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**Platinum nanoparticles and anticancer drugs –
analysis of interactions and biological activity**

Nanocząstki platyny a leki przeciwnowotworowe –
analiza oddziaływań i aktywności biologicznej

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1. Streszczenie

Nowotwory to jedno z najgroźniejszych chorób XXI wieku. Tylko w 2022 zdiagnozowano je u prawie 20 milionów osób, jednocześnie, tego samego roku, z ich powodu zmarło prawie 10 milionów ludzi. Co więcej, według najnowszych prognoz, zapadalność na tę chorobę będzie dalej wzrastać i do 2050 osiągnie blisko 35 milionów nowych zachorowań rocznie, niezależnie od poziomu wskaźnika rozwoju społecznego (HDI) danego kraju. Pomimo znacznego rozwoju terapii przeciwnowotworowych na przestrzeni ostatnich lat, chemioterapia wciąż pozostaje jedną z najczęściej stosowanych metod leczenia. Niestety, pomimo swojej skuteczności, jej stosowanie nie jest obojętne wobec komórek nienowotworowych, prowadząc do skutków ubocznych, w tym tak poważnych jak toksyczność wobec przewodu pokarmowego, mielosupresja, czy kardiotoxyczność. Ponadto, długotrwałe stosowanie chemioterapii często prowadzi do rozwoju lekooporności, co obniża skuteczność terapii. Z tego powodu poszukiwanie czynników modulujących, które mogłyby zwiększyć skuteczność i/lub selektywność działania leków przeciwnowotworowych ma kluczowe znaczenie. Wśród proponowanych kandydatów są nanocząstki, które definiuje się jako cząstki o rozmiarach od 1 do 100 nm. Dzięki swoim właściwościom fizykochemicznym, takim jak zróżnicowany rozmiar, kształt, skład oraz możliwa funkcjonalizacja powierzchni, nanocząstki znajdują zastosowanie w wielu dziedzinach nauki. Oprócz ich własnej aktywności przeciwnowotworowej, wykazują również znaczny potencjał jako modyfikatory aktywności leków przeciwnowotworowych, a także jako istotny czynnik składnik systemów dostarczania leków, których intencją jest zwiększenie skuteczności i selektywności terapii.

Głównym celem niniejszej rozprawy doktorskiej była ocena, czy nanocząstki platyny (PtNPs), o zdefiniowanych średnicach 5, 30, 50 i 70 nm, oddziałują z trzema lekami przeciwnowotworowymi: daunorubicyną (DAU) i idarubicyną (IDA), należącymi do grupy antybiotyków antracyklinowych, oraz mitomycyną C (MMC), reprezentantem grupy antybiotyków alkilujących. Dodatkowym celem tych badań było określenie, czy takie interakcje wpływają na aktywność biologiczną badanych substancji czynnych (API). Aby zrealizować te zadania, zastosowano szerokie spektrum metod fizykochemicznych i biologicznych. Charakterystyka fizykochemiczna obejmowała spektroskopie fluorescencyjną, spektroskopie w podczerwieni z transformacją Fouriera (FTIR), spektroskopię bliskiej podczerwieni (NIR), izotermiczną kalorymetrię

miareczkową (ITC) oraz różnicową kalorymetrię skaningową (DSC). Ponadto, wielkość cząstek i ich potencjalną agregację oceniono z wykorzystaniem dynamicznego rozpraszania światła (DLS), analizy śledzenia nanocząstek (NTA) oraz mikroskopii sił atomowych (AFM). Aktywność biologiczną oceniono przy użyciu testu mutagenności Amesa z wykorzystaniem szczepów *Salmonella enterica* serowar Typhimurium TA98 i TA102 w celu sprawdzenia wpływu PtNPs na potencjał mutageny DAU, IDA oraz MMC. W dodatku przeprowadzono testy cytotoksyczności *in vitro* w dwóch modelach: porównanie wpływu PtNPs na DAU wobec zdrowych komórek HaCaT i nowotworowych komórek MelJuSo, oraz porównanie wpływu PtNPs na IDA i MMC wobec dwóch linii nowotworów piersi, SK-BR-3 i MCF-7.

Wyniki przeprowadzonych badań wykazały, że PtNPs oddziałują ze wszystkimi analizowanymi lekami przeciwnowotworowymi, chociaż charakter i siła tych oddziaływań różniły się w zależności od związku. Analizy termodynamiczne wykazały endotermiczny charakter interakcji, wyraźnie sugerując, że oddziaływania te są napędzane entropowo. Badania spektroskopowe dodatkowo potwierdziły zmiany w środowisku chemicznym badanych leków w połączeniu z PtNPs, natomiast wygaszanie fluorescencji wskazywało na bliskie oddziaływania w układach PtNPs-antracyklina. Co istotne, zaobserwowane interakcje wpływały na aktywność biologiczną badanych związków, ogólnie skutkując obniżeniem potencjału mutagennego, a w kilku układach eksperymentalnych prowadząc do zwiększenia cytotoksyczności leku, jednakże siła i kierunek tych efektów różniły się w zależności od leku, średnicy nanocząstek, i stężenia obu składników.

Podsumowując, wyniki przedstawione w niniejszej rozprawie doktorskiej wskazują, że nanocząstki platyny stanowią interesującą i obiecującą platformę do modulacji aktywności leków przeciwnowotworowych, i mogą stanowić podstawę do opracowania strategii terapeutycznych wspomaganych nanocząstkami.

2. Abstract

Cancer is one of the most dangerous diseases of the 21st century. In 2022 alone, almost 20 million new cases were diagnosed worldwide, while approximately 10 million cancer-related deaths were recorded. Moreover, the global cancer burden is projected to further increase, reaching nearly 35 million yearly diagnoses by 2050, irrespective of the country's human development index (HDI). Although therapeutic approaches have significantly advanced over the years, chemotherapy still remains one of the most widely applied treatment regimens. In spite of its efficacy, it is not indifferent to normal cells, resulting in damage to healthy tissues and adverse effects, some of which as severe as gastrointestinal toxicity, myelosuppression, or cardiotoxicity. In addition to that, prolonged treatment frequently leads to the development of drug resistance, reducing therapeutic effectiveness. Therefore, the search for modulatory agents that would improve the efficacy and/or selectivity of anticancer agents is of considerable importance. Among the proposed modulatory candidates are nanoparticles, which, by definition, are small particles sized between 1 and 100 nm. Due to their physicochemical properties, including a variety of sizes, shapes, compositions, and possible surface functionalisation, nanoparticles have been studied in numerous scientific fields. Besides their intrinsic cytotoxicity towards cancer cells, they have demonstrated a significant potential as modulators of anticancer drug activity, as well as components of drug delivery systems, the design of which is to improve treatment selectivity and efficacy.

The primary aim of this doctoral dissertation was to evaluate if platinum nanoparticles (PtNPs) of distinct diameters, namely 5, 30, 50, and 70 nm, interact with three anticancer agents: daunorubicin (DAU) and idarubicin (IDA), both belonging to the anthracycline antibiotics group, and mitomycin C (MMC), an alkylating antibiotic. The additional aim of these studies was to determine whether such interactions influence the biological activity of the investigated active pharmaceutical ingredients (API). To address these objectives, a combination of physicochemical and biological methods was employed. The physicochemical characterisation methods included fluorescence, Fourier-Transform Infrared (FTIR), and Near-Infrared (NIR) spectroscopies, as well as isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). Furthermore, particle size and aggregation patterns were assessed using dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and atomic force microscopy (AFM). The biological activity was evaluated using the Ames mutagenicity assay with *Salmonella*

enterica serovar Typhimurium strains TA98 or TA102 to assess the influence of PtNPs on the mutagenic potential of DAU, IDA, and MMC, as well as cytotoxicity studies. The latter consisted of *in vitro* analyses using two models: comparison between cancerous MelJuSo and non-cancerous HaCaT cells for the PtNPs-DAU study, and comparison between two breast cancer cell lines, namely SK-BR-3 and MCF-7, for PtNPs-IDA and PtNPs-MMC studies.

The results of all these studies demonstrated that PtNPs interact with all three investigated antineoplastic agents, although the extent and nature of these interactions differed between the compounds. Thermodynamic analyses indicated predominantly endothermic interactions that were most likely driven by favourable entropy changes. Spectroscopic analyses further confirmed changes in the chemical environment of the investigated compounds following combination with PtNPs, while fluorescence quenching indicated close-proximity interactions for the PtNPs-anthracyclines combinations. Importantly, the registered interactions influenced the biological activity of the compounds, generally resulting in a reduced mutagenic potential of the combined APIs, and, in several experimental systems, enhanced cytotoxic effects towards cancer cells. However, the magnitude and direction of these effects varied depending on the drug, nanoparticle sizes, and the concentration of both.

Overall, the findings presented in this dissertation demonstrate that platinum nanoparticles constitute an interesting and promising platform for the modulation of anticancer drug activity and may serve as a basis for the development of nanoparticle-assisted therapeutic strategies.

3. List of abbreviations:

2,7 – DAM - 2,7-diaminomitosen (a MMC reductive metabolite)

AFM – Atomic Force Microscopy

AgNPs – Silver Nanoparticles

ALL – Acute Lymphoblastic Leukaemia

AML – Acute Myeloid Leukaemia

API – Active Pharmaceutical Ingredient

ABC – ATP-binding cassette

AuNPs – Gold Nanoparticles

AuPtNPs – Bimetallic Gold-Platinum Nanoparticles

C₆₀ – Fullerene C₆₀

CAP – Cold Atmospheric Plasma

DAM – Diaminomitosenes

DAU – Daunorubicin

DDS – Drug Delivery System

DOX – Doxorubicin

EPI – Epirubicin

EPR – Enhanced Permeability and Retention

GCO – Global Cancer Observatory

GO – Graphene Oxide

GSH - Glutathione

GST – Glutathione S-transferase

HCPT – 10-hydroxycamptothecin

HDI – Human Development Index

HR – Homologous Recombination

HUS – Haemolytic Uremic Syndrome

IBM – International Business Machines Corporation

IDA – Idarubicin

IONPs – Iron Oxide Nanoparticles (or Fe_3O_4)

MDR – Multidrug Resistance

MMC – Mitomycin C

MNPs – Metallic Nanoparticles

MTX – Methotrexate

NER – Nucleotide Excision Repair

NHEJ – Non-Homologous End Joining

NQO1 – NADPH-quinone dehydrogenase 1

PAA-PtNPs – PolyAcrylic Acid Platinum Nanoparticles

PEG – Polyethylene glycol

PEG-PtNFs – PEGylated Platinum Nanoflowers

PtNPs – Platinum Nanoparticles

PVP – Polyvinylpyrrolidone

ROS – Reactive Oxygen Species

SEM – Scanning Electron Microscopy

STM – Scanning Tunnelling Microscopy

TEM – Transmission Electron Microscopy

VEGF – Vascular Endothelial Growth Factor

XRD – X-ray Diffraction

4. Introduction

4.1. A brief history of cancer

The earliest known written descriptions of conditions resembling cancer come from ancient Egypt and are dated back to 1600 B.C. The script, known as the “Edwin Smith Papyrus”, after an American collector of antiquities, who bought it in 1862, describes 48 surgical case descriptions, several of which concern tumours and other lesions of the breast. One case describes them as “a hard, dense, and cool bulging mass in the breast, spreading beneath the skin” [1]. Although the original author is unknown, the document shows that ancient Egyptian physicians were able to distinguish certain persistent tumours from other inflammatory lesions.

At those times, the medical assistance consisted of a combination of grease, honey and lint in the form of ointments or poultices. In principle, these medications were intended to protect wounds, reduce inflammation, and promote healing. Keeping that in mind, the curation of cancer was severely limited, especially for the deeply located tumours. Legend has it that Atossa, a Persian queen, developed breast cancer, which was properly treated by Democedes, a captive Greek slave who happened to be a physician. In exchange, he demanded that her husband, King Darius I, invade Greece and allow him to join the scouting party. All went to plan, and upon the Persian ships’ arrival to scout the land, Democedes managed to escape the scouting party and soon after returned to his homeland. However, scientists are a bit sceptical of this diagnosis, as the lesion on the breast could have been of bacterial origin, so despite the story having a seemingly happy ending, ancient reports of cancer treatments should be viewed with caution [2].

For centuries, diseases were believed to be a punishment from the gods but around 400 B.C., the Greek physician, a man of many talents and well known as the “Father of Medicine”, Hippocrates, disproved of these ideas and started to claim that diseases, cancer among them, were caused either by excess or deficiencies in one of four essential body fluids: blood, phlegm, yellow bile, and black bile, which was later formalised by the Roman physician Galen. Despite the theory becoming ultimately debunked, Hippocrates laid the foundation for the terminology – he was the first to call it “carcinos”, which was the Greek term for “crab”, as the shape of the observed tumours resembled a crab trying to catch its prey [3].

The cellular nature of organisms did not emerge until the development of microscopy. In 1665, Robert Hooke was the first to introduce the term “cell” after careful examinations of thin layers of cork. However, the recognition that living organisms are composed of cells developed in the 19th century, when Theodor Schwann demonstrated that animal tissues are composed of cells as well. In a similar timeframe, Johannes Müller, who was Schwann’s boss, applied microscopy to investigate tumours. In his work, he presented detailed microscopic observations and illustrations of nearly a hundred distinguishable cancerous cells. Subsequently, Rudolf Virchow established abnormal cell proliferation as a central feature of this disease. These studies significantly contributed to the decline of humoral explanations of cancer [4]. From here on, the progress in microscopy, pathology, surgery, and molecular biology gradually improved the understanding of cancer. These developments allowed for the emergence of modern cancer classification and treatment.

4.2. The cancer problem

In the present day, cancer remains amongst the deadliest diseases worldwide. According to the Global Cancer Observatory (GCO) in 2022 there were nearly 20 million new cases diagnosed, with almost 10 million deaths reported that year (**Figure 1**).

