particles. After penetration into tissues, TiO_2 nanoparticles are not eliminated and accumulate over time, which can lead to very high doses after decades of exposure. It is very difficult to reproduce such chronic exposures, *e.g.*, in rodent models with a short lifetime of no more than 2 years. Therefore, most animal toxicity studies of these nanoparticles use different doses administered at once or for a relatively limited time [19]. Studies have been carried out in which mice are administered titanium(IV) oxide nanoparticles intragastrically for 90 days. As a result, macrophage infiltration was carried out, resulting in spleen apoptosis. In addition, the entire genome was analysed. Exposure in the form of nanoparticles caused significant changes in the expression of over 1,000 genes involved in immune responses, oxidative stress, metabolic processes, ion transport, and others [20]. The titanium(IV) oxide polymorph influences the toxicity of the preparation in which it was applied. Anatase with a particle size below 100 nm is more toxic than rutile due to its catalytic properties and high reactivity. Therefore, the use of this ingredient, *e.g.* in preparations, is more and more cautious for sun protection [21]. As a result of chronic inhalation exposure to titanium(IV) oxide nanoparticles, inflammation may occur, which causes fibrotic and proliferative changes [22]. Drug carriers based on titanium(IV) oxide nanoparticles introduced into the patient's body and, therefore, in contact with body fluids, decompose. During this process, titanium ions are released. They can react with the molecules of the living tissues of the body. If a patient is treated with a titanium implant, its degradation and the release of titanium ions can directly lead to the diagnosis of metallosis. It is a side effect of the implant insertion and the local effects of decay products on the body's tissues. Clinical studies have shown that TiO_2 nanoparticles together with the released ions from the implants accumulate in the peri-implant tissues [23]. Long-term exposure to titanium ions is one of the causes of the allergic response. Allergic reactions and cytotoxic effects of titanium ions released from TiO_2 nanoparticles are directly correlated with their size. The mentioned effects may become visible even after 12 months from the administration of the preparation containing titanium and generate inflammatory reactions. Titanium ions most often accumulate in the liver, lungs, and spleen. Increased amounts of titanium have also been observed in the brain of patients who have been exposed to titanium. Studies were carried out on pregnant mice administered titanium(IV) oxide nanoparticles. Titanium compounds have been detected in the placenta, liver, and foetal brain. Moreover, mice administered with the nanoparticles had a smaller uterus and smaller foetuses than rodents in the control group. When released into the body, titanium may exist as free ions or, due to its unstable nature, will be bound with proteins [24]. The specific properties of titanium(IV) oxide nanoparticles, their use in pharmacy and medicine, and their sensitivity to environmental factors cause their degradation and decomposition with the release of titanium ions. This makes it necessary to control this phenomenon already at the stage of obtaining and producing the desired material.

Esmaeili *et al.* conducted similar studies. The authors took curcumin, a polyphenolic compound that may be found in the *Curcuma longa* plant, as it has various pharmacological benefits. The study focused on using nanosilver

particles modified with glutathione as drug delivery systems for transporting curcumin. The silver nanoparticles were obtained using *Eucalyptus globulus* leaf extract, and then they were modified with glutathione to improve their adsorption capacity and biocompatibility. Nano-Ag particles exhibited spherical shapes with sizes ranging from 5 to 50 nm. The obtained results showed that the nonlinear Langmuir isotherm and pseudo-second-order kinetic models were suitable for describing the adsorption of curcumin on Ag nanoparticles modified with glutathione. The *in vitro* studies indicated that approximately 74% of curcumin was released under simulated intestinal conditions after 20 h [25].

Also, very interesting studies were performed by Hooshyar *et al.* The authors used siliceous magnetised Fe_3O_4 nanoparticles as a carrier for letrozole, a selective nonsteroidal aromatase inhibitor. The nanoparticles were coated with high-branched dendrimers containing *N*-vinyl caprolactam. The process for obtaining the whole construct resulted in the formation of a carrier with an average size of approximately 80 nm. The release of active substances from the carrier was studied in simulated fluid with a pH equal to 7.4. It was found that 95% of letrozole was released in a period of 25 h [26]. Heidarinasab *et al.* proposed another method for improving the delivery of sorafenib, an inhibitor of numerous kinases. The authors obtained allyl glycidyl ether/*N*-isopropylacrylamide-grafted magnetic nanoparticles by performing radical copolymerisation of allyl glycidyl ether and *N*-isopropylacrylamide on silica-coated magnetic nanoparticles. Later, chitosan was coupled with the prepared nanoparticles by opening the epoxy ring of allyl glycidyl ether. The drug release profile demonstrated that 88% of the adsorbed active substance was released within 35 h at 45°C, and the drug release followed a Fickian diffusion-controlled mechanism. The study results confirmed that the obtained construct exhibited a high adsorption capacity at low temperatures and achieved controlled release at a slow rate under high temperatures. Thus, the material is a promising drug carrier for delivery applications [27].

Zifar *et al.,* in their paper, presented very interesting studies. The authors synthesised and performed polymerising of allyl alcohol, *N*-vinylcaprolactam, and sodium alginate on the surface of tungsten disulfide nanoparticles. Later, they conducted drug delivery experiments using flutamide as a model anti-cancer drug. The efficiency of the obtained nanocarrier for controlled release of flutamide was evaluated through *in vitro* drug delivery experiments under simulated human conditions (pH = 7.4) and cancer conditions (pH = 5.6) at different temperatures. During the *in vitro* flutamide delivery investigation, it was found that the drug was released at pH 5.6 and 45°C with a release percentage of 99.54%, while at the same pH but at a lower temperature (37°C), the release percentage was equal to 83.37%. In cytotoxicity tests against PC-3 prostate cancer and HPrEC prostate normal cells, the authors demonstrated that the whole construct kills cancer cells effectively [28].

In their work, Soltani *et al.* presented the study's results, which aimed to create a drug delivery system that could effectively encapsulate and release DOX. To achieve this, the authors used exfoliated molybdenum disulfide nanosheets modified with *N*-isopropyl acrylamide/methyl methacrylate. Additionally, the surface of the carrier was conjugated with glutamine. It was found that maximum DOX adsorption efficiency of 95% could be achieved at pH 8, a contact time of 15 min, and a temperature of 30°C [29].

TiO_2 nanoparticles also show other interesting properties that make it possible to use them in other fields of engineering. For example, Hu *et al.* demonstrated a method for increasing the capabilities of photoelectrochemical cells by using TiO_2 nanowires. Coating a 2–5 nm carbon layer with TiO_2, it was possible to increase the supercapacitance by about 150 times [30]. The assumptions of the study correspond primarily to the criterion of product innovation; however, process innovation can also be seen in it. To the best of our knowledge, despite the numerous range of products based on nanostructured materials used in the pharmaceutical chemistry industry as carriers of medicinal substances, there are no nanocarriers on the market whose properties would not threaten the living organisms that receive them. Based on the literature data, it can be concluded that the market of carriers used in the pharmaceutical chemistry industry is sufficiently saturated with them. Among them, mainly materials based on synthetic and natural polymers prevail [31–35].

The literature review also indicates the wide use of metallic or oxide nanoparticles that effectively act as carriers of medicinal substances. Comparing these two groups of drug carriers, it should be noted that despite the greater biocompatibility of polymeric materials, they are characterised by lower stability in conditions of changing pH, changing concentration of ions present in the environment of their use, worse mechanical strength, and lower resistance to temperature changes [36].

Undoubtedly, one of the biggest drawbacks associated with the use of this type of material is their cytotoxic, genotoxic, and mutagenic activity. The mechanism of the toxic properties of nanoparticles introduced into living organisms is discussed in the Project Description. Taking into account the conclusions of the literature review, which indicate the negative impact of nanoparticles on the work of individual organs, an idea arised to develop innovative nanometric materials, the properties of which would eliminate or reduce their toxic activity.

Thanks to the modification of TiO_2 nanoparticles by introducing organic substances into their structure, it will be possible to reduce or eliminate their toxic properties (cytotoxic, genotoxic, and mutagenic). This will be tantamount to inhibiting the dissolution of nanomaterials, which is evidenced by the release of metals from them in the ionic form. This mechanism is one of the main direct causes of the toxic effects of nanoparticles on living organisms. Nevertheless, it was expected that the addition of substances preventing the release of toxic metal ions would not change the physicochemical properties of the nanocarriers responsible for the success of the targeted therapy. An important feature in this case is the average size of nanoparticles, which should be in the range of 50–800 nm. Then, the nanocarrier has a chance to effectively reach the cancerous tissue, avoiding healthy tissues. In addition, it was expected that the presence of organic substances would not deteriorate the surface properties of the carriers.

Thus, this study aimed to obtain TiO_2 nanoparticles modified with glutathione, which would inhibit the release of titanium ions. This approach would reduce the toxic effect of TiO_2 applied as a drug carrier.

2 Materials and methods

2.1 Materials

In the processes of formation of modified TiO_2 nanoparticles, the following compounds were used: titanium(IV) isopropoxide (TIPO) (97.0%), sodium hydroxide (≥98%), and L-glutathione reduced (GSH) (≥98.0%). Tadalafil was used as a Pharmaceutical Secondary Standard. All compounds were obtained from Sigma Aldrich. All aqueous solutions were prepared using deionised water (Polwater, 0.18 μS). Culture media (F-12K Medium), Chinese hamster ovary (CHO) cell line, and supplements (FBS, antibiotics) were also obtained from Sigma-Aldrich. 5-Bromo-2′-deoxyuridine (BrdU) cell proliferation kit was obtained from Roche, and the LDH cytotoxicity assay kit was provided by Thermo Fisher Scientific. Ketamine, xylazine, buprenorphine, heparin, and saline were obtained and purchased in Sigma Aldrich.

2.2 Methods

2.2.1 Obtaining modified TiO_2 loaded with tadalafil

The process of titanium oxide has three stages and involves basic hydrolysis, polycondensation, and dehydration. In the first step, hydrolysis of TIOP occurred. In the presence of sodium hydroxide, the formation of titanium hydroxide took place. In the second step, the polycondensation of titanium hydroxide occurred. It led to the release of water. The last stage involved the dehydration of condensation product to titanium(IV) oxide and water. The amounts of all reagents were calculated that the final mass of TiO_2 was equal to 2.6978 g (this mass results from the TIPO volume, which was taken into the processes [10 mL]). Also, in some cases, the additional amount of base was used so the fold of NaOH *vs* stoichiometric amount required was equal to 1, 2, or 3.