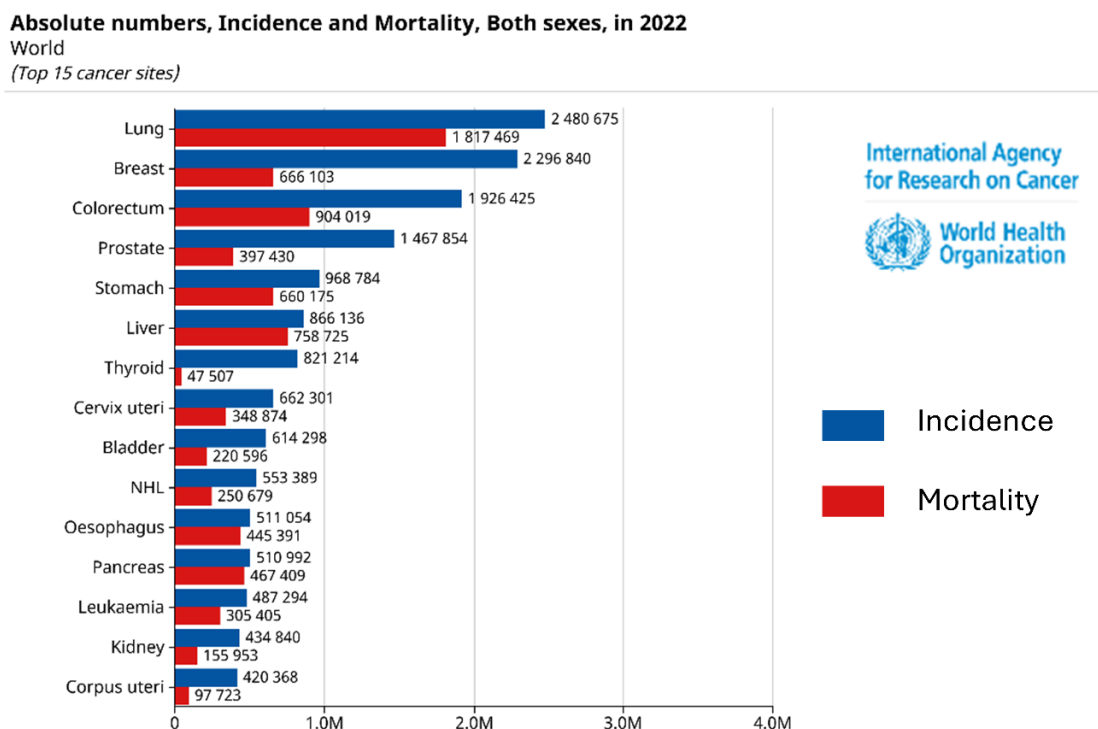


Figure 1. Number of new recorded cases of cancer and deaths caused by it in 2022 (adapted) [5].

The three most prevalent types are lung, breast, and colorectal cancers, accounting for over a quarter of overall diagnoses. In terms of mortality, lung cancer is the leading cause of cancer-related deaths, followed by gastrointestinal cancers. Despite the continuous advances in prevention, diagnosis, and treatment, the global cancer incidence is expected to increase over the next few decades – by 2050 the yearly incidence will increase to roughly 35 million, with proportional mortality of approximately 50%. Importantly, this trend is observed across countries with different levels of the Human Development Index (HDI), indicating that the disease is a concern for everyone (Figure 2).

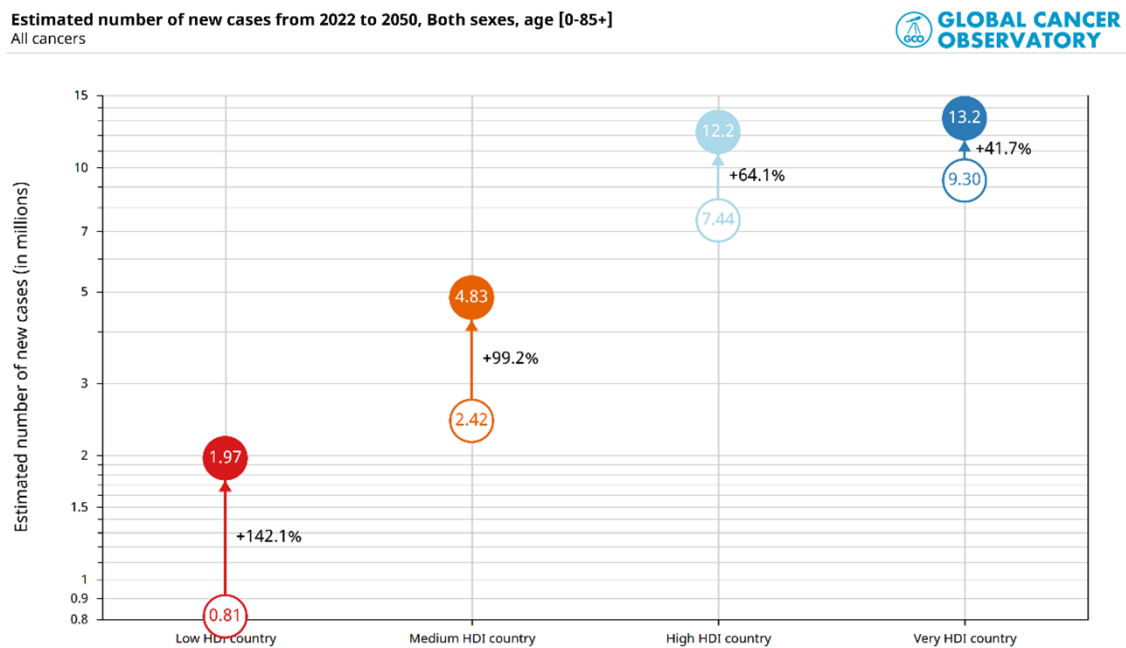


Figure 2. Estimated number of new cases from 2022 to 2050 based on country's HDI (adapted) [5].

4.3. Treatment regimens

Currently, there are various therapeutic strategies to treat cancer, and the selection depends strictly on the tumour type, stage, location, and molecular characteristics. Surgical removal of cancer is one of the principal treatment methods for solid tumours and is particularly effective if the removal of the malignant tissue is feasible. Radiotherapy and chemotherapies are often proposed, either alone or in combination with the surgical removal [6]. However, the main issue with these therapies is that they are not indifferent to healthy cells. Furthermore, cancer cells tend to evade antineoplastic

treatment over time through resistance mechanisms, classical chemotherapy may become insufficient over time, resulting in an unsuccessful therapy outcome.

To address this problem, more selective treatment strategies have emerged. Among them, targeted therapy and immunotherapy have attracted significant attention. In principle, targeted therapy is aimed at molecular pathways or proteins essential for the tumour growth and survival, whereas immunotherapy aims to stimulate or restore the intrinsic ability of the host's immune system to recognise and eliminate malignant cells [6,7]. Although these approaches tend to offer greater tumour specificity, they are not without adverse effects, and their use is dependent on the molecular and immunological characteristics of tumours [8,9].

In principle, chemotherapy relies on the inhibition of cell proliferation, thus limiting the tumour growth, invasion, and metastasis, with the main aim of cancer cell death [8,10]. These agents can work on many levels - their effects may involve disruption of DNA replication, transcription, protein synthesis, or other cellular pathways, ultimately leading to cell-cycle arrest or cell death, most commonly through apoptosis. Due to various mechanisms by which they exert their cytotoxicity, anticancer agents can be classified into several major groups, including alkylating agents, antimetabolites, antimicrotubular agents, topoisomerase inhibitors, and antitumour antibiotics [10,11].

For this doctoral thesis, three anticancer agents were selected: daunorubicin and idarubicin, both of which belong to the anthracycline class, and mitomycin C, which belongs to the general group of antitumour antibiotics.

4.3.1. Daunorubicin and Idarubicin

Daunorubicin (DAU) is an anthracycline antibiotic derived from the *Streptomyces peuceticus* bacterium species, first isolated in 1964 [12,13] (**Figure 3**). Since then, it has been determined to be a potent anticancer agent in many tumours, however, it was proven to be particularly useful in the treatment of acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) [14]. Idarubicin, also used in the treatment of ALL and AML, is a synthetic analogue of DAU, first developed in the 1970s. The main structural difference in comparison to DAU is the removal of the methoxy group at the C-4 position of the anthracycline ring (**Figure 3**). This seemingly minor change alters the

physicochemical and pharmacological properties of this drug significantly. The absence of the methoxy group increases IDA's lipophilicity, potentially improving penetration through biological membranes [15].

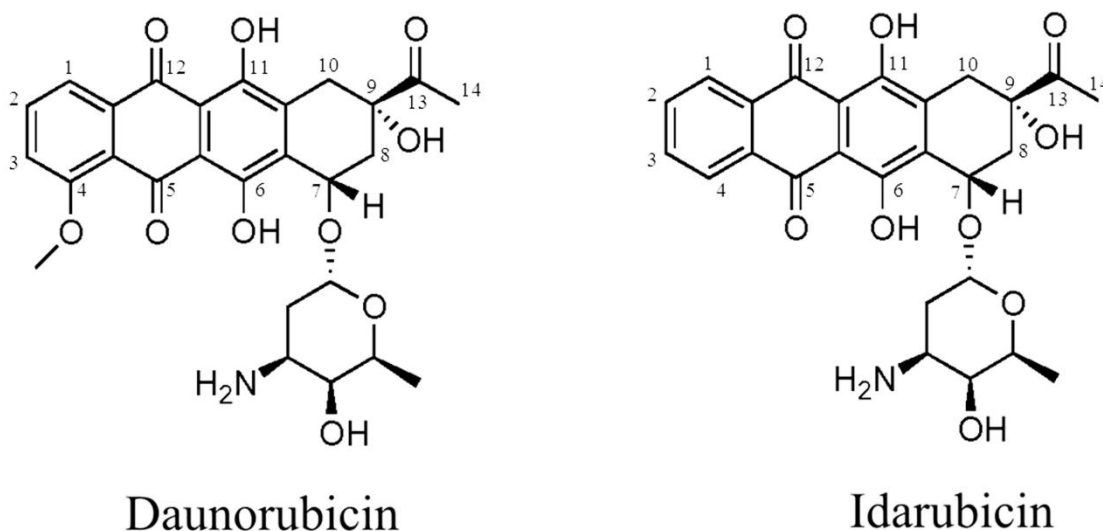


Figure 3. Structural differences between daunorubicin (DAU) and idarubicin (IDA) (adapted) [16].

The anticancer activity of these active pharmaceutical ingredients (API) is mediated through several mechanisms. The first mode of action is the intercalation of the anthracycline ring system between adjacent DNA base pairs, with a preference for GC-rich regions. This interaction disrupts DNA replication and transcription processes, resulting in the inhibition of proliferation. Moreover, they act as a topoisomerase II poison. Under physiological conditions, topoisomerase II introduces transient double-strand DNA breaks that relieve the tension that builds up upon DNA helix unwinding. Anthracyclines stabilise the normally transient topoisomerase II-DNA cleavage complex and prevent relegation of the DNA strands, resulting in accumulation of double-strand breaks and activation of the apoptotic pathway. The last majorly described mechanism of their activity is the generation of reactive oxygen species (ROS). When inside the cell, the quinone moiety undergoes redox cycles, which produces superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$ or $HO\bullet$). Excessive accumulation of these reactive species promotes oxidative damage to DNA, proteins, lipids, and various organelles. Over time, the damage accumulates so much that the repair mechanisms become insufficient, and the cell undergoes either apoptosis or necrosis

[12,17,18]. However, in contrast to daunorubicin, IDA frequently exhibits greater intracellular accumulation and higher cytotoxic potency than DAU [19,20]. By virtue of this, it has been reported that patients treated with IDA had overall greater treatment response than those with DAU treatment, even when the dosages of DAU were elevated. However, no significant differences have been observed in prolonged use, especially in terms of the general cytotoxicity and adverse side effects, suggesting that the relative benefits depend on the treatment regimen, dose, and the disease characteristics [20,21].

Despite their undeniable efficacy, similarly to other chemotherapeutics, the usage of DAU and IDA leads to side effects, with major ones being cardiotoxicity and myelosuppression, as well as gastrointestinal toxicity and possible secondary malignancies [16,22]. The mechanism of the former is combined with the fact that topoisomerase II has two main isoforms: topoisomerase II α and topoisomerase II β . The topoisomerase II α isoform is predominantly expressed in rapidly proliferating cells and is considered an important therapeutic target in malignant tissues. On the contrary, topoisomerase II β is expressed in dormant, non-dividing, and differentiated cells, such as cardiomyocytes [23,24], and because anthracyclines are not specific to either of these isoforms, cardiotoxicity appears as a dose-dependent and overall cumulative effect of using these drugs [25,26].

Unfortunately, cancerous cells can also evade the treatment through acquired drug resistance. One of the most frequently associated mechanisms with this phenomenon is the overexpression of the ATP-binding cassette (ABC) transporters, particularly the P-glycoprotein, MRP1, and BCRP, resulting in a multidrug resistance mechanism (MDR). These ATP-dependent efflux pumps actively transport xenobiotics and chemotherapeutics out of the cell. Through the overexpression of these proteins, the efflux is enhanced, resulting in a lack of efficient accumulation of the drug in the cell. Additional resistance mechanisms include alterations in topoisomerase II, namely its reduced expression, point mutations in the DNA-binding and CAP-like domains of the enzyme, and impaired kinetics of topoisomerase II through an increased ATP requirement for the enzyme to form cleavage complexes. As topoisomerase II binding is one of the key features of DAU and IDA, altogether these drug-evading mechanisms significantly decrease the efficiency of these chemotherapeutic agents. Moreover, cancer cells may acquire resistance through impaired apoptotic pathways, with common examples including mutations/loss of the p53, overexpression of anti-apoptotic proteins from the

BCL-2 family, or the suppression of the caspase cascade signalling. Furthermore, cancerous cells can also upregulate the DNA repair pathways, with the most important being the homologous recombination (HR) and non-homologous end joining (NHEJ). Altogether, these mechanisms enable tumour cells to survive despite the accumulation of drug-induced damages, ultimately reducing the effectiveness of chemotherapy [27–30].

Due to the fact that anthracyclines are still used in chemotherapy to this day, it is worth searching for possible modulators of the activity of these drugs, leading to the formation of better treatments, the results of which would be their increased efficacy and/or decreased off-target cytotoxicity.

4.3.2. Mitomycin C

Mitomycins are a class of antibiotics first isolated from *Streptomyces caespitosus* in 1956. The group consists of several closely related structural analogues named according to alphabetical order, however, the best characterised and widely used is mitomycin C (MMC), so the term “mitomycin” is commonly used to refer specifically to MMC (**Figure 4**) [31]. After its discovery, it has been a successful anticancer agent in both preclinical and clinical studies. It has been used in the treatment of several malignancies, such as gastric, pancreatic, and bladder cancer, often as a part of combination therapies. What is more, it was found to be effective against bladder cancer. However, the activity of MMC is not restricted to cancer treatment only. Due to its nonspecific activity regarding the cell cycle and cell type, it was found also useful as an antifibrotic agent in ophthalmological surgeries, especially in glaucoma surgery [32,33].

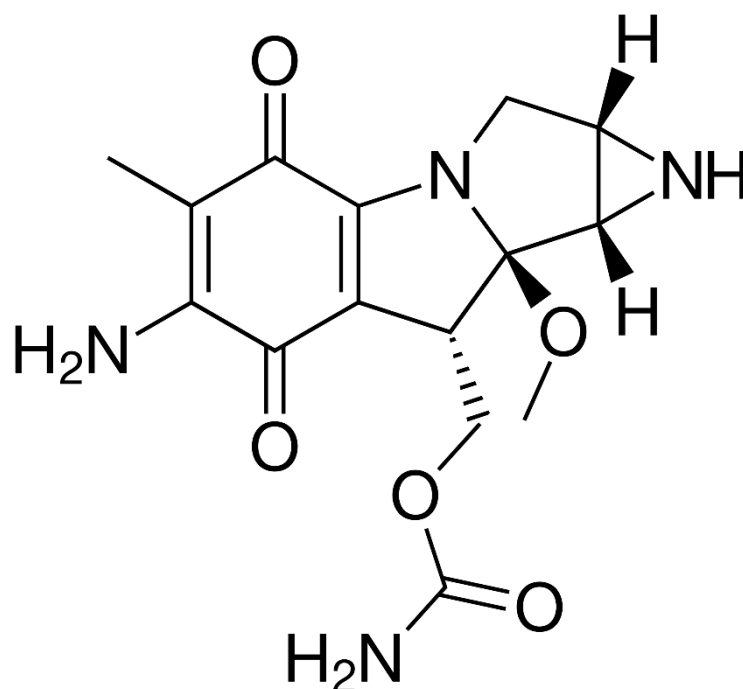


Figure 4. Chemical structure of mitomycin C.

MMC is classified as an antitumour antibiotic with alkylating activity. Its main mode of action involves covalent binding to DNA, with particular preference for the GC-rich regions, resulting in the formation of DNA monoadducts and both intra- and interstrand cross-links. These damages interfere with DNA replication and transcription, ultimately leading to cell-cycle arrest and cell death [32,34]. In contrast to other alkylating agents, MMC is a prodrug that requires enzymatic reduction to fully exert its cytotoxic activity. The reduction of the quinone moiety initiates a series of structural rearrangements, such as opening of the aziridine ring and formation of highly reactive mitosene intermediates. Further on, these intermediates are responsible for the alkylation and cross-linking of DNA. However, the reductive structural transformation of MMC may also generate degradation products, including 2,7-diaminomitosenes (2,7-DAM), which is considered to be a major one. Due to the loss of the aziridine ring and the resulting reduction of alkylating capacity, 2,7-DAM tends to exhibit lesser cytotoxicity in comparison to its parent compound [35,36].

Despite its therapeutic efficacy, the use of MMC also generates a series of adverse effects, with the most considerable being bone marrow toxicity. Furthermore, it has been linked to the occurrence of haemolytic uremic syndrome (HUS). This condition is characterised with the destruction of red blood cells, a decrease in the platelet count, and sudden kidney

failure. Furthermore, the use of MMC can lead to pulmonary toxicity and hepatotoxicity, though these are less frequent than the two aforementioned side effects [33,37].

Similarly to anthracyclines, the use of MMC can be hindered by drug resistance mechanisms. One of the most characteristic and MMC-specific resistance mechanisms is the reduced metabolic activation of the drug. As MMC is a bioreductive prodrug, it requires intracellular enzymatic activation in order to form DNA cross-links. Cancer cells often decrease the expression of activating enzymes required for this process, particularly NADPH-quinone dehydrogenase 1 (NQO1) or cytochrome P450 reductase, resulting in reduced availability of the activated MMC intracellularly. Furthermore, as the main mechanism of MMC's activity is the formation of DNA inter- and intrastrand DNA cross-links, the upregulation of DNA repair pathways, such as homologous recombination (HR) or nucleotide excision repair (NER), is also considered to be one of the major resistance mechanisms. Moreover, MMC may be detoxified before exerting its biological effects by conjugation with glutathione (GSH), a reaction that is frequently catalysed by glutathione-S-transferase (GST). As a consequence, elevated levels of GSH and overexpression of GST have been associated with increased resistance to MMC [38–41].

Therefore, as in the case of anthracyclines, the search and identification of agents capable of modulating MMC's activity is of significant interest, as such combinations may enhance its anticancer efficacy or reduce the toxicity towards the healthy cells.

4.4. Nanoscience

4.4.1. A brief history of nanoscience

Science can be conventionally divided into several disciplines, including physics, chemistry, and biology. The previous statement is important to this work because nanoscience comprises mainly these three fields, though it could be extended to engineering, medicine, and related materials. Broadly speaking, the term “nanoscience” refers to the study of molecules and structures ranging in size from 1 to 100 nm. The origins of nanoscience can be dated back to the 5th century B.C., when the Greek philosopher, Democritus, posed the fundamental materialistic question: is matter continuous, therefore it is infinitely divisible, or is matter composed of smaller particles? Today, we know the latter is true, and the “smaller particles” are called atoms [42].

One of the most frequently cited historical examples of the practical use of nanoscale materials is the Lycurgus cup, formed by the Romans in the 4th century A.D. The cup was made of dichroic glass in such a fashion that when illuminated by reflected light, it appears green, but when light is transmitted through it, it appears red. In the late 20th century, scientists, through transmission electron microscopy (TEM) and X-ray analyses, established that the effect is the result of the presence of 50-100 nm nanoparticles, mainly silver and gold. It is their interaction with visible light that produces wavelength-dependent absorption and scattering, resulting in the unique colour change [43]. Although it is unlikely that Roman craftsmen understood and deliberately utilised these properties, the Lycurgus cup represents an early example of a material production whose properties depend on nanoparticles. Historical records also describe the use of silver-containing materials in e.g. wound care. However, these applications should not be treated as meaningful use of nanomedicine, as any antimicrobial or therapeutic effects may have arisen from the release of metal ions from the bulk material [44,45].

The true exponential growth in interest of nanoscience began in the 19th century, when Michael Faraday synthesised a colloidal solution of gold, which was in fact uniformly sized gold nanoparticles, which, depending on the lighting conditions and the used salt, could yield differently coloured solutions [46]. Through the years of the 20th century, novel methodologies have been developed, among them X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Atomic Force Microscopy (AFM), all of which have allowed us to visualise the world we live in, with great emphasis on the nanoscale [42].

The father of modern nanoscience is thought to be Richard Feynman, who gave a well-cited speech in 1959. He said, quote, “the principles of physics, as far as I can see, do not speak against manoeuvring things atom by atom”. Little did he know that a few decades later, his words would become flesh; researchers at IBM used Scanning Tunnelling Microscopy (STM) to precisely lay 35 xenon atoms on a small nickel plate, depicting the “IBM” logo on it. This achievement became an important symbol of the transition from observing the nanoscale matter to actively controlling and engineering it [47].

4.4.2. Nanoparticles

In nanoscience, nanoparticles (NPs) constitute one of the most intensively studied groups of nanomaterials and represent a major area of interest in modern nanoscience. In principle, nanoparticles are particles ranging from 1 to 100 nm in all three dimensions (X, Y, Z). Depending on their composition, they can be broadly classified into three major categories: organic, inorganic, and carbon-based nanoparticles (**Figure 5**). One of the most distinctive characteristics of nanoparticles is their high surface-to-volume ratio. When the nanoparticle size decreases, a greater proportion of atoms becomes exposed at the surface, resulting in an increased surface availability. This feature distinguishes nanoparticles from their bulk materials and highlights many of their biological and technological applications [48].

Nanoparticles can be synthesised in three major ways. The first approach is the physical, or the so-called top-down approach, where a larger metal is used to produce NPs through mechanical breakdown using e.g. mechanical pressure or thermal energy. The second is the chemical, or the so-called bottom-up approach, where atoms and molecules are assembled into NPs through controlled chemical reactions. The last approach comprises the biological or the “green” synthesis method, which has gained considerable attention recently. These methods employ biological systems, such as microorganisms, plant extracts, or enzymes, to reduce metal ions and generate nanoparticles under relatively mild and ecologically friendly conditions [49].

Due to their diverse size, composition, shape, and possible surface functionalisation, they were found to be of great use in many fields of science and technology, such as electronics, catalysis, diagnostics, environmental studies, and biomedicine. However, a particularly noteworthy area of research, in which nanoparticles are investigated, is cancer treatment, where they are employed as e.g. individual anticancer agents, diagnostic imaging tools, and drug delivery systems, overall aiming at the enhancement of the efficacy and selectivity of the already used therapeutic approaches [50–52].

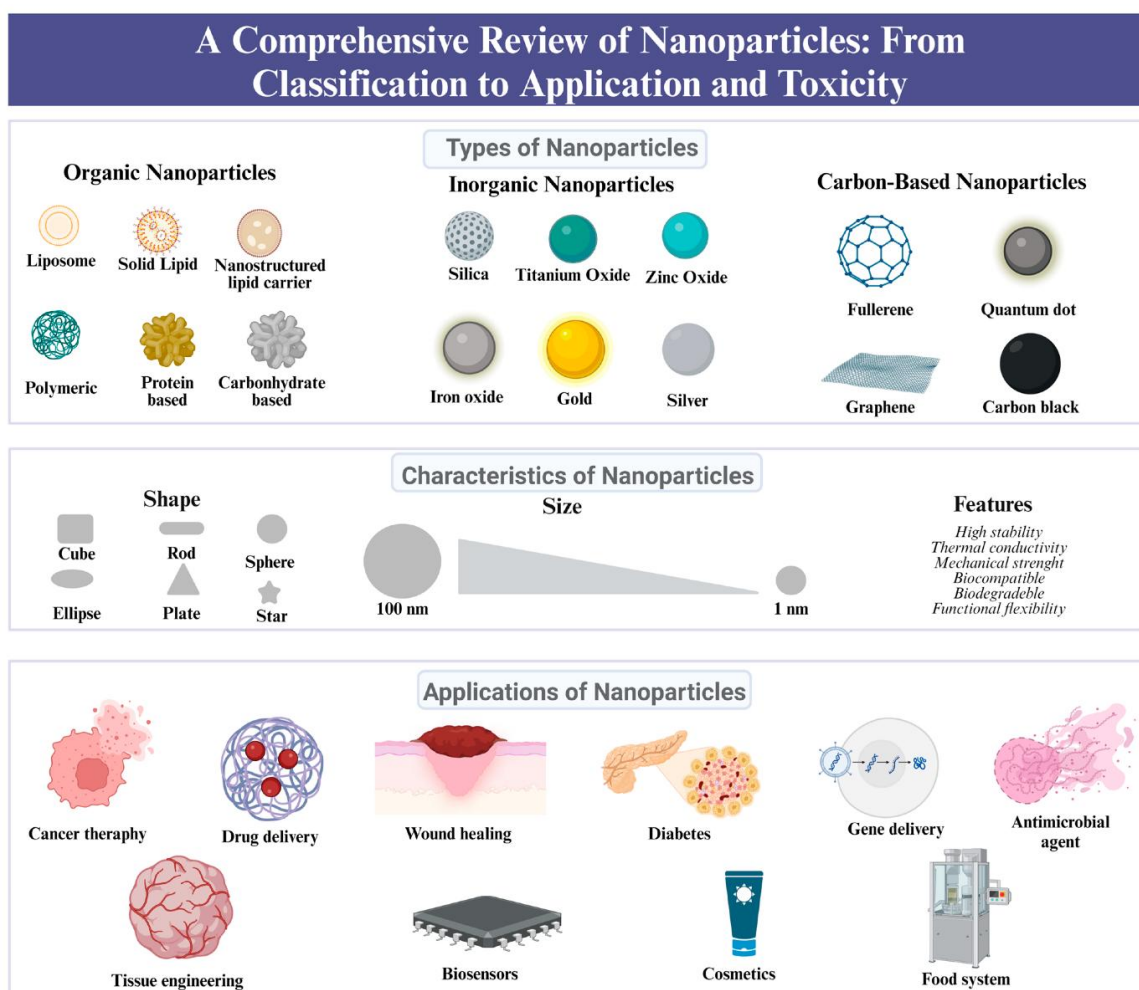


Figure 5. A schematic figure of types, characteristics, and applications of nanoparticles [48].

The inorganic nanoparticles group comprises metallic nanoparticles (MNPs), such as silver (AgNPs), gold (AuNPs), or iron oxide (Fe_3O_4 NPs or IONPs), all of which have been synthesised and examined since the 20th century. Due to their unique optical, thermal, catalytic, and magnetic properties, these nanomaterials have found applications in agriculture, environmental, and energy technologies, electronics, catalysis, and most importantly, medicine [53–55]. In the biomedical field, nanoparticles have attracted particular attention because of their antimicrobial, diagnostic, therapeutic, and drug delivery potential. For example, silver and gold nanoparticles have been extensively studied for antimicrobial applications, including wound healing and crop protection, as well as for cancer therapy. However, platinum nanoparticles (PtNPs), which also belong to the noble metals group, have gained increased attention recently.

4.4.3. Platinum nanoparticles

In contrast to the aforementioned metallic nanoparticles, platinum nanoparticles (PtNPs) have been synthesised for the first time quite recently, in the mid 90's of the 20th century, and soon enough, they have been examined as an excellent tool in many fields of science. They possess great catalytic activity, ability to modulate reactive oxygen species, and are examined as a favourable biocompatibility. All of these features pose them as promising candidates for anticancer applications, therefore, they are currently being studied both as therapeutic agents alone and in combination with chemotherapeutic agents as means of either enhancing these agents' activity or drug delivery systems [56–59]. Similarly to the mentioned silver and gold nanoparticles, PtNPs have been evaluated as great antimicrobial candidates. Ahmed et al. showed that PtNPs sized 2-5 nm inhibited bacterial growth of *Escherichia coli* and *Staphylococcus aureus* in *in vitro* studies. Furthermore, PtNPs were able to eliminate *E.coli* infection in Zebrafish during *in vivo* studies, with the main mechanism established as the generation of ROS and disrupting the bacterial cell membrane integrity [60]. Fanoro et al. observed strong antibacterial activity of biosynthesised PtNPs of approximately 1 nm diameter against *S. aureus* and *Klebsiella spp.*, with a minimal inhibitory concentration (MIC) as low as 1.56 µg/mL [61]. Furthermore, Vukoja et al. demonstrated that 3 nm PtNPs were toxic to *E. coli* and *Klebsiella pneumoniae* strains, and exhibited anti-biofilm activity [62]. In addition to that, they have been shown to be efficient antifungal agents. Godugu et al. synthesised 2-4 nm PtNPs and determined their antifungal activity against *Aspergillus parasiticus* and *Aspergillus flavus* in a dose-dependent manner [63]. Similarly, Kumar et al. demonstrated that greenly synthesised cubic 22 nm PtNPs showed great antifungal properties against, among others, *Candida* species, which is also one of the known human pathogens. Again, the mechanism of their activity was assessed as membrane damage and the generation of ROS, which ultimately led to DNA damage and cell death [64].