Figure 2 presents the schematic diagram of the process for obtaining TiO_2 modified with glutathione loaded with tadalafil. A series of nine products were obtained. Briefly, TIOP was added dropwise into the Teflon vessel in which the aqueous solution of sodium hydroxide had been introduced already. The obtained mixture was then homogenised using an ultrasound homogeniser, Hielscher UP400St, Germany (40 W). After 2 min of homogenisation, an aqueous solution of glutathione was added dropwise to the mixture, and the whole was homogenised for a further 2 min. The concentration of glutathione solution was calculated as the molar ratio of GSH to TiO_2 differed, and it was equal to 0.02, 0.11, or 0.20 (provided in Table 1). As a result, suspension 1 was obtained. In the next step, the active substance was added. For this purpose, the proper amount of tadalafil in a solid form was introduced into suspension 1, and the whole was stirred for 5 min (C-MAG HS 7, IKA). The mass of tadalafil was calculated as the mass ratio of tadalafil to the whole TiO_2–Tad complex was equal to 1.0:3.5. Next, the Teflon vessel was placed in the microwave reactor (Magnum v2, Ertec, Poland). The process temperature differed, and it was equal to 120, 150, or 180°C. After reaching the required temperature, the sample stayed in it for 5 min. The obtained mixture was filtered (0.45 μm). The solid phase was washed with deionised water, and the liquid phase was discarded. The solid product was dried in a laboratory drier at 80°C for 24 h. Pure titanium oxide nanoparticles loaded with tadalafil were the reference material. The specific values of input parameters are provided in Table 1.

The physicochemical properties of the obtained products have been analysed. XRD technique (X'Pert PW 1,752/00, Philips) has been used in order to assess the crystallographic structure of prepared TiO_2 nanoparticles. Based on the Scherrer equation, the crystalline size was calculated. In order to confirm the organic matter coating the surface of TiO_2 nanoparticles, the attenuated total reflectance-Fourier transform infrared (ATR-FTIR) analysis has been performed (Nicolet 380 spectrophotometer, Thermo Fisher). This technique confirmed the presence of glutathione built in the structure of TiO_2 particles. The

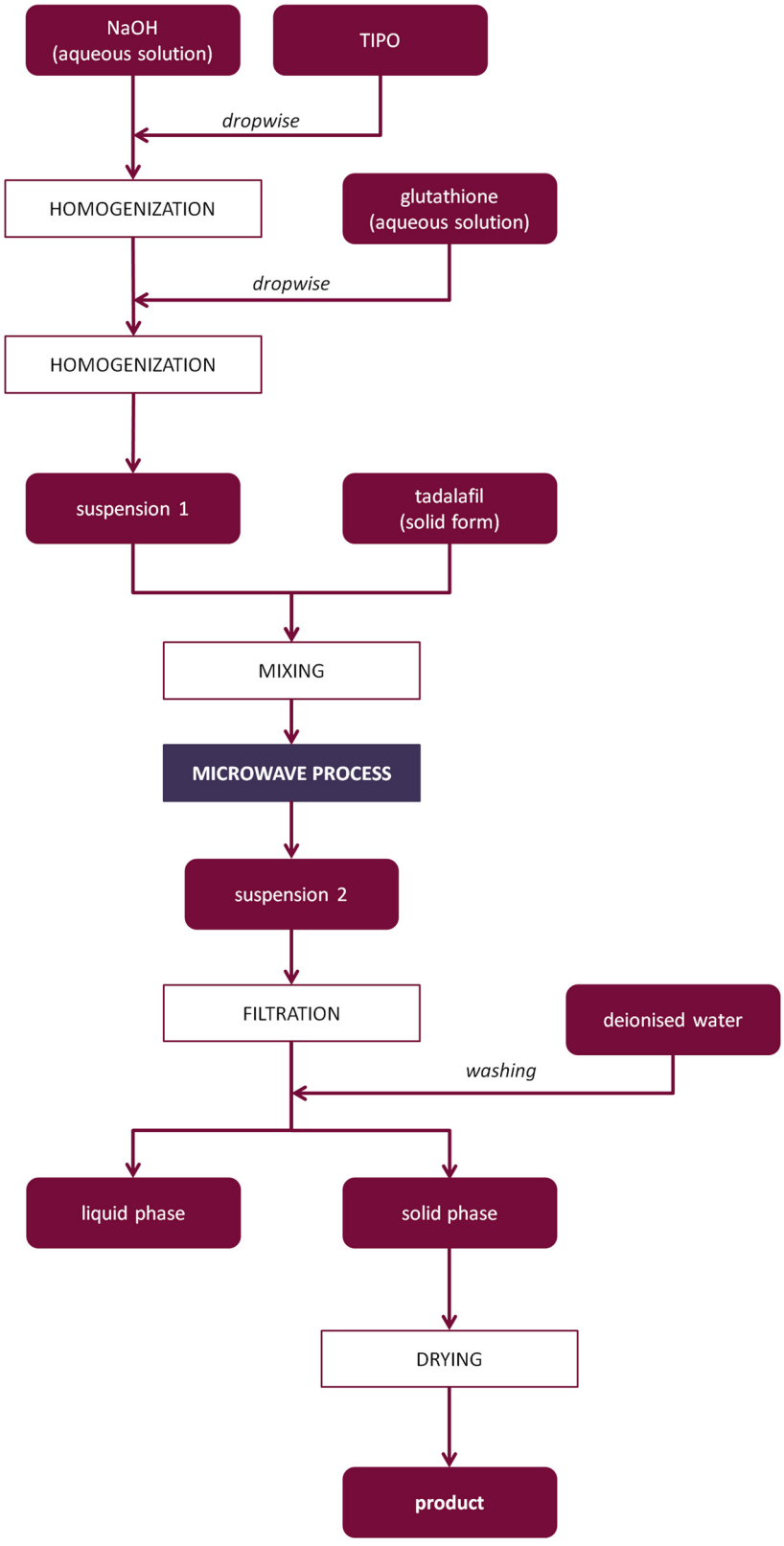

Figure 2: Schematic diagram of the process for obtaining TiO_2 modified with glutathione loaded with tadalafil.

analysis of nanoparticle size was conducted using the DLS technique (Zetasizer Nano ZS, Malvern Instruments Ltd). Also, this method assessed the stability of the aqueous suspensions by providing the electrokinetic potential, ζ. The concentration of the analysed suspension was equal to 10 mg/L. The suspensions were homogenised for 1 min prior to analysis (Hielscher UP400St, Germany, 40 W). The specific surface area, pore volume, and size were assessed using the low-temperature nitrogen sorption (ASAP2010 apparatus from Macromeritics, USA). The samples were desorbed at 200°C before measurement in helium flow for 6 h and then under vacuum to a final pressure of 0.001 Torr. The size and shape of the prepared materials were analysed based on TEM-EDS microscopy (Tecnai TEM G2 F20X-Twin 200 kV, FEI).

2.2.2 Analysis of titanium elution from the prepared complexes

The analysis of titanium elution from the prepared samples was conducted in the aqueous environment. For this purpose, a specific amount of the tested sample (0.1500 g) was taken to the glass beaker with analytical accuracy. Next, a specific amount of deionised water, which played the role of the leaching agent, was introduced to the powder. The ratio of substance mass to volume of the leaching agent ratio was equal to 0.1 g:2.0 mL. The prepared suspensions were mixed on a magnetic stirrer with temperature control. The elution process was led at 37°C. After the specific time of mixing (0, 1, 3, 5, 10, 20, 40, and 80 min), the suspension was filtered through the syringe filters (φ = 0.45 μm). The concentration of the leached titanium was analysed by Atomic Absorption Spectrometry (Perkin Elmer)

2.2.3 Analysis of active substance elution from the prepared complexes

The analysis of tadalafil released from the prepared samples was conducted in the aqueous environment. For this purpose, a known mass of the tested sample (0.1500 g) was taken to the glass beaker with analytical accuracy. The appropriate amount of the leaching agent (Ringer's fluid or simultaneous body fluid [SBF]) was later introduced to this. The purpose of using Ringer's fluid or SBF was to simulate the conditions of the biological environment. The composition of Ringer's fluid (water solution of chlorides, sodium, potassium, and calcium) makes it neutral for the human body. The composition of SBF is similar to the composition of the plasma in the human body. Both fluids are widely used in biochemical studies to simulate the application environment [37]. The mass-to-volume of the leaching agent ratio was equal to 0.1 g: 2.0 mL. The obtained suspensions were mixed on a magnetic stirrer with temperature control. The elution process was led at 37°C. After the specific time of mixing (0.5, 1, 3, 5, 10, 20, 30, 40, 50, 60, 120, and 180 min), the suspension was filtered through the syringe filters (φ = 0.45 μm). The concentration of the eluted tadalafil in the obtained filtrates was analysed using a spectrophotometer (Rayleigh UV-1800) at λ_{max} against the reference sample (pure Ringer's fluid or SBF).

2.2.4 *In vitro* cell viability assay

In this study, the influence of prepared TiO_2 nanoparticles (both in basic and in modified form) on cytotoxicity and proliferation of CHO cells was analysed. In the studies, the following materials were used: CHO cells (Sigma-Aldrich, Cat. No. 85051005), grown according to the producer's

Table 1: Process parameters

Sample	Input parameters				
	n GSH:*n* TiO_2	Fold of NaOH *vs* stoichiometric amount	Process temperature (°C)	Process time (min)	*m* TAD (g)
A1	0.02	1	120	5	1.079
A2	0.02	2	180		
A3	0.02	3	150		
A4	0.11	1	180		
A5	0.11	2	150		
A6	0.11	3	120		
A7	0.20	1	150		
A8	0.20	2	120		
A9	0.20	3	180		
A/Base	0.00	1	150		

instructions, were used; f-12 K medium (Sigma-Aldrich, Cat. No. N4888) supplemented with foetal bovine serum (Thermo Fisher Scientific no. 10270106) and antibiotics (Sigma-Aldrich, Cat. No. P4333). The investigated cultures were grown at 37°C and 5% CO_2, and cells were passaged when reaching 80% confluence 2–3 times per week. The lactate dehydrogenase (LDH) test which is a colorimetric method of analysing the cytotoxic effect of materials on cells was conducted. This test made it possible to measure the amount of coloured formazan formed from tertazoline (measurement of absorbance at 490 nm). This reaction is catalysed by LDH, in the presence of NAD+. As a result of the disruption of the integrity of the cell membranes, LDH is released from dead cells into the culture medium. Analysis of cytotoxicity was performed as follows. CHO cells were plated in 96-well plates at 9×10^3 cells per well in 150 µL of medium. Cultures were stabilised for 24 h, and after that time, the medium was replaced with a fresh one, which contained the tested nanomaterials. The concentrations of the nanoparticles in the applied suspensions were 30, 50, 70, and 80 µg/mL (the control sample did not contain any nanoparticles). The cytotoxicity analysis of the prepared materials was performed using the Pierce LDH cytotoxicity kit (Thermo Fisher Scientific, Cat. No. 88954), according to the protocol provided by the manufacturer using a Multiskan GO microplate reader (Thermo Fisher Scientific) at two wavelengths −490 nm (formazan absorbance) and 680 nm (background absorbance). In order to calculate the cytotoxicity, the following equation was used:

$$\%\text{Cytotoxicity} = \frac{\text{Compound} - \text{treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100.$$

The BrdU proliferation analysis assay serves as a colorimetric test for measurement of the amount of BrdU incorporated into DNA. Chemically, BrdU is a synthetic analogue of thymidine nucleoside incorporated into the DNA of a dividing cell during the S phase of division. In order to measure the amount of incorporated BrdU, the enzymatic reaction of the enzyme conjugated with the anti-BrdU antibody and the substrate in previously fixed cells is carried out. A low signal indicates an inhibition of division, while a high signal indicates a high proliferative activity of cells.

For proliferation analysis, CHO cells were plated in 96-well plates at 9×10^3 cells per well in 150 µL of medium. Cultures were stabilised for 24 h, and after that time, the medium was replaced with a fresh one that contained the tested nanomaterials. The control sample did not contain any nanoparticles. Cells were let to grow for 24, 48, and 72 h. Assessment of cell proliferation in the presence of nanomaterials was performed using the Cell Proliferation ELISA kit, BrdU (Roche, Cat. No. 11647229001), according to the protocol provided by the producer. The readings were taken at two wavelengths −450 nm (product absorbance) and 690 nm (background absorbance) (Multiskan GO microplate reader, Thermo Fisher Scientific).

The comet test (CT) enables the detection of DNA damage at the level of a single cell. The analysed cells are mounted in agarose on a microscope slide. DNA remains after the proteins are digested. The slide is electrophoresed and stained with a fluorescent substance. One obtains an image in the form of "comets." The "head" is where the cell immobilises before lysis, the "tail" is the damaged DNA fragments. The measure of the level of DNA damage is the length of the tail and the amount of DNA it contains. In the analysis, CHO was seeded in 12-well plate cells in the amount of 80,000 cells per well and grown in 1 mL of culture medium for 24 h. Then, the medium was replaced with fresh, containing nanomaterials and cultured for 24 h. After incubation, cells were harvested and suspended in 1% low melting point agarose (Eurx, Cat. No. E0303) and poured onto glass slides pre-coated with 1% agarose (Eurx, Cat. No. E0301). Slides with cells suspended in congealed agarose were placed in lysis buffer (pH 10, 2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 200 mM NaOH) for 1 h at 4°C. The slides were then placed in an electrophoresis chamber containing buffer (pH > 10, 300 mM NaOH, 1 mM EDTA). The electrophoresis was performed at 18 V (0.5 V/cm) for 1 h. After the separation was completed, the slides were washed with distilled water and placed in a solution containing SYBR™ Gold dye (ThermoFisher Scientific cat. No. S11494). Detection was performed with a ZOE Fluorescent Cell Imager fluorescence microscope, and genotoxicity assessment was performed with CometScore 2.0 software.

3 Results

3.1 Modified TiO_2 loaded with tadalafil

The diffraction angles and their corresponding planes in the XRD results are presented in Figure 3a. Based on these results, one may observe that not all prepared products had a crystalline structure. Samples 2, 3, 4, 5, 9, and base exhibited strong diffraction peaks at 25.1, 37.4, 47.5, and 54.1° of the 2θ angle. The amorphic structure had samples 1, 6, 7, and 8. Samples 1, 6, and 8 were obtained at the lowest temperature, which suggests that 120°C was not enough for the crystalline structure of TiO_2. In the case

of sample 7, which was obtained at 150°C one may observe that a weak diffraction peek around 25° of 2θ angle appears. This suggests that despite the highest glutathione content, the crystallisation process begins at this temperature. The size of crystallites has been calculated based on the Scherrer equation:

$$d_{Sch} = k\lambda/\beta \cos\theta,$$

where d_{Sch} is the crystallite size, k constant depends on the shape of the crystallite size, β is the width at half maximum peak describing the material, λ is the wavelength of CuKα radiation, and θ is the Bragg diffraction angle. Figure 3b presents the results of the analysis. All crystallites are below 10 nm, which suggests that the specific surface area is highly expanded. However, one may observe that the size of crystallites in the basic sample (not modified) is less than in the rest of the materials. That may indicate that the modifier molecules may clog the pores and thus make the surface area not much expanded.

Figure 4 presents the results of ATR-FTIR spectroscopy. The presence of Ti–O is confirmed by peaks around 700 cm^{-1}. This region is characteristic of metal–oxygen bonding. Weak peaks in the region around 3,300 cm^{-1} may be attributed to the hydroxyl group of adsorbed water. The presence of glutathione is confirmed by peaks at 1,385 and 1,516 cm^{-1}, which correspond to the δ_{N-H} and $\nu_{C=O}$ stretching bands in NH_3^+ and carboxylic groups [38].

Results of the size of nanoparticles measurement by the DLS technique are presented in Figure 5. The size of nanoparticles in most prepared samples was between 40 and 50 nm. Only in sample 8, the nanoparticles were smaller, and their dimension was 20 nm. Also, the electrokinetic potential of some of them was measured. It ranged between −40.7 and −50.8 mV. The absolute values of this parameter are far higher than 20 mV, which confirms that the aqueous suspensions of the obtained products are kinetically stable.

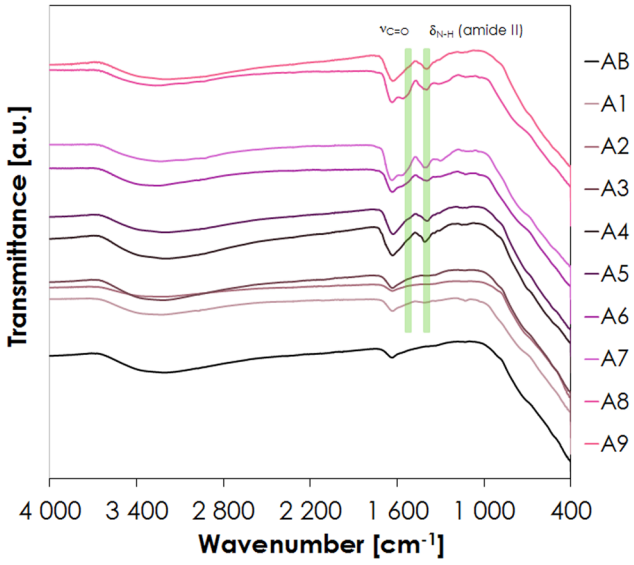

Figure 4: ATR-FTIR spectra of obtained materials.

Figure 6 presents the hysteresis loops along with the type of pores identified based on the hysteresis loop type. Based on hysteresis loops, one may assign their types to the

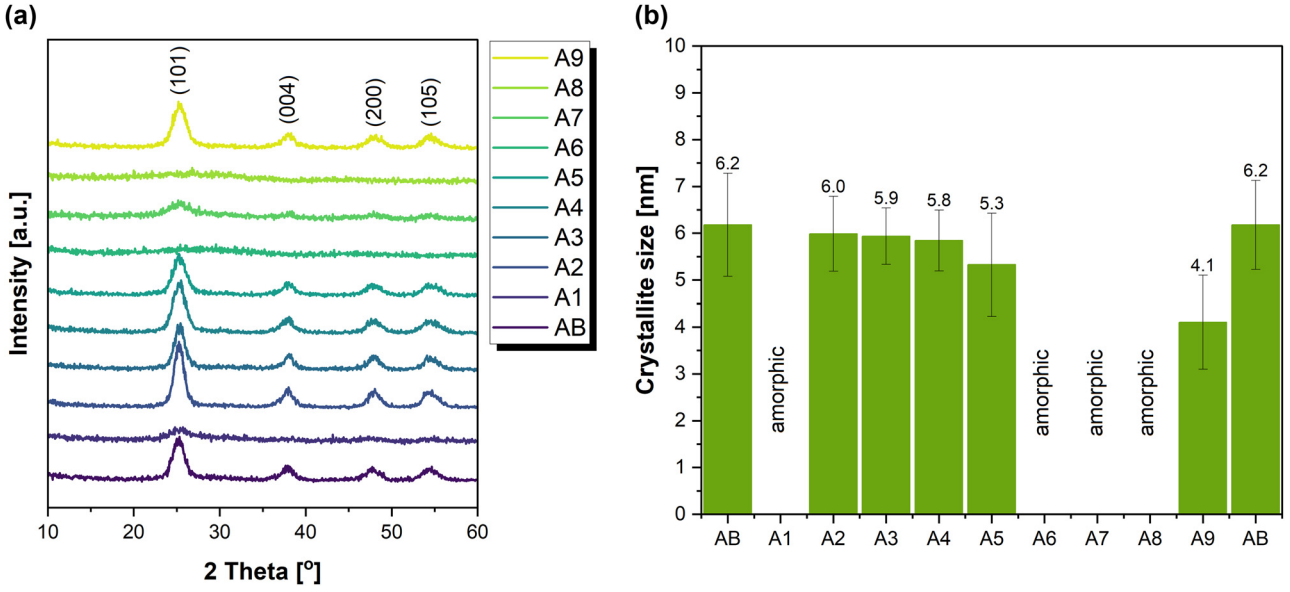

Figure 3: (a) XRD diffractograms of all prepared products, and (b) crystallite size calculated based on the Scherrer equation.

shape of pores. The reference sample and A4 material had the same hysteresis loop type, which was H2. A1 material had an H3 hysteresis loop. That means that reference and A4 materials had neck-like and wide body pores or ink bottle-like pores. Contrary to that, material A1 had groove pores of a nonrigid generation formed by flaky particles, which was confirmed by the H3 hysteresis loop type [39,40]. Table 2 shows the surface properties by indicating the specific surface area, pore volume, and pore size. The results are in line with above-described observations. Material A1