4.4.4. Anticancer properties of PtNPs

Despite the undeniable broad activity of platinum nanoparticles, the anticancer properties seem to be the most interesting and important ones in the context of medicine. In regard to that, platinum has been used as an anticancer drug in the form of cisplatin, carboplatin, and oxaliplatin. These agents are used in the treatment of a multitude of cancers, including

testicular, ovarian, and gastrointestinal cancers [65–67]. Although their efficacy is indisputable, their use is often limited by severe systemic toxicity, and thus platinum nanoparticles have emerged as a promising alternative. They have been tested to some extent, and the current literature suggests that they exhibit anticancer activity of their own.

PtNPs' cytotoxicity

Baskaran et al. synthesised PtNPs using *Streptomyces spp.* The nanoparticles, with an average diameter of 45 nm, exhibited a significant dose-dependent cytotoxicity, with an IC₅₀ value of roughly 31 µg/mL after 24h of incubation [68]. Similarly, Kutwin et al. investigated PtNPs ranging from 2 to 19 nm in diameter on the U87 glioblastoma cell line. The nanoparticles were again found to exhibit concentration-dependent cytotoxicity. The TEM analysis revealed that PtNPs accumulated around the mitochondria and nuclei, while the results of the comet assay showed an increase in DNA damage. Furthermore, the PCR analysis revealed elevated *p53* and *caspase-3* expression upon cultivation with PtNPs, indicating activation of apoptotic pathways. Importantly, *in vivo* experiments showed a significant reduction in tumour size and weight compared to the untreated controls, and the effect exceeded that observed for cisplatin [69]. Further evidence of the intrinsic anticancer activity of PtNPs was given by Gurunathan et al., who evaluated PtNPs of approximately 50 nm in diameter and examined their cytotoxic properties towards the A549 lung cancer cell line. The viability assay revealed that these nanoparticles were toxic mostly via the promotion of the oxidative stress, and in a dose-dependent manner. Importantly, the addition of PtNPs resulted in a significant increase in exosome secretion. Because exosomes can contain molecules reflecting the molecular status of tumour cells, the observed increase in exosome secretion may potentially be exploited in future diagnostic applications [70].

All of the aforementioned examples show that PtNPs possess intrinsic anticancer activity across a variety of tumour models, and the demonstrated efficacy highlights their potential as standalone anticancer agents and provides a justification for their further employment in cancer therapy.

Mechanism of PtNPs activity

One of the most commonly proposed mechanisms underlying the cytotoxic activity of PtNPs is the generation of ROS, which, upon accumulation of cellular damage, leads to apoptosis. However, the influence of PtNPs on cancer cells is reportedly different to the one on healthy cells, with indications of anti-oxidant properties. Selvi et al. examined the cytotoxicity of 335 nm PtNPs, synthesised using *Tragia involucrata* extract, on HeLa cancerous cells. Fluorescence analysis revealed significantly elevated intracellular ROS levels in cells treated with the IC₅₀ concentration of PtNPs compared with untreated controls, suggesting that oxidative stress was a major contributor to the observed apoptosis [71]. Similarly, Asharani et al. comparatively examined the effects of 5-8 nm PtNPs on the non-cancerous IMR-90 fibroblasts and U251 glioblastoma cells. At a concentration of 40 µg/mL, no significant increase in ROS levels was observed in either cell line. However, increasing the nanoparticle concentration to 160 µg/mL resulted in a four-fold elevation of ROS levels in the glioblastoma cells compared to the fibroblasts. Furthermore, the Western blot analysis revealed increased expression of p53 in cancer cells, whereas no significant changes were observed in the fibroblasts, suggesting that cancer cells may be more susceptible to PtNP-induced oxidative stress [72].

Evidence of the antioxidant properties of PtNPs have also been reported. Watanabe et al. investigated the potential for ROS scavenging of PolyAcrylic Acid Platinum Nanoparticles (PAA-PtNPs) in *Caenorhabditis elegans*. The construct, measuring roughly 2 nm, was able to scavenge AOO•, a form of residual peroxy radical, with nearly 50% efficiency at concentration of 50 µM, and the effect further increased with the increase of PtNPs concentration. Importantly, the PAA alone did not exhibit any radical-scavenging activity, confirming that the observed antioxidant effect was only due to the activity of platinum nanoparticles [73]. Further support of this PtNPs feature was provided by Gunes et al., who investigated the influence of PtNPs on the cancerous U251 glioblastoma and non-cancerous HEK293 cell lines alone, and in combination with Cold Atmospheric Plasma (CAP) treatment. Although PtNPs exhibited cytotoxicity at higher concentrations, lower doses did not significantly affect cell viability. Instead, nanoparticles effectively protected both cell lines from CAP-induced oxidative stress by reducing intracellular ROS accumulation and preserving mitochondrial function. Importantly, PtNP-treated cells displayed improved resistance to ROS-mediated

cytotoxicity, thus demonstrating the potent antioxidant and protective properties of PtNPs under conditions of excessive oxidative stress [74].

Drug delivery systems

The above-mentioned studies highlight the dual redox activity of PtNPs, and pose them as a good candidate for combined therapies as a means of reducing the potential off-target cytotoxicity. Depending on their physicochemical properties, concentration, and biological environment, PtNPs may induce oxidative stress and apoptosis in cancer cells while simultaneously exhibiting antioxidant and protective effects in non-malignant cells. This dualism may also be the result of the increased permeability of cancer, the so-called Enhanced Permeability and Retention (EPR) effect. The principle of this phenomenon lies in the voracious behaviour of cancer cells. Rapid tumour growth creates a demand for oxygen and nutrients, which stimulates extensive angiogenesis through the secretion of e.g. Vascular Endothelial Growth Factor (VEGF). The overproduction of this protein results in a rapid, chaotic, and structurally abnormal vascular growth, characterised by defective and poorly aligned endothelial cells with wide fenestrations, and impaired functional receptors for angiotensin II, a regulator of vasoconstriction. As a result, the blood vessels around the tumour present abnormal molecular and fluid transport dynamics. In turn, macromolecules, such as nanoparticles, can extravasate from the bloodstream into the tumour faster and more easily than into healthy cells. Furthermore, tumours are distinguished by ineffective lymphatic drainage, therefore once nanoparticles enter the tumour, they tend to remain there for prolonged periods, in contrast to healthy cells [75–77]. Altogether, the EPR effect allows for a passive targeting, therefore nanoparticles are also employed as Drug Delivery Systems (DDS) in order to enhance the selectivity of the drug, as well as to exploit the synergic cytotoxicity of the drug alone and the nanoparticles [78,79].

Kalindemirtaş et al. synthesised ~22 nm spherical PtNPs and subsequently loaded them with DOX. The results of a comparative MTT assay on cancerous MCF-7 and HCT116 cells revealed that the PtNPs-DOX conjugates exhibited significantly greater cytotoxicity than DOX alone, even at low concentrations. Analysis of the IC₅₀ values showed an ~eight-fold enhancement of DOX activity following conjugation with PtNPs. Importantly, no significant cytotoxic effects were observed on non-cancerous HUVEC

cells at low concentrations, suggesting a degree of selectivity towards malignant cells [80]. Similarly, Stavropoulou et al. constructed bimetallic gold-platinum nanoparticles (AuPtNPs), which were further coupled with quinazoline (Q), a compound of anticancer properties. The end construct, QAuPtNPs, measuring approximately 40 nm, was then examined on three different glioblastoma cell lines, as well as on a non-cancerous HEK-293 cell line. The viability of the cancerous cells decreased in a dose-dependent manner of the nanoparticle construct in comparison to AuPtNPs alone. Importantly, no cytotoxic effect was observed for the healthy cell line when combined with QAuPtNPs [81]. Similarly, Patel et al. investigated the effects of PtNPs on the activity of DOX in MCF-7 and MDA-MB-231 breast cancer cell lines. The constructed PtNPs-DOX system exhibited a pH-responsive drug release, with approximately 15% greater DOX release at pH 4.5 than at pH 7.4 after 48 hours of incubation. This feature is particularly advantageous for cancer therapy, as acidic conditions are characteristic of the tumour microenvironment. Furthermore, the PtNPs-DOX conjugates displayed lower IC₅₀ values than DOX alone, indicating enhanced anticancer activity. Ultimately, the construct demonstrated its efficacy in breast cancer apoptosis through the generation of ROS and attenuation of the mitochondrial potential, altogether showing that the combination of PtNPs with an antineoplastic agent can be a promising enhancer of the therapy [82].

Interactions in the nanoparticle-drug systems

The interaction between nanoparticles and therapeutic agents plays a crucial role in determining the physicochemical properties, stability, and biological activity of nanoparticle-based drug delivery systems. These interactions may involve either a covalent or a non-covalent association. While covalent conjugation often provides high stability, non-covalent interactions have gained great attention due to their simplicity, reversibility, and the ability to retain the chemical integrity of the therapeutic agent. The typical non-covalent bonds described for nanoparticle-drug interactions are hydrogen bonds, hydrophobic, and electrostatic forces, as well as van der Waals interactions. Despite their singular weakness, when in a greater number, their combined forces may lead to the formation of stable nanoparticle-drug conjugates [83–85].

Rafik et al. conjugated gold nanoparticles coupled with methotrexate (MTX), an API used in the treatment of e.g. rheumatoid arthritis, with the final construct measuring 20-30 nm. The loading of the drug was confirmed via FTIR, which revealed the formation of hydrogen bonds. A comparative study on the efficacy of MTX and AuNPs alone, as well as MTX-AuNPs conjugates on joint inflammation, vascular reactivity, and systemic inflammation, revealed that the MTX-AuNPs were superior and demonstrated synergistic activity [86]. Similarly to this study, Borowik et al. investigated the fullerene C₆₀ interactions with an acridine mutagen ICR-191. The aggregation studies revealed that these two form aggregates, and the molecular modelling studies suggested that the interactions between C₆₀ and ICR-191 are likely due to electrostatic forces and π - π stacking interactions. These interactions significantly reduced the mutagenic potential of ICR-191 in comparison to the compound alone, as evidenced by a colony count identical to that of the negative control in the Ames mutagenicity assay [87]. Wu et al. investigated graphene oxide (GO) as a means of a DOX carrier. The adsorption of DOX on GO was assessed as spontaneous and endothermic in nature. The FTIR analysis revealed various shifts in the wavenumbers of GO-DOX complexes in comparison to DOX alone, with major indications that the key factor here is the formation of hydrogen bonds, as well as electrostatic interactions between the negatively charged GO and the positively charged DOX molecules, overall yielding a successful combination of the two [88].

Surface modifications for improvement of nanoparticles' efficacy

One of the major issues with metallic nanoparticles in general is that once in the bloodstream, the immune system tends to quickly recognise the intruder and capture these nanoparticles. To overcome this obstacle, nanoparticles are often modified so that their recognition by the immune system is delayed, allowing for a longer retention period, making them more biocompatible. These modifications often consist of coating the surface of PtNPs with polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP) to increase the biocompatibility and retention time, as well as antibodies or other biomolecules to increase the specificity [89,90].

Mukherjee et al. generated PEGylated PtNPs of approximately 45 nm in diameter and conjugated them with DOX. The viability assays demonstrated that the PEG-PtNPs were non-toxic to several non-cancerous cell lines, as well as no significant changes to the

blood vessels in *ex vivo* chicken embryo angiogenesis process were determined, suggesting their notable safety and biocompatibility. However, when PEG-PtNPs were combined with DOX, the construct was severely toxic to the B16-F10 melanoma cell line and A549 lung carcinoma, and the efficacy was greater than that of DOX alone [91]. Similarly, Li et al. constructed PEGylated PtNPs with diameters in the range of 30-60 nm, and conjugated them with Rutin, a herbal flavonoid exhibiting e.g. anticancer properties. After confirming with FTIR analysis a successful coupling, they examined the influence of PEG-PtNPs on the activity of the herbal compound. The constructs were further analysed for their cytotoxicity on cancerous MCF-7 and non-cancerous MCF-10A cell lines. While the PEG-PtNPs exerted cytotoxicity on their own, the conjugated Rutin-PEG-PtNPs showed even further decreased IC₅₀ values than that of coated nanoparticles alone, indicating a great synergistic effect of the two [92].

Interestingly, such conjugates can be further utilised by combining them with other types of therapies, such as phototherapy or radiotherapy. Yang et al. constructed PEGylated platinum nanoflowers (PEG-PtNFs) with a hydrodynamic diameter of approximately 35 nm. The construct exhibited dose and time-dependent cytotoxicity towards the HeLa cancer cell line, showing the maximal surviving fraction of around 65% at the highest concentration. When irradiated with increasing doses of γ -rays, the survivability of HeLa cells incubated with PEG-PtNFs decreased by an additional ~20% [93]. Fu et al. generated PEGylated PtNPs of approximately 90 nm diameter and loaded them with DOX. When incubated with DOX at 1 mg/mL, the MCF-7 cells viability was reduced by 19%. At the same time, when an identical concentration of DOX from PEG-PtNPs-DOX conjugate was present, the cell's viability reduced by an additional 10%, which indicated synergistic cytotoxicity of PtNPs. Furthermore, the application of NIR laser at a power of 1 W/cm² further decreased the survivability of cancer cells, killing approximately 84% at DOX concentration of 8 μ g/mL [94]. This result proposes that the therapies could be combined not only by combining a drug with nanoparticles, but also by cross-linking several types of therapies, creating multifarious treatment regimens for maximum efficiency. Similarly, Samadi et al. investigated the efficiency of commercially available platinum nanoparticles of diameters 30, 50, and 70 nm. While no significant cytotoxicity was observed on the ovarian cancer SK-OV-3 cell line irrespective of the PtNPs concentration. When the cells were treated with an NIR laser of 45 W/cm² power for 5 minutes, no changes were observed. However, when cells were exposed to PtNPs prior

to photothermal therapy, a significant cytotoxicity was observed in comparison to non-treated cells. Interestingly, the effect was observed only for 50 and 70 nm PtNPs, but with no difference between these two [95].

Size is the determining factor

One of the key physicochemical parameters determining nanoparticle activity is their diameter. Numerous studies investigating a variety of nanomaterials, including metallic nanoparticles, have shown that particle size strongly influences biodistribution, tumour penetration and cellular uptake, ultimately defining the therapeutic efficacy. One proposed explanation is that ultrasmall nanoparticles are more likely to leak out of the abnormally formed vasculatures, characteristic for the tumour tissues, compared to the healthy blood vessels [96].