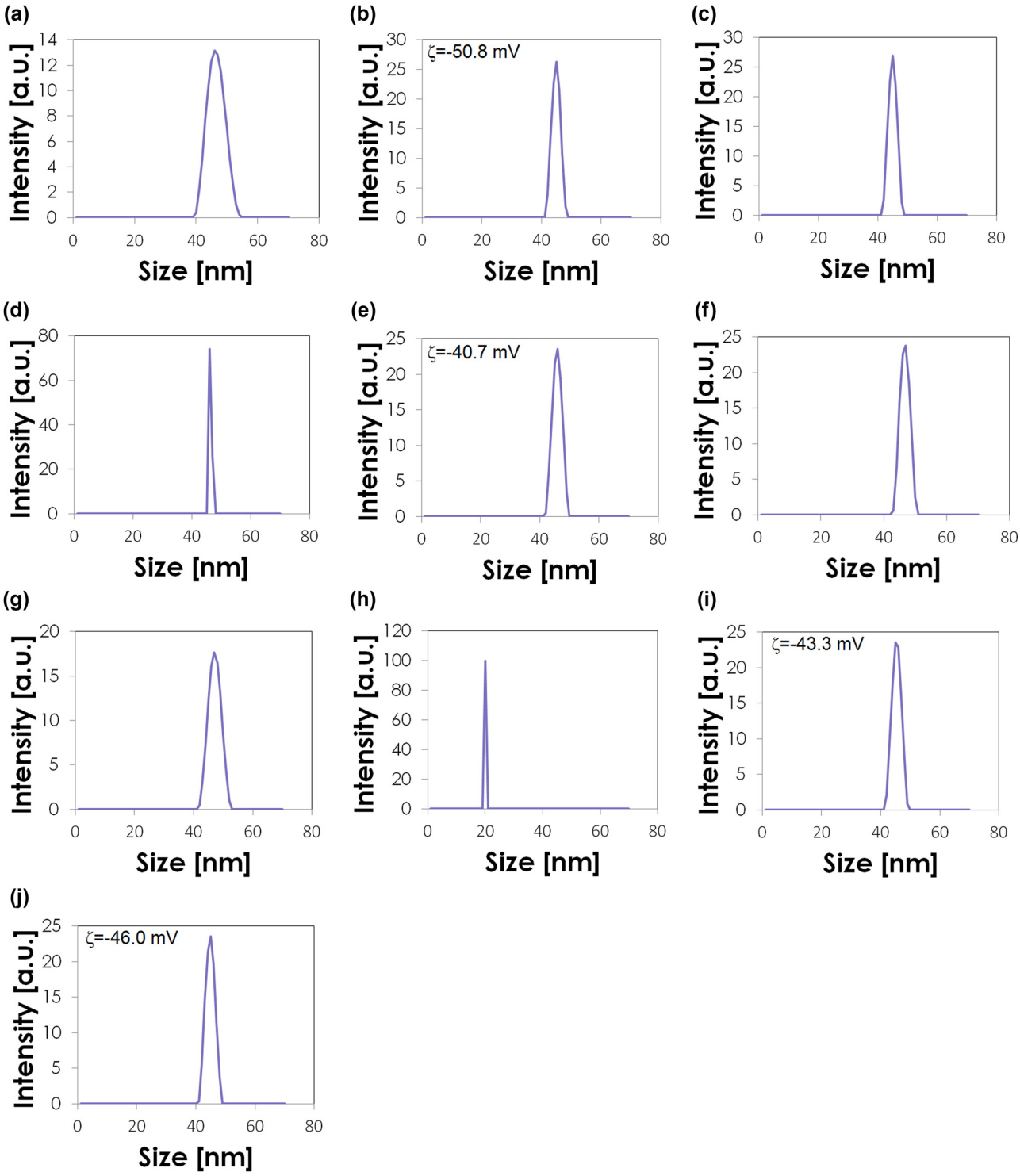

Figure 5: Results of DLS analysis presenting size of nanoparticles along with electrokinetic potential: (a) A/Base, (b)–(j) A1–A9.

was characterised by the most developed specific surface area. It also had the largest pores volume and the smallest pores size. The specific surface area in all materials was above 230 m^2/g, which suggests that the obtained products have a great load capacity.

Figure 7 presents the results of the TEM analysis. This was performed for A/Base (Figure 7a), A1 (Figure 7b), and A4 (Figure 7c) samples. It may be clearly seen that material A1 is characterised by the smallest particles (around 5 nm), which is in line with the results obtained *via* surface properties analysis. Both reference product and A4 material have TiO_2 nanoparticles whose size does not exceed 10 nm. A1 material was obtained at the lowest temperature (120°C). This parameter seems to be essential in the nanoparticle formation process.

Figure 8 shows the results of TEM-EDS analysis. It has revealed that pure TiO_2 nanoparticles, which were not modified consisted of titanium and oxygen only (Figure 8a). Based on this analysis, one may observe that titanium oxide, which was modified with glutathione (material A4), had in its structure titanium, oxygen, and carbon, which origins from the organic matter (Figure 8b).

Table 2: Results of analysis of surface parameters

Material	SS (m^2/g)	V (cm^3/g)	Size (nm)
A/Base	263.8	0.4409	1.291
A1	383.2	0.6382	1.096
A4	231.9	0.3129	1.552

3.2 Titanium elution from the prepared complexes

The results of the analysis of titanium released from the prepared materials are presented in Figure 9. Reducing the leaching of titanium was one of the main aims of the studies. This was due to the fact that reduced titanium (in both metallic and ionic form) poses a threat to living organisms. After being incorporated into tissues, they may accumulate in their structure and induce the formation of tumours. Thus, the release of metals from the drug carrier systems should be eliminated. The black curve represents the titanium releasing profile from the reference sample (not modified). As one may observe, four materials released

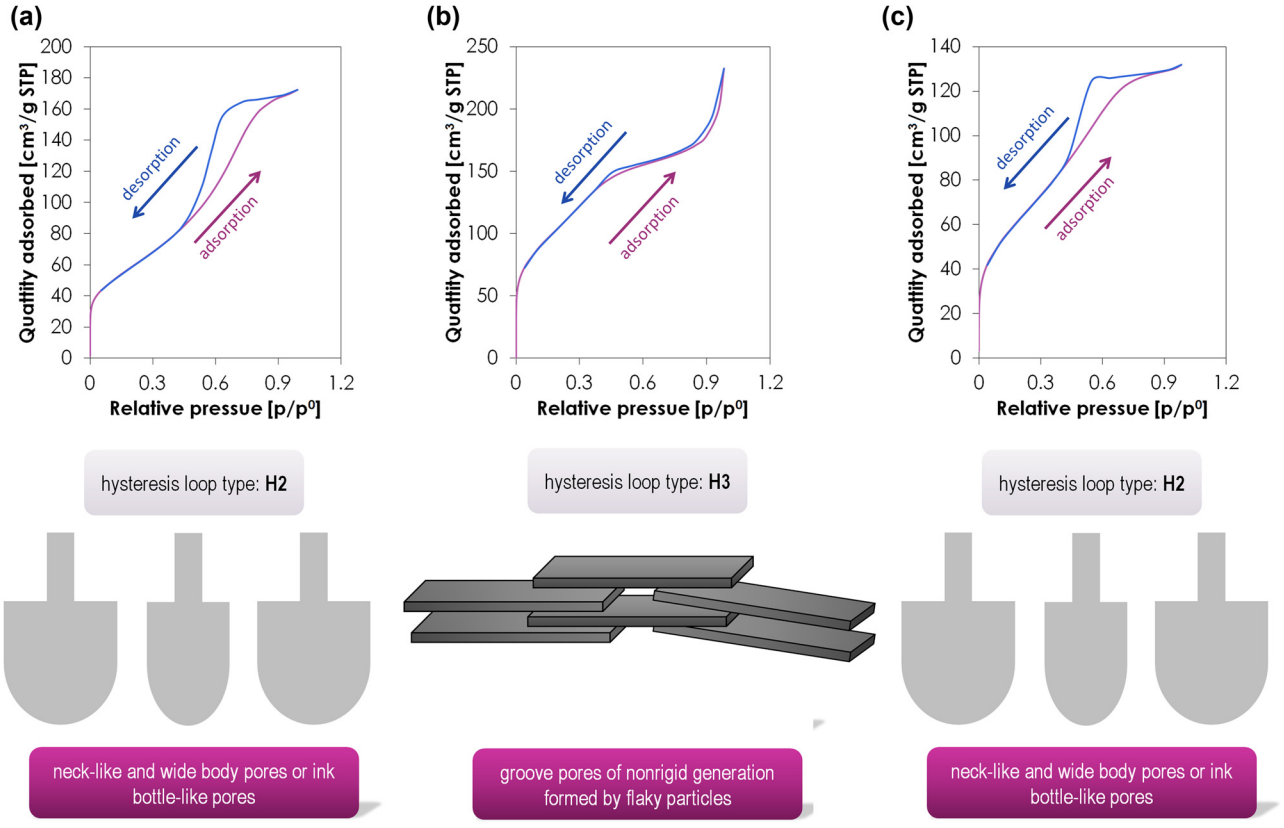

Figure 6: Hysteresis loops with the shape of identified pores: (a) A/Base, (b) A1, and (c) A4.

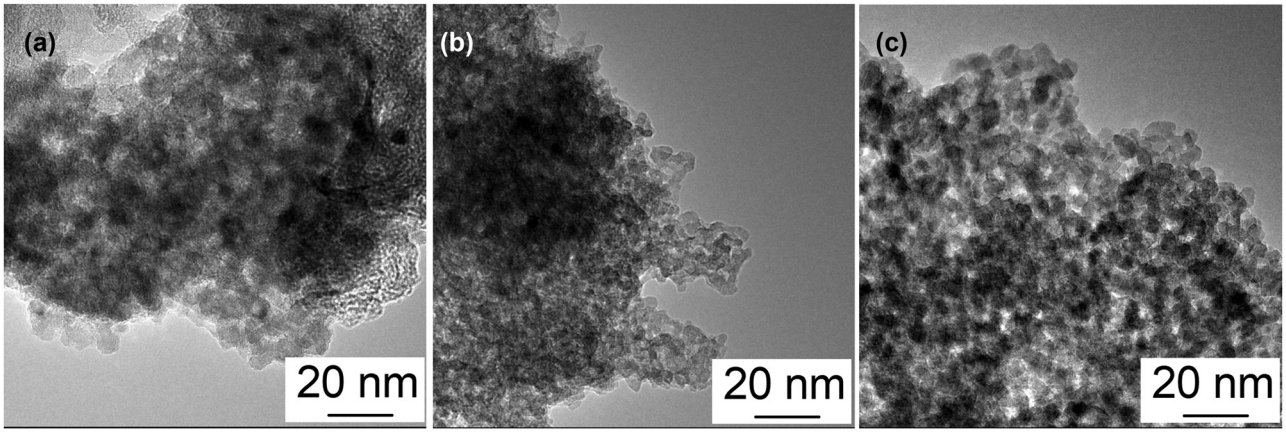

Figure 7: Results of the TEM analysis: (a) A/Base, (b) A1, and (c) A4.

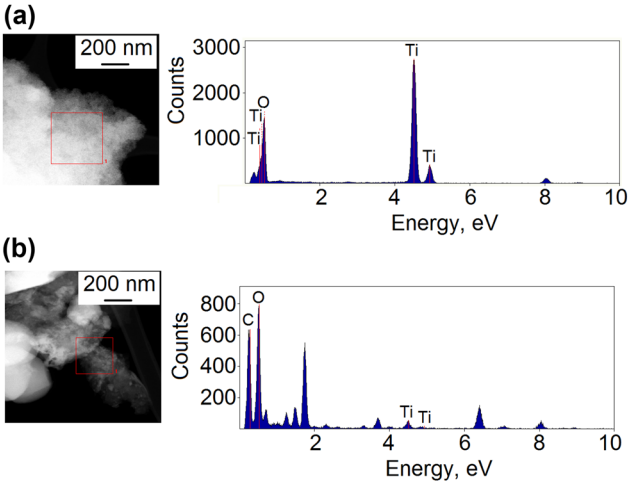

Figure 8: Results of the TEM-EDS analysis: (a) A/Base and (b) A4.

less titanium than the reference sample did (A4, A6, A7, and A8). Samples 7 and 8 had minimal elution of titanium, which is the most desired result. These were obtained when the molar ratio of glutathione to TiO_2 was the highest and was equal to 0.2:1.0. The same ratio had sample 9, and the profile releasing is similar to the reference one. That means that coating with glutathione inhibits the release of titanium indeed.

3.3 Tadalafil elution from the prepared complexes

Figure 10 presents the results of tadalafil elution analysis. The calibration curves are presented in Figure 10a and b. Figure 10a shows the curve obtained in Ringer's fluid and B – in SBF. The wavelength with peak maximum for tadalafil is λ = 284 nm. In both cases, the determination factors are above 0.999, which confirms a good curve fit to empirical points. The tadalafil solution in Ringer's and SBF's fluid shows linearity in the concentration range from 5 to 60 µg/mL. The linear nature of the tadalafil solutions was within the 95% confidence interval. The black curve presents the elution profiles from reference material (not modified). In both cases, they confirm the strongest elution of active substance from the carrier. TiO_2 modified with glutathione did not release tadalafil as fast as in the case of reference samples (in both tested environments). In the case of A4 material which was put into Ringer's fluid, one may observe a curve drop after 120 min. This may result in again adsorption of tadalafil on the surface of the material. There are many known mechanisms by which the active substance is released from the transport system [41]. The behaviour of the released active substance

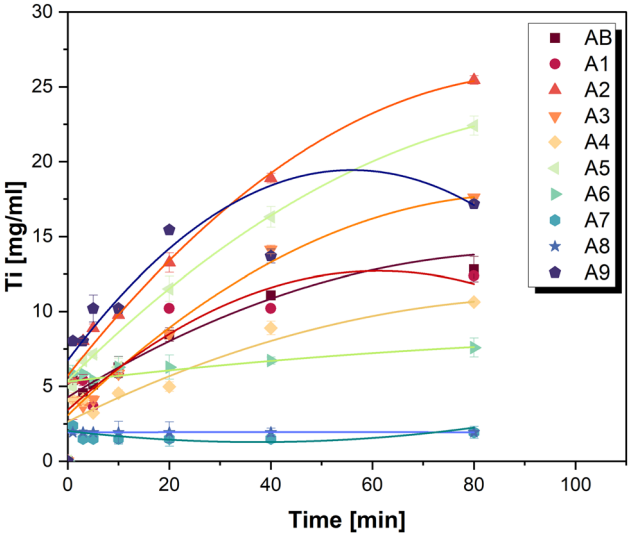

Figure 9: Profiles of titanium releasing from the prepared materials.

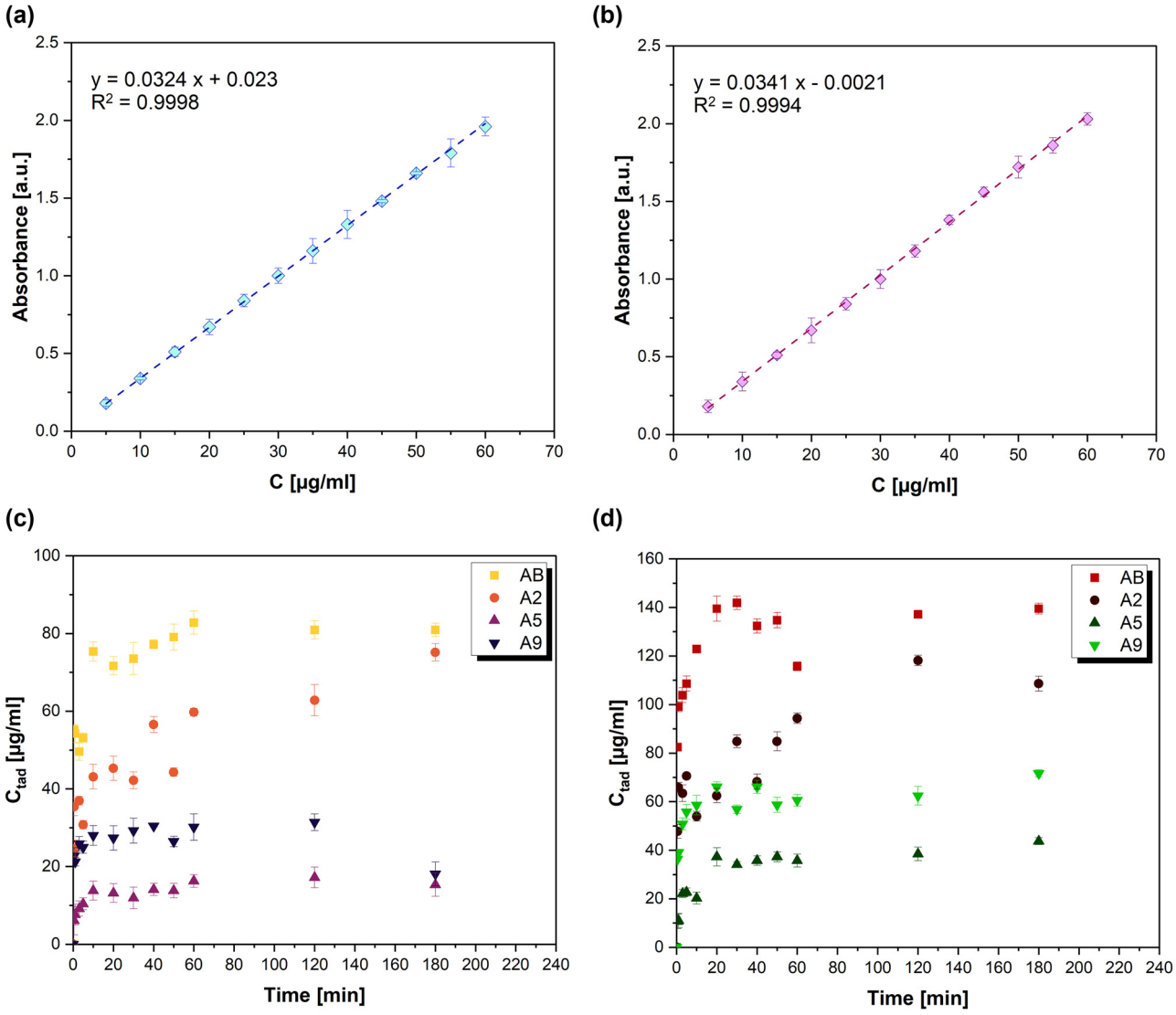

Figure 10: Results of tadalafil elution analysis: (a) calibration curve obtained in Ringer's fluid, (b) calibration curve obtained in SBF, (c) tadalafil elution profiles obtained in Ringer's fluid, and (d) tadalafil elution profiles obtained in SBF.

depends on its stability as well as the physicochemical properties of the nanocarrier. Components of the release process are (1) desorption of the surface-bound drug; (2) diffusion of the drug from the carrier surface; (3) carrier erosion; and (4) a further combination of erosion and diffusion processes [42]. The release profiles shown in Figure 9c and d follow the typical diffusion profile that is common for nanoparticle-based drug carriers [43]. The diffusion mechanism is appropriate for systems in which the diffusion of the drug is faster than the degradation of the carrier, which is consistent with the nature of the type of carrier used in the work (metal oxide). An initial rapid release, called "bursting release," and a further "sequential" release were observed in the studies. This profile is attributed to complexes in which the drug is adsorbed or weakly bound to the carrier surface. It should be noted that when SBF was used as an acceptor medium, the concentration of eluted tadalafil was higher than when Ringer's solution was used. This is in line with the theoretical conditions. The drug release rate may be influenced by ionic interactions between the carrier and secondary components present in the acceptor medium. The composition of SBF is much more varied than that of Ringer's. In its environment, the interaction of the active substance with the carrier matrix is weakened, as there is a competitive electrostatic interaction between the carrier and the surrounding ions, which explains the increase in drug release in this environment.