Several studies support the idea that reducing nanoparticle size enhances tumour penetration. Sykes et al. generated differently sized spherical gold nanoparticles coated with either PEG, for passive targeting, or transferrin, for active targeting. In MDA-MB-435 tumour xenografts, the actively targeted nanoparticles accumulated approximately five times faster and exhibited roughly two times greater tumour accumulation in comparison to the passively-targeted nanoparticles within the 60 diameter range for the MDA-MB-435 tumour xenografts [97]. Similarly, Huang et al. determined that ultrasmall gold nanoparticles, measuring 2 and 6 nm, penetrated deeply into tumour spheroids, whereas identically prepared 15 nm nanoparticles showed limited penetration. Furthermore, the ultrasmall nanoparticles were later detected in both the cytoplasm and the nucleus of cancer cells *in vitro* and *in vivo*, whereas 15 nm AuNPs were found only in the cytoplasm in the form of aggregates [98].

Despite these observations, the relationship between nanoparticle size and therapeutic efficacy is not straightforward. While smaller nanoparticles exhibit great penetration capabilities in general, various reports state that particles in the 30-70 nm diameter range generally provide the most favourable balance between penetration and therapeutic efficacy, with ~50 nm frequently described as the optimal size for anticancer medicinal applications. Beldzińska et al. evaluated PtNPs ranging from 5 to 70 nm in diameter as modulators of DOX activity. The Ames mutagenicity assay revealed that combining them with API resulted in a reduced mutagenicity, and the effects were more pronounced with

the increase in the diameter of particles. Furthermore, the comparative cytotoxicity assay on non-cancerous HaCaT and cancerous MelJuSo cell lines revealed that combining DOX with PtNPs elevated the toxic effects of DOX on the cancerous cells, simultaneously exerting protective effects towards the healthy cells. Surprisingly, the greatest enhancement in cytotoxicity was observed for the largest, 70 nm PtNPs [99]. Similarly, Bao et al. examined the possible enhancement of 10-hydroxycamptothecin (HCPT) by 10, 25 and 50 nm gold nanoparticles. The HCPT-AuNPs combination resulted in greater cytotoxic effects towards the MDA-MB-231 cell line in comparison with an identical dose of HCPT alone. Interestingly, the greatest therapeutic effect was achieved using 50 nm nanoparticles, despite the smaller particles being expected to show superior penetration characteristics [100]. These findings demonstrate that nanoparticle size influences multiple biological processes simultaneously, such as cellular uptake, retention, and interactions with therapeutic agents. As a result, the optimal nanoparticle diameter is strictly dependent on the specific nanoparticle-drug system being investigated, and the results observed in one formulation should not be directly extrapolated to other systems.

Summary

Taken together, platinum nanoparticles are an excellent tool for various biomedical applications. Due to their diversity in sizes, shapes and possible surface functionalisation, they can be used both as standalone anticancer agents or in combination with other chemotherapeutics, with the main aim of enhancing the efficacy of these therapeutics or reducing their off-target activity. However, as the results of studies are ambiguous in terms of e.g. optimal nanoparticle size, combinations of PtNPs with different anticancer agents need to be studied separately.

5. Aims of the doctoral thesis

Cancer remains one of the leading causes of death worldwide, and current prognoses indicate the global burden will increase over the next decades. Despite the undeniable advances in medical techniques and treatment methods, chemotherapy remains the most widely applied treatment regimen. Although it is effective, it lacks the selectivity towards cancer cells, leading to a series of side effects: from mild ones like diarrhoea, vomiting, or hair loss, to very severe ones, such as myelosuppression, nephrotoxicity, cardiotoxicity, or even secondary malignancies. One of the ways to overcome this burden is to attempt to combine the already existing chemotherapeutics with other agents in order to improve their cytotoxicity and/or minimise their off-target toxicity. Nanotechnology has developed significantly over the years, showing promising results in the improvement of, among others, the cytotoxic activity of the drugs. Furthermore, they have been used as drug delivery systems, with the aim of increasing the selectivity of therapies. Due to the variety in sizes, shapes, compositions, and possible surface functionalisation, nanoparticles seem to be a perfect tool for therapy enhancement. Therefore, the primary aim of the presented work was to verify if and how platinum nanoparticles of distinct sizes 5, 30, 50 and 70 nm interact with three chosen anticancer agents, namely daunorubicin, idarubicin, and mitomycin C. An additional aim of the study was to assess if the possibly found interactions influence the activity of these antineoplastic agents, and if so, what is that influence regarding various factors, such as the size of nanoparticles and the concentration ratio.

6. Results

6.1. The effect of platinum nanoparticles on the physicochemical properties of daunorubicin – published in September 2025 in *RSC Advances* (Royal Society of Chemistry, IF 4.6)

This study was the first attempt to verify if platinum nanoparticles of distinct sizes 5, 30, 50, and 70 nm interact with daunorubicin, the first described anticancer agent from the anthracycline group.

To preliminary asses whether combining PtNPs with DAU alters the hydrodynamic diameter of nanoparticles in the sample, dynamic light scattering (DLS) was employed. While PtNPs alone exhibited a size in the range declared by the manufacturer, the addition of DAU resulted in an increase of the observed hydrodynamic diameter, and the effect was dose-dependent. To further inspect this phenomenon visually, nanoparticle tracking analysis (NTA) was used. The analysis revealed visible changes in the size of the particles in the colloidal suspension, and further supported the DLS data. Next, knowing that DAU is fluorescent, we performed fluorescence spectroscopy. The results showed that upon the addition of increasing amounts of PtNPs to the solution containing DAU, the fluorescence gradually decreased. In the literature, the metal-enhanced fluorescence (MEF) effect is known to appear if the fluorophore is in the distance range 5 – 90 nm from the nanoparticle, however, if the distance is below 5 nm, then quenching is the dominant effect. Therefore, to evaluate the nature of these interactions, we employed more insightful spectroscopic methods, namely Fourier Transform Infrared Spectroscopy (FTIR) and Near Infrared Spectroscopy (NIR). Both these analyses showed structural changes of the drug when coupled with PtNPs, thus confirming the existence of PtNPs-DAU interactions, and solidifying the already obtained results. The proposed interaction types were hydrogen bonds, van der Waals forces, and possible weak electrostatic interactions. The isothermal titration calorimetry (ITC) showed that the interaction is endothermic, although most likely entropy-driven, with the calculated enthalpy consistently increasing with the diameter of nanoparticles, with roughly 0.5 kcal/mol for 5 nm up to almost 4.4 kcal/mol for 70 nm PtNPs. The differential scanning calorimetry (DSC) revealed that PtNPs altered the thermal properties of DAU, once more supporting the previous results. To inspect if the observed interactions affect the kinetics of the drug release whilst mimicking various cellular environments, an *in vitro* drug release through

a membrane was performed at distinct pH levels of 5.4, 6.4, and 7.4. The main results revealed that while DAU alone was released with a hundred percent efficacy, when combined with PtNPs this decreased to ~70% overall. The Ames mutagenicity assay on *Salmonella enterica* serovar Typhimurium TA98 strain showed that while DAU alone was a potent mutagen, the addition of PtNPs decreased this potential significantly in a dose-dependent manner. Finally, the influence of PtNPs on DAU's cytotoxicity was assessed using *in vitro* cellular studies on a cancerous MelJuSo cell line and a non-cancerous HaCaT cell line. Despite the undeniable physicochemical interactions proven by a couple of methods and a biological effect on the mutagenicity towards bacteria, no significant overall change in the efficacy of DAU towards the cells was observed.

In summary, this study has shown that PtNPs interact with DAU through the formation of diverse heteroaggregates. The most probable type of bonds are weak, non-covalent bonds, and the interaction is likely entropy-driven and influences the thermal properties of DAU, as well as the kinetics of the drug's release at various pH. Despite these interactions influencing the mutagenicity of the drug, no significant change in the cytotoxicity of PtNPs-DAU conjugates in respect to DAU alone was observed.

6.2. Platinum nanoparticles as modulators of idarubicin activity: a physicochemical and in vitro biological study (manuscript)

The aim of this work was to determine if platinum nanoparticles (PtNPs) of distinct sizes 5, 30, 50, and 70 nm interact with idarubicin (IDA), an anthracycline antibiotic with anticancer properties, and if so, how would these interactions alter the drug's physicochemical and biological properties.

The first method used for a preliminary study of interaction was dynamic light scattering (DLS). The results were similar to those of the PtNPs-DAU study – upon addition of IDA to the solution with PtNPs, a shift in the registered size appeared, suggesting formation of aggregates in all tested samples. Again, the increase in size was dose-dependent, with greater overall size of aggregates observed after the highest concentration of IDA was applied. Importantly, the polydispersity index (PDI) lowered, especially in the case of 5 nm PtNPs, suggesting that the formed aggregates were of uniform size. To visualise the formed aggregates, atomic force microscopy (AFM) was employed. PtNPs alone were visualised mostly as either single dots or small clusters, but upon addition of IDA,

formation of aggregates was observed, which further supported the data obtained by DLS. The fluorescence spectroscopy results showed a significant quenching of IDA's fluorescence upon addition of PtNPs in a dose-dependent manner, suggesting direct and close-proximity interactions between PtNPs and IDA. To further inspect the nature of these interactions, Fourier Transform Infrared Spectroscopy (FTIR) and Near-Infrared Spectroscopy (NIR) were used. Both these analyses concluded that spectral shifts in PtNPs-IDA conjugates in comparison to IDA alone, suggesting formation of weak non-covalent bonds, most likely hydrogen bonds, and possibly carbonyl groups. To inspect the thermal nature of the assessed interactions, isothermal titration calorimetry (ITC) was employed. The calculated enthalpy was in the range of approximately 1 kcal/mol for the smallest nanoparticles, up to almost 4 kcal/mol for the 70 nm PtNPs. Despite the endothermic enthalpy, the interactions were most likely entropy-driven. The differential scanning calorimetry (DSC) revealed that PtNPs do not unanimously alter the thermal behaviour of IDA. Nevertheless, out of all the tested diameters, the interaction of PtNPs with 70 nm PtNPs showed the greatest change in the thermal behaviour of the drug. Overall, the physicochemical part of this study confirmed that PtNPs interact with IDA in close proximity, the reaction is probably entropy-driven, and the basis of it is most likely through weak non-covalent interactions. To examine the influence of the aforementioned interactions on the biological activity of IDA, Ames mutagenicity assay on *Salmonella enterica* serovar Typhimurium TA98 was employed. The results revealed that whilst PtNPs alone were neither mutagenic nor cytotoxic, they significantly reduced the mutagenicity of the drug in a dose-dependent manner of the PtNPs concentration. Interestingly, as the concentration rose, the mutagenicity of PtNPs-IDA complexes seemed to return to the baseline of the positive control (IDA alone). This phenomenon could be explained by the ratio between PtNPs and IDA; at low concentrations of PtNPs, the API-to-nanoparticle ratio is high, therefore, PtNPs are heavily coated with IDA molecules, which in turn reduces the bioavailability of the drug, but with the rise in PtNPs concentration, the ratio decreases, and it may result in an increased homoaggregation of nanoparticles, as well as the ratio of PtNPs and IDA molecules in heteroaggregates. Thus, in the highest PtNPs concentration, the concentration of free IDA increases, resulting in restoration of the mutagenic activity, and therefore greater revertant colony count. Lastly, the influence of PtNPs on IDA's cytotoxicity was assessed in *in vitro* analysis on two breast cancer cell lines, namely the SK-BR-3 cell line (ER-, PR-, HER2+), and the MCF-7 cell line (ER+, PR+, HER2-). Almost all of the tested PtNPs sizes and concentrations

exhibited anticancer activity towards both cell lines. When it comes to SK-BR-3, at lower concentrations of the drug, the majority of PtNPs elevated the toxicity, however, when the concentration of the drug was increased, the additional cytotoxicity effects either diminished or, on the contrary, more viability was observed. In the case of MCF-7, the cytotoxicity was greater only when the smallest concentration of PtNPs was applied, in the rest, either no improvement was observed, or a significant increase in the viability of the cells was registered. These results suggest that the synergy of these two agents exists, as both IDA and PtNPs were evaluated as toxic to cells, but there is a thin line between synergy and possible antagonism, and that line is the dosage of the two: the main hypothesis is that at greater concentrations, especially of PtNPs, the formation of PtNPs-IDA aggregates is so elevated, that the drug is no longer bioavailable, and such theorem would find proof in our aggregation studies.

6.3. Comparative analysis of platinum nanoparticles interactions with mitomycin C and its activated metabolite (manuscript)

The last drug tested for interactions with platinum nanoparticles (PtNPs) of distinct sizes 5, 30, 50, and 70 nm PtNPs was mitomycin C (MMC), which belongs to the alkylating chemotherapeutic group. MMC is a prodrug, and one of its major metabolites is 2,7-diaminomitosene (2,7-DAM), therefore, the additional aim of this work was to study the interaction between both intact MMC and the aforementioned metabolite, and PtNPs.

The initial investigation consisted of dynamic light scattering (DLS). When PtNPs were combined with MMC dispersed in DMSO, no significant changes were observed. The addition of identical volumes of DMSO also did not result in any meaningful changes, however, when 2,7-DAM was added to the solution, a significant change in the registered diameter was observed. Once again, it was dose-dependent, though not as pronounced as in the case of the previously tested anthracyclines. To further study this matter, atomic force microscopy (AFM) was employed. Again, nanoparticles alone were visualised as either single dots or small clusters. With the addition of both MMC and 2,7-DAM, the aggregate formation was visible, but it was more pronounced in the case of 2,7-DAM. The discrepancy between DLS and AFM could be explained by the environmental features of these methods; DLS measures the changes in the colloidal state, which is more native for a suspension, rather than the AFM study, in which the samples were dried prior

to the procedure. Nevertheless, the AFM data supported the formation of aggregates, so further evaluation of the interactions was conducted using Fourier Transform Infrared Spectroscopy (FTIR) and Near-Infrared Spectroscopy (NIR). Both these analyses revealed spectral shifts due to the combination of PtNPs with MMC, however, these results were not as significant as with any of the tested anthracyclines, with the suggestion that the interaction is primarily through weak non-covalent bonds, most likely hydrogen bonds. The DSC analysis supported the spectroscopic results, exhibiting only minor endothermic effects. However, when tested on isothermal titration calorimetry (ITC), the enthalpy of PtNPs-MMC interactions was very low in the range from 0 to 0.3 kcal/mol, but when PtNPs were combined with 2,7-DAM, the calculated enthalpy significantly increased, from roughly 0.4 kcal/mol for 5 nm PtNPs up to almost 3 kcal/mol. This difference could be attributed to the structural changes between MMC and 2,7-DAM; through metabolic reduction, the aziridine ring of MMC opens, exposing additional amine and carbonyl groups along with hydroxyl groups, all of which may contribute to the formation of weak non-covalent bonds. Despite these differences, both these agents were mutagenic in *Salmonella enterica* serovar Typhimurium TA102, and the addition of PtNPs resulted in a decrease of this mutagenicity in a dose-dependent manner. The observed phenomenon was significantly more pronounced in the case of 2,7-DAM, where the combination of the highest PtNPs concentration resulted in decreasing the mutagenicity to the level of the negative control. Ultimately, cytotoxicity assays with MCF-7 and SK-BR-3 cell lines were applied. Both MMC and 2,7-DAM were toxic, though the latter was overall less potent. Almost all tested nanoparticle concentrations improved the cytotoxicity of lower concentrations of MMC towards the MCF-7 cell line, however, as the MMC concentrations increased, the effects started to diminish. As for the SK-BR-3 cell line, all tested combinations improved the drug's toxicity. On the contrary, when PtNPs were combined with 2,7-DAM, either no significant enhancement of cytotoxicity or a significant improvement of cell viability was observed. The discrepancy between these outcomes may be attributed to an unambiguous aggregation pattern. While PtNPs improve MMC's cytotoxicity, they reduce the toxic potential of 2,7-DAM, most likely through absorption, aggregation, or sequestration.

7. Conclusions

The aim of the doctoral thesis was to assess if platinum nanoparticles of distinct sizes 5, 30, 50, and 70 nm interact with three chosen anticancer agents, namely daunorubicin, idarubicin, and mitomycin C, and if so, how these interactions influence the biological activity of these APIs. The overall results can be summarised in two parts: physicochemical and biological.

Physicochemical:

- Both DAU and IDA form aggregates with all tested PtNPs sizes
- MMC does not aggregate with PtNPs in the colloidal state, however, its metabolites do
- The DLS analyses showed the greatest change in hydrodynamic diameter overall for 5 nm PtNPs, with a possible stabilising effect by typically reducing the polydispersity index when combined with the tested APIs
- The interactions in PtNPs-DAU and PtNPs-IDA systems are of close-proximity, as evidenced by a significant quenching of these anthracyclines' fluorescence
- The spectroscopic analyses have examined nanoparticles to interact with all tested agents, as evidenced by spectral shifts with respect to the chemotherapeutics alone
- Thermal analyses have concluded that PtNPs interact in an endothermic manner with DAU and IDA, and the calculated enthalpy increases with the increase in the PtNPs' diameter
- MMC's interaction with PtNPs was of low and negligible endothermic enthalpy, however, MMC's metabolites presented enthalpy comparable to that of PtNPs-anthracycline systems
- The observed aggregation in the PtNPs-DAU study significantly reduced the drug's release through the membrane *in vitro*, and overall, the mean release percentage of DAU from PtNPs-DAU mixtures was the lowest at pH 7.4 and the highest at pH 6.4.
- Adding to that, 30 nm PtNPs were most prominent by means of a concise decrease in the amount of API released irrespective of the pH

Biological:

- All tested PtNPs sizes and concentrations were neither cytotoxic nor mutagenic to both *Salmonella enterica* serovar Typhimurium TA98 and TA102 strains, and combining them with DAU, IDA, MMC, and MMC's metabolites decreased the mutagenicity of these APIs
- This effect was dose-dependent, besides the PtNPs-IDA study, where the increasing concentration of PtNPs resulted in bringing back the revertant colony number nearly to the state of positive control (IDA alone)
- All tested PtNPs sizes and concentrations were nontoxic to MelJuSo and HaCaT cell lines in the PtNPs-DAU study, and their combination with DAU did not significantly alter the drug's cytotoxicity
- On the contrary, PtNPs alone were mostly cytotoxic to both SK-BR-3 and MCF-7 cell lines, irrespective of the PtNPs diameter and the applied concentration
- PtNPs significantly enhanced IDA cytotoxicity against SK-BR-3 cells at low concentrations of IDA, but the effect started to diminish at higher concentrations of IDA, especially with 5 µg/well of PtNPs
- On the MCF-7 cell line, 0.2 µg/well of PtNPs showed overall greatest enhancement of IDA cytotoxicity, whereas at 5 µg/well PtNPs, most of the combinations yielded greater cancerous cells viability, irrespective of the PtNPs size, which was especially pronounced for the two highest IDA concentrations
- In general, 5-50 nm PtNPs likely increased IDA's cytotoxicity, whereas 70 nm PtNPs mostly did not, and the results indicate a strong concentration-dependent balance between synergy and antagonism for PtNPs-IDA combinations
- In the PtNPs-MMC study, nearly all PtNPs sizes and concentrations significantly increased the cytotoxic potential of mitomycin C, however, with MMC's metabolites, PtNPs either did not affect the toxicity or, on the contrary, improved the viability of cancerous cells
- Overall, the efficiency of PtNPs gradually decreased with the increase of their diameter: 5 nm PtNPs exhibited the greatest improvement of MMC's activity towards both cell lines, with almost no significant effect on the MMC's metabolite
- Conversely, 70 nm PtNPs had the least influence on MMC's cytotoxicity, though still statistically significantly elevated it, whereas they almost consistently increased SK-BR-3 and MCF-7 cells viability combined with MMC's metabolites

In summary, these results show that PtNPs interact with all the investigated anticancer agents, although the strength and nature of these interactions vary between compounds. The obtained evidence suggests these interactions are predominantly mediated by weak non-covalent forces, most likely hydrogen bonds and other intermolecular interactions. Importantly, the influence of these interactions was observed in both bacterial and eukaryotic systems. Taken together, these results highlight the potential of PtNPs as modulators of chemotherapy and provide a basis for further studies aimed at developing more effective nanoparticle-assisted therapeutic strategies.

8. References

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9. List of figures

Figure 1. Number of new recorded cases of cancer and deaths caused by it in 2022 (adapted).

Figure 2. Estimated number of new cases from 2022 to 2050 based on country's HDI (adapted).

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Figure 4. Chemical structure of mitomycin C.

Figure 5. A schematic figure of types, characteristics, and applications of nanoparticles.

10. Publications constituting the core of this dissertation

1. **Zakrzewski M.**, Bełdzińska P., Gajdowska F., Gołuński G., Gackowska K., Strankowska J., Jamrógiewicz M., Wyrzykowski D., Grzyb K., Gutowska-Owsiak D., Synak A., Bojarski P., Piosik J. The effect of platinum nanoparticles on the physicochemical properties of daunorubicin. RSC Adv. 2025 Sep 25;15(42):35407-35420. DOI: 10.1039/d5ra02827b.



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The effect of platinum nanoparticles on the physicochemical properties of daunorubicin

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Cancer is a difficult disease to cure due to its diversity and complexity. Despite novel therapeutic approaches having been proposed in recent years, chemotherapy remains one of the most common treatment regimens to this day. Unfortunately, its anticancer effects are often quelled by the lack of selectivity; hence alteration of the activity of already existing drugs by combining them with potential modulators e.g. metallic nanoparticles has been proposed as a possible strategy to overcome this problem. Herein, we verified if platinum nanoparticles (PtNPs) of various sizes interact with daunorubicin (DAU), a chemotherapeutic agent used for the treatment of leukaemia. Utilizing DLS and NTA we observed nanoparticle aggregation upon addition of increasing concentrations of DAU. Furthermore, we noticed that PtNPs quench DAU's fluorescence, suggesting their direct interaction. Next, we used FTIR and NIR spectroscopies and registered significant changes in the obtained spectra in the presence of PtNPs. The ITC and DSC analyses showed that all tested sizes of PtNPs interact with DAU in an endothermic manner, with the enthalpy change between 0.47 and 4.36 kcal mol⁻¹. The release analysis employing the dialysis bag method evidenced that PtNPs influence DAU's diffusion kinetics, decreasing its release from 100% (when alone) to ca. 70% when combined with PtNPs. All tested sizes of PtNPs reduce DAU's mutagenicity towards *S. enterica* serovar Typhimurium TA98 strain, however, no significant influence on DAU's cytotoxicity was observed on neither MeJuSo nor HaCaT eukaryotic cell lines. Overall, these results indicate that PtNPs may affect DAU's biological activity and warrant further biological studies.

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1 Introduction

Cancer prevalence is increasing worldwide due to the aging populations and lifestyle changes, and is among the three leading causes of death in the age group 30–60 years old in almost all countries.¹ By 2050, the gross number of all new cases diagnosed annually is expected to increase from 20 to approximately 35 million.¹ Currently diverse treatment regimens are employed, including both surgical intervention and pharmacotherapy. However, while the number of approved anti-cancer agents has increased almost every year over the course of the past decade, according to the 2021 FDA report the percentage of new therapeutics with novel mechanisms of action within a given type of cancer has declined.² Despite numerous advancements in the field (incl. immunotherapy), chemotherapy remains the main mode of treatment, especially in newly diagnosed patients.³ Regardless of how effective it may be, it is not indifferent to the healthy cells, contributing to major side effects, such as cardiotoxicity, neurotoxicity, or myelosuppression, thus making the therapy unsustainable in the long run.⁴ A potential solution to this problem is the

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incorporation of nanoparticles into the drug formulation, creating Nanoparticle Drug Therapy (NDT). This approach aims to enhance the efficacy, selectivity, and bioavailability of the drug within the tumour.

Nanoparticles (NPs) have received interest from the scientific community since the last century.⁵ In general, they can be characterized as structures ranging from 1 to 100 nm in size and can be divided into three groups, *i.e.*, inorganic, organic, and carbon-based nanoparticles. Due to the variety of size, shape, composition, optical properties, and, additionally, possible surface functionalization,^{5–7} NPs exhibit a broad spectrum of features. As a consequence, they were found to be important in numerous scientific and clinical applications, including medicine, as antibacterial substances, biosensors, or anticancer agents, acting either directly or as drug carriers.⁶ Among metallic nanoparticles, platinum nanoparticles (PtNPs) have gained interest in recent years. They have been shown to be cytotoxic against various cancer cell lines,^{8–11} mainly through the production of reactive oxygen species (ROS) and causing cell damage to those lines, but with no significant cytotoxic effects to the healthy cells.^{10,12,13} PtNPs have also been applied as nanocarriers, leading to the possibility of lowering the dose of the drug whilst maintaining its efficacy.^{14,15} Furthermore, since the pH of tumour cells is known to be lower than that of healthy cells,^{16,17} there is an additional opportunity through combining the drug with nanoparticles allowing for its increased selectivity through a pH dependent release.^{18,19}

Daunorubicin (DAU), an anthracycline antibiotic discovered in mid-60's, was soon recognized as a potent anticancer agent, especially in the treatment of lymphoblastic and myeloblastic leukaemia. The three main modes of its activity are the production of ROS, intercalation to DNA, and inhibition of topoisomerase II activity, ultimately leading to cell death.^{20,21} Unfortunately, DAU is also associated with significant side effects, mainly cardiotoxicity and bone marrow suppression.²⁰ Cardiotoxic effects may persist for up to five years after treatment.²² Additional adverse effects include therapy-related tumorigenesis and gonadotoxicity.²³

Unfortunately, the current knowledge regarding the possible influence of nanoparticles on cancer chemotherapy is still limited and requires detailed studies. Bearing in mind the numerous properties of metallic nanoparticles, we decided to study whether platinum nanoparticles of different sizes interact with daunorubicin and if so, what are the *in vitro* biological implications of these interactions. To do so, we employed various physicochemical methods, as well as biological assays.

2 Experimental

2.1 Materials

Platinum nanoparticles (PtNPs) at concentrations 0.05 mg mL⁻¹ and 1 mg mL⁻¹ were obtained from nanoComposix (San Diego, California, USA). The nanoparticles were suspended in 2 mM sodium citrate for 5, 30 and 50 nm PtNPs and in 4 mM sodium citrate for 70 nm PtNPs. Daunorubicin hydrochloride (DAU) was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). The stock solutions were prepared by dissolving DAU in

water and the desired concentration was validated *via* spectroscopic methods with the molar extinction coefficient of $\epsilon_{545} = 5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The phosphate-citrate buffers with pH 5.4, 6.4 and 7.4, were prepared according to the buffers for pH and metal ion control²⁴ protocol, utilizing citric acid monohydrate from WVR Chemicals (Radnor, Pennsylvania, USA) and sodium phosphate from Sigma-Aldrich (Saint Louis, Missouri, USA). The chemicals were dispersed in deionized water and their pH was validated using pH-meter from Mettler Toledo (Columbus, Ohio, USA). The sodium citrate tribasic dihydrate was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). The Spectra/ Por 7 dialysis tubing of 10 000 MWCO were bought from Repligen (Waltham, Massachusetts, USA).

Salmonella enterica serovar Typhimurium TA98 strain was purchased from Xenometrics AG (Allschwil, Switzerland). Nutrient Agar, Nutrient Broth and Biological Agar media were purchased from BioMaxima S.A. (Gdansk, Poland). Histidine, biotin, and ampicillin were acquired from Sigma Aldrich Chemical Company (St. Louis, Missouri, USA). Human keratinocyte cell line (HaCaT) and human melanoma cell line (Mel-JuSo) were obtained from the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute (Stockholm, Sweden) and the Department of Medicinal Microbiology, Leiden University Medical Center (Leiden, The Netherlands), respectively. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium, and 10% fetal bovine serum (FBS), 4 mM L-glutamine, glucose and antibiotic-antimycotic solution were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA).

2.2 Dynamic light scattering (DLS)

The hydrodynamic diameter of the PtNPs and the change in registered sizes upon the addition of DAU were evaluated in polystyrene cuvettes at 25 °C utilizing Zetasizer Nano S from Malvern Panalytical (Malvern, Great Britain). The initial concentration of PtNPs was 2.38 µg mL⁻¹, the concentration range of DAU was 4.2–42 µM. All of the measurements were done in triplicates, each consisting of six measurements, 10 seconds per measurement. The data was processed by Zetasizer software.

2.3 Nanoparticle tracking analysis (NTA)

To visualize how platinum nanoparticles interact with daunorubicin in aqueous environment and to further evaluate their size alone and in combination with the drug, we performed a nanoparticle tracking analysis using Nanosight NS300 from Malvern Panalytical (Malvern, Great Britain), with a sCMOS camera and a blue 488 nm laser. PtNPs, at concentration 0.0025 mg mL⁻¹, were added to the cell, and measured in a stationary manner, with 5 recorded videos, 1 minute each. Next, after rinsing the cell with deionized water, a solution of 0.0025 mg mL⁻¹ of PtNPs and 21.7 µM DAU was added to the measurement cell, and recorded as previously described. Due to the limitations in the apparatus' range of measurements, 5 nm PtNPs had to be excluded from the procedure. The nanoparticles were diluted in deionized water.