3.4 *In vitro* cell viability assay

The results of *in vitro* cell viability analysis are presented in Figure 11. Figure 11a shows the dependence of cytotoxicity on the concentration of the analysed suspensions. All tested materials modified with glutathione induced stronger proliferation of CHO cells than in the case of using a reference sample (not modified, basic TiO_2). One may note that TiO_2 nanoparticles modified with glutathione even promote the development of CHO cells. Compared to the reference

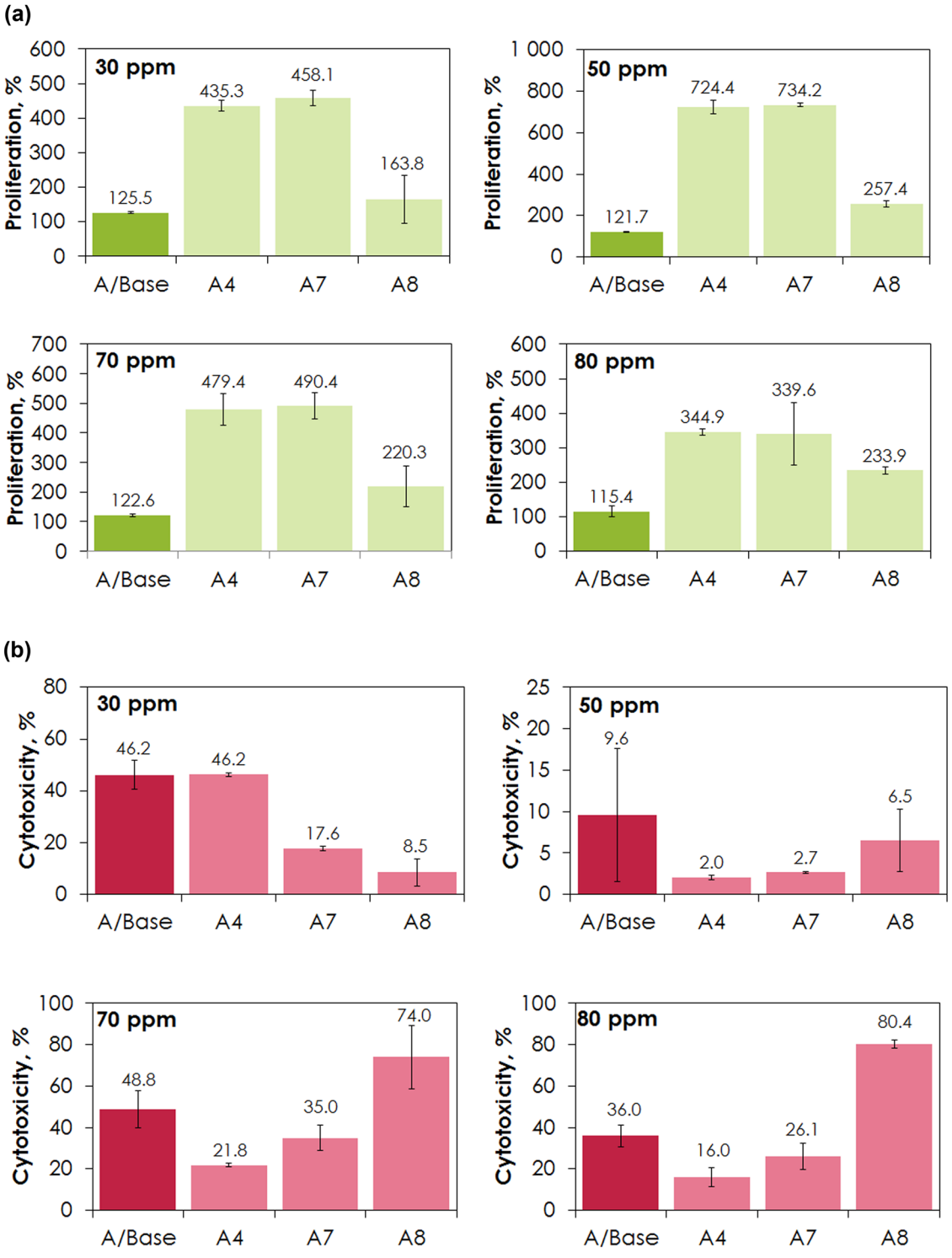

Figure 11: (a) Results of proliferation analysis and (b) results of cytotoxicity analysis.

material, weaker cytotoxic properties exhibited modified materials A4 and A7 (Figure 11b). It was achieved thanks to the presence of on their surface glutathione, which inhibited the release of Ti ions, which would induce ROS formation and cell apoptosis.

The CT, also known as single-cell gel electrophoresis or microgel electrophoresis, is used in studies such as genetics, toxicology, biomonitoring, ecogenotoxicity, molecular epidemiology, nutrigenomics, DNA repair system studies, assessment of genotoxicity and mutagenicity of nanomaterials, and integrity assessment DNA in mesenchymal stem cells. The CT detects breaks in the DNA strands, which are visualised by the increased migration of free DNA segments, resulting in comet-like images. DNA breaks, both double-stranded and single-stranded, are associated with chromosomal aberrations and genome instability. Genome instability is directly related to neoplastic processes. The CT allows the detection of aneugenic and clastogenic substances with high sensitivity *in vivo* and *in vitro* in populations exposed to irritants. Its use allowed for the assessment of genotoxic and mutagenic properties of the obtained products. The advantage of the CT over the micronucleus test is that the micronucleus test only analyses genetic damage in mitotic cells, while the CT detects DNA damage in both the interphase and mitotic cells. Single-cell gel electrophoresis is the basic method of analysing the degree of DNA fragmentation as a result of genotoxic and mutagenic factors. This method identifies single-stranded and double-stranded DNA breaks, and any chemical and enzymatic modifications that can transform into DNA breaks or chromatids. It was found that compared to the reference material (unmodified nanoparticles), the tested products induced shorter comets corresponding to cells undergoing apoptosis (Figure 12). This means that the modified TiO_2 induces DNA damage to a lesser extent and shows lower genotoxicity and mutagenicity. Figure 12d–f presents these observations in numerical terms. For reference material, the values of all parameters tested (tail length, DNA tail, olive moment) are higher than the values of the modified TiO_2. The expected result of the research indicates the limitation of genotoxic and mutagenic properties of the tested materials.

Other authors' investigations also confirm that modified drug carriers are less toxic to the analysed cell lines. The effect of drugs encapsulated with a micellar drug carrier (curcumin or quercetin) on the viability of CHO-K1 cell lines was investigated. Dose-dependent cell suppression was observed. Curcumin inhibited CHO-K1 cell proliferation more strongly than quercetin. Significant inhibition appeared at a concentration of 12.5 µg/mL of curcumin [44]. The modification of PAMAM dendrimer by glycosylation was checked in terms of its cytotoxic activity. The drug carrier was loaded with methotrexate. The cell lines (HeLaT and MDA-MB-231) were subjected to the action of both non-modified drug carriers loaded with the active substance and

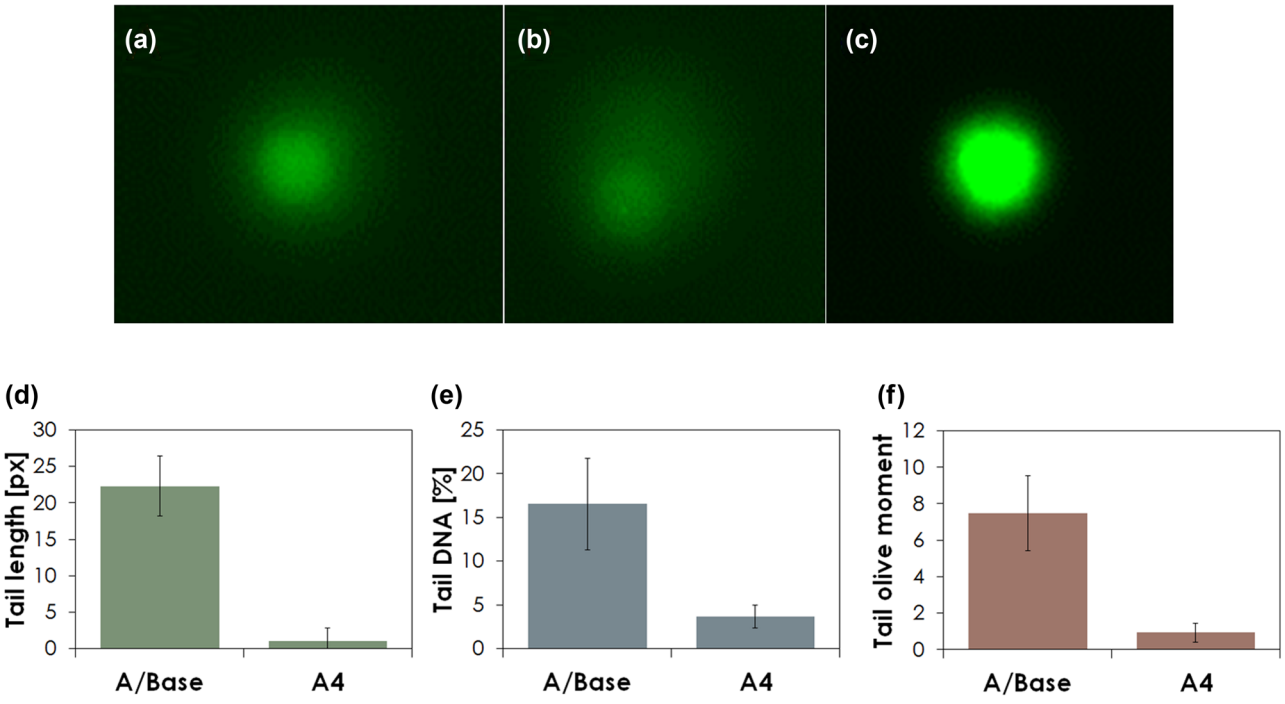

Figure 12: CT results: (a) positive control, (b) A/Base, (c) A4, (d) tail length, (e) DNA tail, and (f) moment olive.

drug carriers modified with glucose loaded with the active substance. The study showed that glucose conjugation led to a 50% reduction of MDA-MB-231 cells compared to non-modified material. In the case of HaCaT cells, this reduction was slightly lower [45].

4 Discussion

Table 3 presents the critical assessment of the developed method by providing its advantages and disadvantages. One may note that the presented method has more pros than cons. The process is carried out in the field of microwave radiation, where its efficacy stands in stark contrast to the traditional practice of heating reaction mixtures. Microwave radiation excels at generating thermal energy more efficiently, offering a marked advantage. In this innovative approach, aqueous solutions of both reactants come into play. Water, acting as a dipole solvent, emerges as an ideal medium, swiftly and effectively facilitating the transfer of thermal energy engendered by the microwave field. Through the dipole polarisation mechanism, heat diffuses uniformly across the entire reaction mixture. Notably, the infusion of microwave energy substantially expedites chemical processes, ushering in accelerated reactions. This fact directly affects the shortening of the time needed for an effective reaction. Under microwave radiation, the temperatures from the range of 120–180°C are high enough for performing the polycondensation of titanium hydroxide and dehydration of condensation product to titanium(IV) oxide and water. The great advantage is that the reagents used in the process do not pose a threat to the environment. They are neither irritating nor harmful to living organisms. This applies mainly to the modifying substance glutathione. There is no need to manage solid waste as it is not generated in the process. On the other hand, the process may seem to be quite expensive since the unit costs of the used reagents are relatively high. The step that guarantees the formation of fine nanoparticles is the homogenisation of the reaction mixture before the microwave process. This step can be seen as requiring additional energy input, which is economically undesirable.

Table 3: Potential advantages and disadvantages of the developed method

Advantages	Disadvantages
Microwave-assisted process	High cost of the reagents
Short time of the process	Need to homogenise the reaction mixture
Low temperatures of the microwave process	
No need for TiO_2 curing	
No toxic reagents	
Safe and environment-friendly modifying agent	
No solid waste	

In the works of other scientists, one may find information on obtaining less toxic titanium oxide nanoparticles, which may be used in drug delivery systems. Bhullar *et al.* obtained stable TiO_2 nanoparticles by performing the following steps. A precise quantity of Titanium TetraIsopropoxide was introduced into 50 mL of ethanol while maintaining continuous stirring. Subsequently, a gradual, dropwise addition of 40 mL of deionised water along with a controlled amount of HNO_3 was carried out. The solution was then subjected to a heat treatment stage at 60°C, upheld for a duration of 3 h. The ensuing step involved the calcination of the samples. Materials were calcined at 1,000°C [46]. In the presented method, we avoid using organic solvents such as ethanol, which from the ecological point of view is highly recommended. Moreover, thanks to the performing microwave-assisted processes, we omit the calcination stage, which consumes a lot of energy.

Silica coating serves as a prevalent chemical technique for altering the surface properties of TiO_2 nanoparticles, offering several advantages, including enduring stability, biocompatibility, and hydrophilicity. The Stöber technique stands out as the most widely utilised for incorporating silica onto bare TiO_2 nanoparticles. In the typical Stöber process, TiO_2 nanoparticles are uniformly dispersed within an ethanol solution. Sequentially, tetraethoxysilane (TEOS) and aqueous ammonia solution are introduced. Ammonia functions as a basic catalyst, overseeing the hydrolysis of TEOS and controlling silica thickness to yield particles with uniform morphology. The hydrolysis reaction involves the conversion of $–Si–OC_2H_5$ groups from TEOS to silanol groups (–Si–OH). Subsequently, a condensation reaction takes place between these –Si–OH groups and the surface –OH groups of TiO_2 nanoparticles, resulting in the formation of chemical Ti–O–Si bonds. This sol–gel process culminates in establishing a three-dimensional silica network enveloping the core of TiO_2 nanoparticles [47].

In order to improve the biocompatibility and tolerances of a specific material and thus increase the effectiveness of its potential applications, specific structures are subjected to modification of their surface. This is especially important in the case of biomedical and pharmaceutical applications, where materials used, for example, as drug carriers, must meet several restrictive requirements. Using additional compounds such as proteins, sugars, or polymers ensures no accumulation or non-toxicity. In addition,

it is often desirable to have specific functional groups on the surface that effectively bind the active ingredient to the carrier and maintain its therapeutic properties. The types of surface changes that, for example, drug carriers based on nanoparticles are subjected to can be broadly divided into physicochemical and biochemical. The former causes changes in the chemical composition of the surface layer, while biochemical methods are based on the attachment of organic compounds to the surface [48].

The properties of the (modified) material obtained depend primarily on the substrates used. Their degree of conversion and other process parameters are important. The modification is most often performed *in situ*, and then the modifying substance is added at the synthesis stage. The modifier can also be added *ex situ*, *i.e.* after the synthesis is finished, when the finished, pure product is subjected to modification [49]. Among the many purposes of modifying materials for biomedical applications is to ensure the ability of the material to function properly in conditions of low pH and body fluids. It is also necessary to ensure that the recipient's body is not adversely affected. The use of modifiers also affects biofunctionality. It consists in fulfilling specific functions *in vivo* for the assumed time or resistance to degradation. So far, no suitable biomaterial that would meet all the requirements set for it has been developed. Disintegration under relatively aggressive conditions faced by nanoparticle-based carriers is inevitable. Another priority in the use of modifiers is counteracting destruction in order to prevent negative effects caused by decay products, such as toxic, carcinogenic, mutagenic, or inflammatory effects [48]. Covalent carriers linked to an active substance are called conjugates. Depending on the conjugated substance, the following systems are distinguished: carrier–drug, carrier–protein, and carrier–DNA. Modifying substances attached to the carriers can, for example, change their solubility. Interestingly, they can play the role of the so-called tropic molecules that are responsible for recognising target tissues. The first conjugate of this type to enter the clinical trial phase was the combination of DOX with *N*-(2-hydroxypropyl) methacrylamide and galactosamine, which acted as a tropic molecule [50].

The use of modifications in the case of nanomaterials is also aimed at reducing agglomeration, controlling hydrophobic properties, or facilitating self-organisation. Organic compounds are most often used for this purpose. Among them, mention may be made of carboxylic acids, silanes, thiols, surfactants, and amines. Polymers for unconventional functionalisation (coating the surface of nanoparticles) are also often used, *e.g.* low-molecular PEG [50]. Galactose can be successfully used as a modifier of drug carriers in anti-cancer therapy. Its effectiveness is mainly based on the fact that carrier molecules loaded with saccharides on the surface are more readily captured by neoplastic cells. This is due to the fact that they have a high energy demand, which ultimately increases the effectiveness of targeted therapy [1]. Xia *et al.* [51] used galactose in their research to modify selenium nanoparticles. The tumour-targeted "delivery system" created in this way was covered with an anti-cancer drug. DOX was added to the surface of the modified nanoparticles to improve its antitumour efficacy in treating hepatocellular carcinoma (HCC). Further studies showed that the thus prepared, functionalised drug delivery system showed effective cellular uptake and penetrated them through endocytosis. The selenium–galactose–DOX nanoparticle system showed significant activity in inducing apoptosis of HCC cells *in vitro* [51]. Targeted drug delivery can be improved by drug loading on nanoparticles to which an appropriate recognition particle can be added for efficient transport to specific tumour cells. Galactose is a good example of such a molecule because it enhances uptake by pathogenic cells, but other recognition mechanisms such as ligand–receptor or antibody–antigen interaction can also be used. Also, in their research, Cardoso *et al.* designed nanoparticles coupled with galactose. The anti-cancer drug used in this case was also DOX. Modification with galactose showed a huge improvement in the therapeutic effectiveness of its action. The synthesised structures allowed the active base to be trapped in a hydrophobic inner core during the nanoparticle formation process. As a result, a carrier with a high drug content was obtained, which effectively recognises neoplastic cells [52].

5 Conclusion

A series of TiO_2 nanoparticles modified with glutathione was prepared. Physicochemical properties of the obtained products were analysed. Also, the analysis of releasing of both titanium ions and active substance was performed. The materials were assessed *via in vitro* studies in which the cytotoxicity, proliferation, and mutagenicity in relation to CHO cells were checked. Material A4 was found to meet all assumed requirements. It was characterised by a crystalline structure with crystallite size equal to 5.8 nm. The specific surface area of A4 material was equal to 231.9 m^2/g, the pore volume was 0.3129 cm^3/g, and their average size was 1.5519 nm. According to DLS analysis, the nanoparticles size was equal to 46 nm, however, based on TEM microphotographs, it did not exceed 10 nm. One must take into account that the DLS technique is based on

Mie's theory, and according to that, the elongated particles are equated to a sphere, which may lead to erroneous size analysis by this technique. Product A4 was obtained when the molar ratio of glutathione to TiO_2 was middle (0.11), the fold of NaOH was used in a stoichiometric amount, the process temperature was the highest (180°C), and the process time was equal to 5 min. Such prepared material has a great potential to be checked in further studies as a solid drug carrier.

Further research should focus on examining the safety of the developed material in the context of clinical trials. Therefore, after the production of a medicinal product in the form of an active substance combined with a nanocarrier, preclinical, and clinical trials should be carried out, which will include the following tests:

- Safety pharmacology
- Pharmacodynamics
- Toxicokinetics
- Acute toxicity
- Single dose toxicity
- Repeated dose toxicity
- Reproductive toxicity
- Carcinogenicity
- Genotoxicity.

The product must be subjected to tests specified in ISO 10993, such as sensitising properties, irritating properties/intradermal reactivity, acute systemic toxicity (animal model), and genotoxicity. The next stage necessary to carry out are *in vivo* tests and clinical trials that should be performed for a test series of products. The tests must be performed by an entity that meets the requirements of the Notifying Body, *e.g.* will have implemented the EN ISO 17025 standard. It is necessary to carry out a conformity assessment and obtain a CE certificate.

Acknowledgments: The authors are thankful to the National Centre for Research and Development, Poland, for the grants and financial support to this research.

Funding information: This work is a part of the project "A method of producing non-toxic carriers of active substances based on nanomaterials," supported by the National Centre for Research and Development, Poland, under the agreement LIDER/20/0080/L-9/17/NCBR/2018.

Author contributions: JPP: concept, design, and writing of the manuscript, OD: analysis of crystallographic data, AS: analysis of physicochemical properties of obtained products, PR: analysis of titanium releasing from the prepared materials, DD: performing of the cytotoxic studies, writing of the manuscript, MB: interpretation of the obtained results. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: The authors state no conflict of interest.

Data availability statement: The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

References

[1] Hossen S, Hossain MK, Basher MK, Mia MNH, Rahman MT, Jalal Uddin M. Smart nanocarrier-based drug delivery systems for cancer therapy and toxicity studies: A review. J Adv Res. 2019;15:1–18.

[2] Kadhum WR, Al-Zuhairy SAS, Mohamed MBM, Abdulrahman AY, Kadhim MM, Alsadoon Z, et al. A nanotechnology approach for enhancing the topical drug delivery by newly developed liquid crystal formulations. Int J Drug Deliv Technol. 2021;11(3):716–20.

[3] Xu Y, Zhao M, Zhou D, Zheng T, Zhang H. The application of multifunctional nanomaterials in Alzheimer's disease: A potential theranostics strategy. Biomed Pharmacother. 2021;137:111360.

[4] Liu D, Yang F, Xiong F, Gu N. The smart drug delivery system and its clinical potential. Theranostics. 2016;6(9):1306–23.

[5] Shah M, Fawcett D, Sharma S, Tripathy SK, Poinern GE. Green synthesis of metallic nanoparticles via biological entities. Mater (Basel). 2015;8(11):7278–308.

[6] Jayaseelan C, Rahuman AA, Roopan SM, Kirthi AV, Venkatesan J, Kim S-K, et al. Biological approach to synthesize TiO2 nanoparticles using Aeromonas hydrophila and its antibacterial activity. Spectrochim Acta – A: Mol Biomol Spectrosc. 2013;107:82–9.

[7] Ovais M, Khalil AT, Ayaz M, Ahmad I, Nethi SK, Mukherjee S. Biosynthesis of metal nanoparticles via microbial enzymes: a mechanistic approach. Int J Mol Sci. 2018;19(12):4100.