2.4 Fluorescence spectroscopy

The fluorescence measurements were performed in quartz cuvettes at temperature 25 ± 0.1 °C using Jasco FP-8500 (Jasco, Easton, Maryland, USA) with a Peltier thermostat. In each sample the initial concentration of DAU was $34.7 \mu\text{M}$ followed by titration with PtNPs in the concentration range 0 – 10.62 mg mL^{-1} . All the measurements were performed in 2 mM sodium citrate for 5 , 30 and 50 nm PtNPs and in 4 mM sodium citrate for 70 nm PtNPs. For the negative control the DAU was titrated with analogous volumes of appropriate sodium citrate solution. The excitation was set at 480 nm , and the emission was registered withing 400 – 800 nm range.

2.5 Fourier transform infrared spectroscopy (FTIR) and near infrared spectroscopy (NIR)

FTIR and NIR spectra of complexes formed between daunorubicin and platinum nanoparticles were recorded on Jasco-4700 instrument (IR: 4000 – 400 cm^{-1} NIR: 8200 – 4000 cm^{-1}) with 32 scans, 4 cm^{-1} resolution; (Jasco Company, Tokyo, Japan). All prepared samples were lyophilized prior to the procedure. NIR Transmission technique was applied using dry salt KBr and dry samples of DAU, PtNPs, and sodium citrate obtained in the process of lyophilization. The materials were compressed with potassium bromide to form a disc. All spectra were recorded as a background and analysis of spectra was performed using Spectra Analysis software (Jasco Company, Tokyo, Japan).

2.6 Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry measurements were performed at 25 °C on AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, Massachusetts, USA). The volumes of the sample and reference cells were 1.4491 mL . The experiment consisted of 15 injections of $10.02 \mu\text{L}$ with a $2 \mu\text{L}$ for the first injection only of DAU solution (concentration range 0.002 – 0.029 mM) into the sample cell containing PtNPs (initial concentration 0.05 mg mL^{-1}). Background titrations were performed by injecting DAU into the appropriate sodium citrate solution and appropriate sodium citrate solution to PtNPs. The results of the background titrations were subtracted from each experimental result to account for the heat of dilution. Using linear regression, we calculated the exact values of enthalpy. Each injection lasted 20 s . To reach a homogenous mixing in the cell, the stirrer speed was kept constant at 300 rpm .

2.7 Differential scanning calorimetry (DSC)

The thermal behaviour of the samples was measured by Differential Scanning Calorimetry (DSC), STAR-1 System, Mettler Toledo (Greifensee, Switzerland), which was calibrated with indium. The analysis was carried out in aluminum crucibles $40 \mu\text{L}$ at the temperature range from 20 °C to 300 °C. For performing the DSC, 1 – 5 mg of the appropriate sample was weighed. DSC curves were recorded and analysed by the STAR-1 System (Mettler Toledo, Greifensee, Switzerland) combined

with the intercooler system. Samples were heated at the heating rate of 10 °C min^{-1} and a nitrogen flow rate 60 mL min^{-1} . All tests were standardized to each sample size.

2.8 Release analysis using dialysis bag technique

The release analysis of daunorubicin from PtNPs-DAU aggregates was conducted using dialysis bags from Repligen (Waltham, Massachusetts, USA) with a Molecular Weight Cut Off (MWCO) of 10 kDa , at room temperature. Prior to the procedure, a DAU-PtNPs mixture was prepared; $1800 \mu\text{L}$ of PtNPs at a concentration of 0.05 mg mL^{-1} and $200 \mu\text{L}$ of DAU at a concentration of 2 mg mL^{-1} were added to an Eppendorf tube and left to mix for 24 hours at room temperature. A beaker with a 50 mL capacity was filled with 28 mL of citrate-phosphate buffer at the desired pH, and a magnetic stirrer was placed inside. A dialysis bag of appropriate length was prepared, 2 mL of the incubated mixture was added and the membrane was closed tightly. 2 mL of citrate-phosphate buffer at the desired pH was added to a quartz cuvette with an optical path length of 1 cm , and absorption was measured in a spectrophotometer as a baseline. The prepared dialysis bag was placed in the beaker with the buffer, and at desired intervals 2 mL of the solution was taken from the beaker and placed in a new measurement cuvette with the same parameters as above for absorption measurements. The solution from each timepoint was returned to the beaker.

The concentration of released daunorubicin was calculated using the molar extinction coefficient of DAU which was spectrophotometrically obtained prior to the experimental procedure. The absorption results of each concentration were averaged and then converted to concentration using the Lambert-Beer law: $A = \epsilon \times c \times l$, where A is absorption, ϵ is the molar extinction coefficient ($\epsilon_{345} = 5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), c is the concentration of the substance (mol L^{-1}), and l is the optical path length (cm). The analysis of the release process was performed based on two mathematical models, Korsmeyer-Peppas and Hopfenberg, to the experimental release curves. The models are explained in detail in the SI.

2.9 Ames mutagenicity assay

The influence of PtNPs on the mutagenic activity of DAU was assessed using the Ames mutagenicity plate assay with *Salmonella enterica* serovar Typhimurium TA98 strain, according to the procedure described by Wozniowicz *et al.*²⁵ A mixture containing $100 \mu\text{L}$ of the overnight bacteria culture, $50 \mu\text{L}$ of 3% NaCl, and $100 \mu\text{L}$ of the tested solution (or sterile distilled water as a negative control) was incubated for 4 h in darkness at 37 °C and 220 rpm . Afterwards, the mixture was centrifuged at $11\,840 \text{ g}$, the bacterial pellet was washed with 0.75% NaCl, and resuspended in $300 \mu\text{L}$ of 0.75% NaCl solution containing $0.1 \mu\text{M}$ histidine and $0.1 \mu\text{M}$ biotin. The bacterial suspension was spread on a glucose minimal agar plate and incubated at 37 °C in darkness. After 48 h incubation, the number of revertant colonies per plate was calculated. All experiments were performed in triplicate.



2.10 Cell culture and cytotoxicity assay

The HaCaT cell line was cultivated in DMEM containing 4500 mg L⁻¹ glucose, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, and 0.25 µg mL⁻¹ amphotericin B. The MelJuSo cell line was cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, and 0.25 µg mL⁻¹ amphotericin B. Both cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

HaCaT and MelJuSo cells were seeded on a 96-well plate (2×10^4 cells/well) and incubated overnight in humidified atmosphere at 37 °C and with 5% CO₂. Next, the cells were washed thrice with appropriate medium devoid of FBS. Subsequently, PtNPs alone, at concentrations 0.2 and 1 µg per well or with 25 µM DAU, were added to cell cultures (90 µL per well), each sample in three replicates and incubated for 20 h. After that, 10 µL of alamarBlue (BioRad, Hercules, California, USA) was added to each well, and the plates were further incubated for 4 h in conditions described above. The absorbance was measured at 570 nm and 600 nm, and the percentage of alamarBlue reduction was calculated as a difference between treated and

untreated cells, according to the protocol provided by the manufacturer, using the data gathered from 570 and 600 nm scans.

2.11 Computational and statistical analysis

All of the graphs were prepared in SigmaPlot 14.5 from Systat Software Inc (San Jose, California, USA). The release modelling was prepared using Origin from OriginLab (Northampton, Massachusetts, USA). Statistical analysis was conducted using Statistica 14.0 (TIBCO Software Inc., Palo Alto, California, USA) software. For the Ames mutagenicity test and release analysis, one-way variance analysis (ANOVA) followed by the post-hoc Tukey's HSD test was applied. For the cytotoxicity assay U-Mann Whitney test was used. The significance level for all the analyses was established at $\alpha = 0.05$.

3 Results and discussion

3.1 DAU-PtNPs aggregation

Dynamic light scattering (DLS) has proven to be a reliable method for particle size analysis, however, the results available in the literature vary when it comes to the interactions between

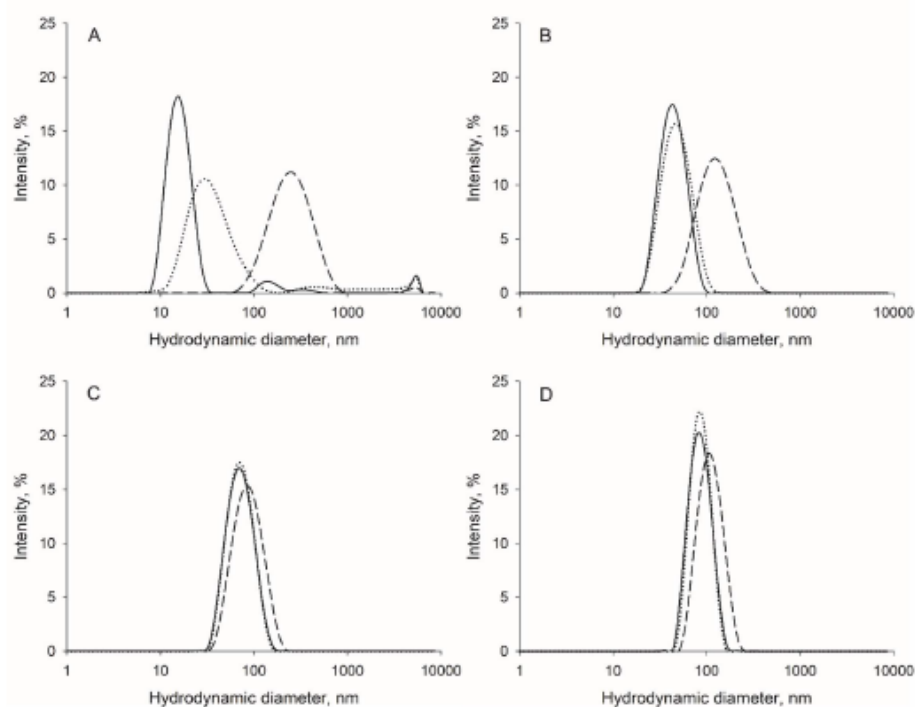


Fig. 1 Hydrodynamic diameter distribution of platinum nanoparticles (PtNPs) alone and in combination with daunorubicin (DAU). (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs. Solid line – nanoparticles alone, dotted line – nanoparticles with 4.2 µM DAU, dashed line – nanoparticles with 42 µM DAU; PtNPs initial concentration 2.5 µg mL⁻¹.



nanoparticles (NPs) and chemicals. In our previous study, we observed the formation of heteroaggregates between doxorubicin (DOX) and 5 nm and 50 nm silver nanoparticles in a dose-dependent manner,²⁶ as well as aggregation of fullerene C₆₀ with DOX²⁷ and heteroaggregation between platinum nanoparticles (PtNPs) and ICR-191,³⁰ all of them resulting in a change of biological activity of the analysed active substance. On the other hand, cisplatin, did not promote significant change in registered diameter for 5 nm and 50 nm PtNPs based on DLS data, yet the remaining results suggested their interaction.⁴¹ Herein, we observed a shift towards greater sizes upon the addition of daunorubicin (DAU) to PtNPs in all tested samples (Fig. 1). 5 nm PtNPs presented a polydisperse distribution, with three distinct peaks of 16.2, 201 and 5199 nm (Table 1). The addition of DAU resulted in a shift towards greater hydrodynamic diameters; *i.e.*, 36.9, 437.1 and 5165 nm for 4.2 μ M DAU, and to 273.9 and 4825 nm for 42 μ M DAU. In the case of 30, 50 and 70 nm PtNPs the nanoparticles alone were monodisperse and the change in registered size was mainly visible with the addition of 42 μ M DAU, which was calculated as a 3.06-fold, 1.22-fold and 1.3-fold increase in the hydrodynamic diameter, respectively. Overall, the acquired results suggest formation of heteroaggregates between PtNPs and DAU and, possibly, homoaggregates of PtNPs alone. It is worth noting that

the polydispersity index (Pdl) remained below 0.3 in all the experiments, indicating the reliability of the obtained results.²⁸

Among the available methods for particle diameter and distribution examination, nanoparticle tracking analysis (NTA) has significantly developed throughout the last two decades. Even though both DLS and NTA focus on determining the size of registered particles, the advantage of using the latter is that it can provide an approximate concentration of the measured particles, offering valuable insight into the kinetics of possible interactions within the sample.²⁸ The average size of 30 nm PtNPs was recorded as 38.8 nm by the NTA analysis, with a concentration of approximately 1×10^9 particles per mL. Upon the addition of DAU, the average diameter changed to 189.3 nm, with the concentration decreasing to 9.9×10^7 particles per mL, which suggests increased NPs aggregation. For 50 nm PtNPs, the addition of daunorubicin shifted the diameter from 61.3 nm to 125.8 nm, simultaneously lowering the concentration from 5.5×10^8 particles per mL to 1.1×10^8 particles per mL. In the case of 70 nm PtNPs, the mean size changed from 86.5 nm to 160.2 nm, with the concentration dropping down from 2.2×10^8 particles per mL to 6.8×10^7 particles per mL (Table 2).

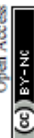
A representative video of 30 nm platinum nanoparticles alone and in combination with daunorubicin is available in the supplementary materials (SI Video 1 and SI Video 2).