[8] Kurutas EB. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. Nutr J. 2016;15:71.

[9] Sagadevan S, Imteyaz, S, Murugan B, Lett JA, Sridewi N, Weldegebrieal GK, et al. A comprehensive review on green synthesis of titanium dioxide nanoparticles and their diverse biomedical applications. Green Process Synth. 2022;11(1):44–63.

[10] Singh J, Soni RK. Fabrication of hydroxyl group-enriched mixed-phase TiO2 nanoflowers consisting of nanoflakes for efficient photocatalytic activity. J Mater Sci Mater Electron. 2020;31:12546–60.

[11] Basavarajappa L, Patil PS, Ganganagappa SB, Reddy N, Raghu KR, Reddy AV, CV. Recent progress in metaldoped TiO2, non-metal doped/codoped TiO2 and TiO2 nanostructured hybrids for enhanced photocatalysis. Int J Hydrog Energy. 2020;45:7764–78.

[12] Ghumro SS, Lal B, Pirzada T. Visiblelight-driven carbon-doped TiO2-based nanocatalysts for enhanced activity toward microbes and removal of dye. ACS Omega. 2022;7:4333–41.

[13] Singh J, Soni RK. Tunable optical properties of Au nanoparticles encapsulated TiO2 spheres and their improved sunlight mediated photocatalytic activity. Colloids Surf A: Physicochem Eng Asp. 2021;612:126011.
[14] Singh J, Tripathi N, Mohapatra S. Synthesis of Ag–TiO2 hybrid nanoparticles with enhanced photocatalytic activity by a facile wet chemical method. Nano-Struct Nano-Objects. 2019;18:100266.
[15] Singh J, Kumar Verma A. Plasmonic-TiO2 nanohybrid for environmental and energy applications. Bejaoui B, editor. In:Updates on Titanium Dioxide. London: InTech Open; 2022. p. 1–24.
[16] Zhang H, Wang C, Chen B, WangInt X. Daunorubicin-TiO2 nanocomposites as a "smart" pH-responsive drug delivery system. J Nanomed. 2012;7:235–42.
[17] Zienta D, Czarczynska-Goslinska B, Mlynarczyk DT, Glowacka-Sobotta A, Stanisz B, Goslinski T, et al. Titanium dioxide nanoparticles: prospects and applications in medicine. Nanomaterials. 2020;10(2):387.
[18] Wu F, Hicks AL. Estimating human exposure to titanium dioxide from personal care products through a social survey approach. IEAM. 2019;16(1):10–6.
[19] Valentini X, Rugira P, Frau A, Tagliatti V, Conotte R, Laurent S, et al. Hepatic and renal toxicity induced by TiO2 nanoparticles in rats: a morphological and metabonomic study. J Toxicol. 2019;2019(1):5767012.
[20] Lappas CM. The immunomodulatory effects of titanium dioxide and silver nanoparticles. Food Chem Toxicol. 2015;85:78–83.
[21] Bojarowicz H, Bartnikowska N. Kosmetyki ochrony przeciwsłonecznej. Część I Filtry UV oraz ich właściwości Probl Hig Epidemiol. 2014;95(3):596–601.
[22] Skowroń J, Konieczko K. Narażenie zawodowe na związki chromu (VI). Med Pr. 2014;65(3):407–27.
[23] Romanos GE, Fischer GA, Delgado-Ruiz R. Titanium wear of dental implants from placement, under loading and maintenance protocols. Int J Mol Sci. 2021;22(3):1067.
[24] Tibau AV, Grube BD, Velez BJ, Vega VM, Mutter J. Titanium exposure and human health. Oral Sci Int. 2019;16(1):15–24.
[25] Esmaeili A, Moniri E, Safaeijavan R, Panahi HA. Green synthesis of $AgNO_3$/glutathione nanoparticles by *Eucalyptus Globulus* extracts as a novel nanocarrier for using as drug delivery system: study of nonlinear isotherms and kinetics. Polym Bull. 2023;80:10843–61. doi: 10.1007/s00289-022-04583-7
[26] Hooshyar SP, Mehrabian RZ, Panahi HA, Jouybari MH, Jalilian H. Synthesis and characterization of magnetized-PEGylated dendrimer anchored to thermosensitive. polymer for letrozole drug delivery. Colloids Surf B. 2019;176:404–11.
[27] Heidarinasab A, Panahi HA, Faramarzi M, Farjadian F. Synthesis of thermosensitive magnetic nanocarrier for controlled sorafenib delivery. Mater Sci Eng C. 2016;67:42–50.
[28] Zifar M, Panahi HA, Asli MD, Eftekhari-Sis B, Rafiee A. Fabrication of pH and thermosensitive polymer conjugated tungsten disulfide for photo-thermal prostate cancer therapy under near-infrared laser irradiation: In vitro cytotoxicity study. Eur Polym J. 2023;192:112066.
[29] Soltani ER, Panahi HA, Moniri E, Fard NT, Raeisi I, Beik J, et al. Construction of a pH/Temperature dual-responsive drug delivery platform based on exfoliated MoS2 nanosheets for effective delivery of doxorubicin: Parametric optimization via central composite design. Mater Chem Phys. 2023;295:127159.
[30] Hu Z, Guo W. New insight into the effect of interface supercapacitance on the perforamce of titanium dioxide/carbon nanowire array for photoelectrochemical water oxidation. Chin Chem Lett. 2021;32(11):3359–63.
[31] Seymour LE, Ferry DR, Kerr DJ, Rea D, Whitlock M, Poyner R, et al. Phase II studies of polymer-doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer. Int J Oncol. 2009;34(6):1629–36.
[32] Terwogt JMM, ten Bokkel Huinink WW, Schellens JH, Schot M, Mandjes IA, Zurlo MG, et al. Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymer-conjugated prodrug of paclitaxel. Anticancer Drugs. 2001;12(4):315–23.
[33] Bissett D, Cassidy J, de Bono JS, Muirhead F, Main M, Robson L, et al. 2Phase I and pharmacokinetic (PK) study of MAG-CPT (PNU 166148): a polymeric derivative of camptothecin (CPT). Br J Cancer. 2004;91:50–5.
[34] Singer JW, Shaffer S, Baker B, Bernareggi A, Stromatt S, Nienstedt D, et al. Paclitaxel poliglumex (XYOTAX; CT-2103): An intracellularly targeted taxane. Anticancer Drugs. 2005;16(3):243–54.
[35] Homsi J, Simon GR, Garrett CR, Springett G, De Conti R, Chiappori AA, et al. Phase I trial of poly-L-glutamate camptothecin (CT-2106) administered weekly in patients with advanced solid malignancies. Clin Cancer Res. 2007;13(19):5855–61.
[36] Safari J, Zarnegar Z. Advanced drug delivery systems: Nanotechnology of health design A review. J Saudi Chem Soc. 2014;18(2):85–99.
[37] Sobczak A, Kida A, Kowalski Z, Wzorek Z. Evaluation of the biomedical properties of hydroxyapatite obtained from bone waste. Pol J Chem Tech. 2009;11(1):37–43.
[38] Huang P, Jiang Q, Yu P, Yang L, Mao L. Alkaline post-treatment of Cd(II)–glutathione coordination polymers: toward green synthesis of water-soluble and cytocompatible CdS quantum dots with tunable optical properties. ACS Appl Mater Interfaces. 2013;5(11):5239–46.
[39] Chen K, Zhang T, Chen X, He Y, Liang X. Model construction of micro-pores in shale: A case study of Silurian Longmaxi Formation shale in Dianqianbei area. SW China Petrol Explor Dev. 2018;45(3):412–21.
[40] Chang SS, Clair B, Ruelle J, Beauchêne J, Di Renzo F, Quignard F, et al. Mesoporosity as a new parameter for understanding tension stress generation in trees. J Exp Bot. 2009;60(11):3023–30.
[41] Bruschi ML. Main mechanisms to control the drug release. In: Bruschi ML, editor. Strategies to modify the drug release from pharmaceutical systems. Amsterdam: Elsevier; 2015. p. 37–62.
[42] Lu XY, Wu DC, Li ZJ, Chen GQ. Polymer nanoparticles. Prog Mol Biol Transl Sci. 2011;104:299–323.
[43] Lavielle N, Hébraud A, Thöny-Meyer L, Rossi RM, Schlatter G. 3D Composite assemblies of microparticles and nanofibers for tailored wettability and controlled drug delivery macromol. Mater Eng. 2017;302(8):1600458.
[44] Sheth U, Bahadur A. *In-vitro* evaluation of cytotoxic and antioxidant properties of drugs solubilized in EO-PO star block copolymer micelles. Colloids Surf B. 2018;171(1):343–50.
[45] Torres-Pérez SA, Torres-Pérez CE, Pedraza-Escalona M, Pérez-Tapia SM, Ramón-Gallegos E. Glycosylated nanoparticles for cancer-targeted drug delivery. Front Oncol. 2020;10:605037.
[46] Bhullar S, Goyal N, Gupta S. A recipe for optimizing TiO2 nanoparticles for drug delivery applications. OpenNano. 2020;8:100096.
[47] Haghighi FH, Mercurio M, Cerra S, Salamone TA, Bianymotlagh R, Palocci C, et al. Surface modification of TiO2 nanoparticles with organic molecules and their biological applications. J Mater Chem B. 2023;11:2334–66.

[48] Przybyszewska-Doroś I, Okrój W, Walkowiak B. Modyfikacje powierzchni implantów metalicznych. Inż Biomat. 2005;8(43–44):52–62.
[49] Lee CY, Hu SM, Christy J, Chou FY, Ramli TC, Chen HY. Biointerface coatings with structural and biochemical properties modifications of biomaterials. Adv Mater Interfaces. 2023;10(10):2202286.
[50] Odrobińska J, Neugebauer D. Nowe rozwiązania i modyfikacje najpopularniejszych polimerowych nośników substancji aktywnych. Wiad Chem. 2019;73(9–10):481–501.
[51] Xia Y, Zhong J, Zhao M, Tang Y, Han N, Hua L, et al. Galactose-modified selenium nanoparticles for targeted delivery of doxorubicin to hepatocellular carcinoma. Drug Deliv. 2019;26(1):1–11.
[52] Cardoso MM, Peça IN, Raposo CD, Petrova KT, Barros MT, Gardner R, et al. Doxorubicin-loaded galactose-conjugated poly(D,L-lactide-co-glycolide) nanoparticles as hepatocyte-targeting drug carrier. J Microencapsul. 2016;33(4):315–22.