Table 1 Mean size of platinum nanoparticles (PtNPs) alone and in combination with daunorubicin (DAU). Pdl – polydispersity index, SD – standard deviation

Sample	Pdl $\pm$ SD	Mean size, nm $\pm$ SD	Area, % $\pm$ SD
5 nm PtNPs	0.26 $\pm$ 0.06	16.2 $\pm$ 0.4 201 $\pm$ 11.3 5199 $\pm$ 3001.6	91.8 $\pm$ 5.9 5.6 $\pm$ 4.8 2.6 $\pm$ 4.6
5 nm PtNPs + 4.2 μ M DAU	0.26 $\pm$ 0.09	36.9 $\pm$ 9.2 437.1 $\pm$ 252.4 3597 $\pm$ 1812.17	90.1 $\pm$ 4.3 2.8 $\pm$ 4.9 7.1 $\pm$ 3.5
5 nm PtNPs + 42 μ M DAU	0.2 $\pm$ 0.04	273.9 $\pm$ 55.4 4825 $\pm$ 2785.7	98.8 $\pm$ 2.1 1.2 $\pm$ 2.1
30 nm PtNPs	0.09 $\pm$ 0.01	45.4 $\pm$ 0.9	100
30 nm PtNPs + 4.2 μ M DAU	0.12 $\pm$ 0.01	49.8 $\pm$ 0.8	100
30 nm PtNPs + 42 μ M DAU	0.15 $\pm$ 0.01	138.8 $\pm$ 28.2	100
50 nm PtNPs	0.09 $\pm$ 0.02	74.1 $\pm$ 1.9	100
50 nm PtNPs + 4.2 μ M DAU	0.08 $\pm$ 0.01	74 $\pm$ 1.9	100
50 nm PtNPs + 42 μ M DAU	0.11 $\pm$ 0.02	90.7 $\pm$ 0.3	100
70 nm PtNPs	0.08 $\pm$ 0.03	87.2 $\pm$ 1.1	100
70 nm PtNPs + 4.2 μ M DAU	0.04 $\pm$ 0.01	87.6 $\pm$ 0.8	100
70 nm PtNPs + 42 μ M DAU	0.08 $\pm$ 0.01	113.2 $\pm$ 5.3	100

Table 2 Mean size, mode, and concentration of platinum nanoparticles (PtNPs) alone and in combination with daunorubicin (DAU) obtained from NTA. SD – standard deviation

Sample	Mean size, nm $\pm$ SD	Mode, nm $\pm$ SD	Concentration, particles/mL $\pm$ SD
30 nm PtNPs	38.8 $\pm$ 1.4	29.8 $\pm$ 0.7	$1 \times 10^9 \pm 3.3 \times 10^7$
30 nm PtNPs + 21.7 μ M DAU	189.3 $\pm$ 9.4	124.4 $\pm$ 17	$9.9 \times 10^7 \pm 1.2 \times 10^7$
50 nm PtNPs	61.3 $\pm$ 0.5	59.2 $\pm$ 0.5	$5.5 \times 10^8 \pm 2.8 \times 10^7$
50 nm PtNPs + 21.7 μ M DAU	125.8 $\pm$ 6.5	86.7 $\pm$ 5.5	$1.1 \times 10^8 \pm 1.5 \times 10^7$
70 nm PtNPs	86.5 $\pm$ 1.1	73.8 $\pm$ 0.8	$2.2 \times 10^8 \pm 1.2 \times 10^7$
70 nm PtNPs + 21.7 μ M DAU	160.2 $\pm$ 7.1	110 $\pm$ 8.4	$6.8 \times 10^7 \pm 6 \times 10^6$



Interestingly, the observed values were greater compared to the DLS results, however, the dissimilarity is possibly caused by different way of obtaining and processing of the data between these two methods. As mentioned above, particles concentration lowered after the DAU addition within all tested diameters of PtNPs, which was also visible as a change in the size and particle movement trajectories in the recorded movies, thus once more suggesting that DAU promotes the aggregation of PtNPs.

3.2 PtNPs influence on DAU spectra

Daunorubicin has two aromatic rings in its structure, therefore we used fluorescence spectroscopy to further confirm if the platinum nanoparticles interact with daunorubicin. Upon titrating DAU with increasing volumes of PtNPs (solid lines and triangles), the fluorescence significantly quenched compared to the negative control (solid lines and dots), where the drug was titrated with appropriate sodium citrate buffer (Fig. 2). The observed pattern was consistent and confirmed through additional independent experiments research (data not shown).

It is known that metallic nanoparticles can induce an effect known as metal-enhanced fluorescence (MEF), resulting in an enhanced fluorescence of a fluorophore. However, this phenomenon depends strictly on the distance between the

analysed particles; MEF typically occurs when the distance is between 5 to 90 nm, but if the distance is lower, the metallic nanoparticle interaction can result in the quenching of the fluorescent signal.^{30,31} Hence, our results suggest that daunorubicin is most likely localized in a very close proximity to the platinum nanoparticles, and as such, its fluorescence intensity is reduced upon titration. Similar effects were observed for PtNPs-DOX interactions,³¹ another anthracycline agent, and PtNPs-ICR-191 interactions.³² All these compounds share significant similarities in the chemical structure that include aromatic rings as well as reactive nitrogen residues. Therefore, the mechanism of their interactions with the PtNPs is probably analogous. Moreover, according to literature, other metallic nanoparticles, such as silver or gold also promote such effects,^{33–34} therefore we conducted FTIR and NIR analyses to further inspect the nature of the bonding between PtNPs and DAU.

Infrared spectroscopy (IR) is a valuable tool for chemical identification and interpretation of intermolecular interactions.³⁵ Based on the obtained FTIR spectra we detect either important or interesting physicochemical changes that occur in the sample prepared with sodium citrate solution (Fig. 3).

The most significant spectral changes upon interaction between DAU and PtNPs were observed at 2976 cm^{-1} , corresponding to C-H (CH_2) stretching alkane. In pure DAU this

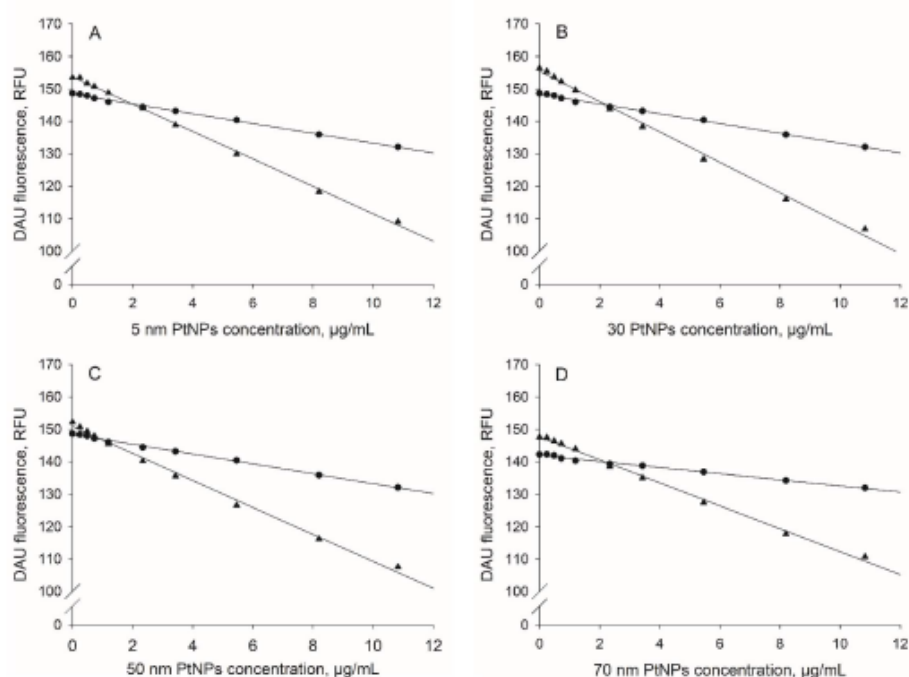


Fig. 2 Fluorescence intensity for platinum nanoparticles (PtNPs) interactions with daunorubicin (DAU) at 590 nm. (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs; circle – appropriate sodium citrate solution, triangle – platinum nanoparticles. DAU initial concentration $34.7\text{ }\mu\text{M}$.

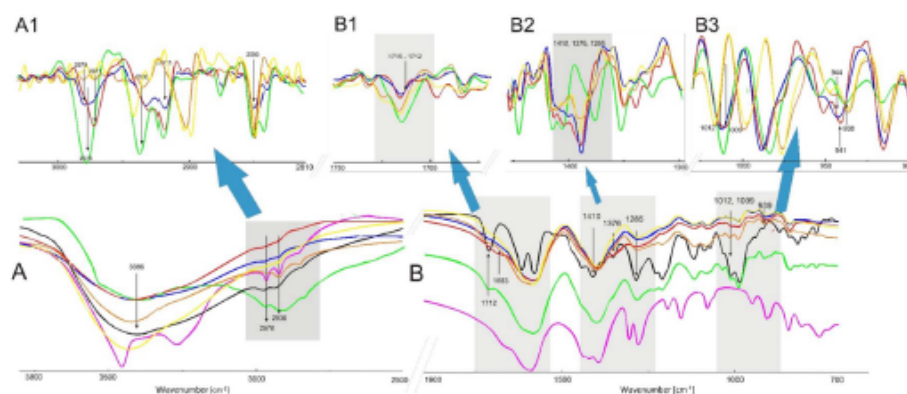


Fig. 3 FTIR spectra of the complexes of daunorubicin (DAU) and platinum nanoparticles (PtNPs) at (A) region 3800–2500 cm^{-1} and at (B) region 1900–700 cm^{-1} and its second derivatives (A1, B1, B2, B3, respectively). 5 nm PtNPs with DAU – blue, 30 nm PtNPs with DAU – orange, 50 nm PtNPs with DAU – red, 70 nm PtNPs with DAU – yellow, sodium citrate – pink, DAU solution in sodium citrate lyophilised – green, DAU alone – black. Arrows indicate the characteristic bands and interactions described in the text. All the samples were lyophilised prior to the experiment.

vibration appears as a single band, but the addition of PtNPs causes a shift to 2965 cm^{-1} , corresponding to decreased energy of this bond. Similarly, O–H stretching region (2930 cm^{-1} to 2840 cm^{-1}) also shifts to lower wavenumbers, suggesting a decrease in energy through interactions between daunorubicin and platinum nanoparticles in all tested sizes.

Significant changes were also recorded for the $\text{C}=\text{O}$ asymmetric ketone vibrations (1713 cm^{-1}), which shifted to 1683 cm^{-1} , most notably for 50 nm PtNPs. Another similar effect is observed at the 1088 cm^{-1} and 1070 cm^{-1} area, as the complexes are influenced by nanoparticles and decrease the energy of C–O stretching bond, visible as shift to 1078 cm^{-1} and 1063 cm^{-1} . Surprisingly, the bending alkene $\text{C}=\text{C}$ substituted, visible as a broad band at 1579 cm^{-1} and in 954–943 cm^{-1} range is also influenced by different sized nanoparticles but there are shifts of bands proving higher energy of this system. Second derivative analysis of spectra was used to better visualize these subtle fluctuations. Complete infrared spectroscopic characterization of DAU and its complexes is shown in Table S1 (SI). Overall, the most prominent interactions were observed for 50 and 70 nm PtNPs-DAU complexes in comparison to the spectrum of sodium citrate solution of DAU, nevertheless 5 and 30 nm PtNPs also induced notable spectral changes, indicating direct interactions with DAU.

Near infrared spectroscopy (NIR) has been found to be advantageous in a wide range of areas in the biotechnological and chemical field, from drug discovery to product development and manufacturing. NIR spectroscopy is sensitive to changes in hydrogen bonding and packing in the crystal lattice, so it can be applied to solid-state analysis, allowing for understanding of molecular interactions.²⁶ In the near-infrared area mainly vibrations of CH, OH, NH and the SH bonds are observed as overtones or combinations of the fundamental IR bands, resulting in overlapping absorption bands in the spectra.²⁷ The OH group demonstrates a strong tendency to

interact with the chemical neighbourhood, e.g., to form a hydrogen-bond network. The existence of an OH group (as in the DAU compound) is a major spectrum-forming factor in NIR, and therefore many physicochemical influences might be found.²⁸ Most significant differences in the interaction forces of the PtNPs and DAU were observed in wavenumber region 7000–6300 cm^{-1} as well as near 5176 cm^{-1} which can be assigned to O–H alcohol and free or hydrogen bonded water, respectively²⁹ (Fig. 4, panel A and B2).

Additional band shifts were observed for O–H/C–O and C–H vibrations, with a change from 4347 cm^{-1} to 4352 cm^{-1} upon PtNPs-DAU complexes formation with 5 nm PtNPs and 70 nm PtNPs. Similar effects were observed at 5176 cm^{-1} , where a shift to lower wavenumbers was recorded for most PtNPs, whereas for 50 nm PtNPs the band shifted upward to 5184 cm^{-1} . The detailed characteristics of NIR analysis are shown in Table S2 (SI). To sum up, these interactions with nanoparticles are responsible for O–H assigned to molecular water or N–H from amine combination bending.

3.3 Endothermic character of DAU-PtNPs interactions

To verify the thermodynamic parameters of PtNPs interactions with DAU, we performed an isothermal titration calorimetry. For 5 nm PtNPs, the registered enthalpy turned out endothermic, with a value of $0.47 \pm 0.21 \text{ kcal mol}^{-1}$. The registered total heat increased throughout the remaining 30 nm, 50 nm and 70 nm PtNPs combinations with DAU, yielding in the enthalpy changes of $1.71 \pm 0.16 \text{ kcal mol}^{-1}$, $1.89 \pm 0.14 \text{ kcal mol}^{-1}$, and $4.36 \pm 0.22 \text{ kcal mol}^{-1}$, respectively (Fig. 5). The thermograms are available in the SI (Fig. S1).

The positive values of ITC results, showing that the interactions between PtNPs and DAU are endothermic, can lead to an assumption that the studied interactions are entropy driven processes. Interestingly, the enthalpy increases with the nanoparticles size, as their surface increases and possibly more DAU



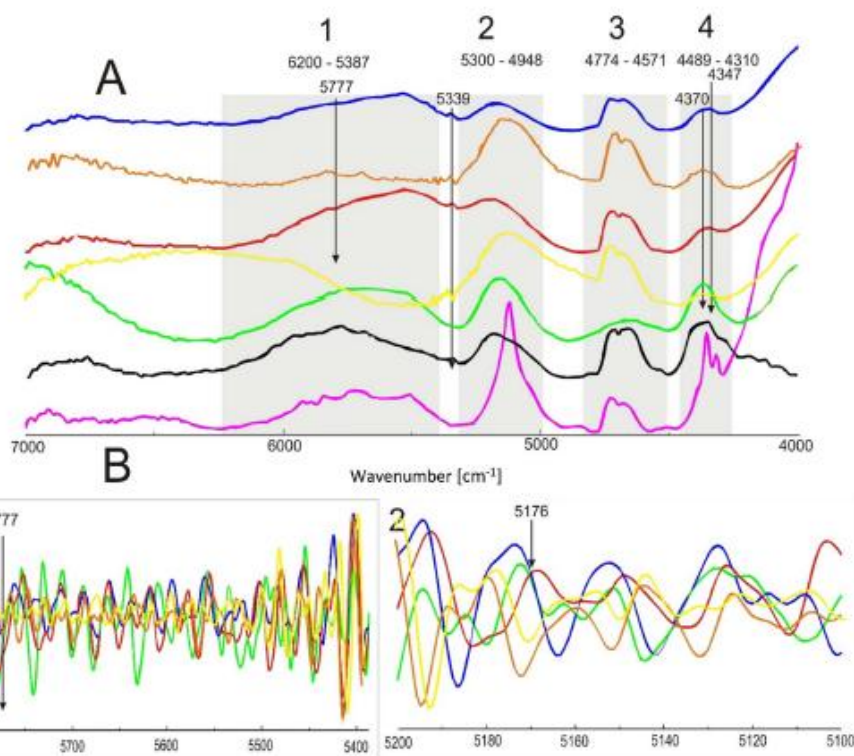


Fig. 4 NIR spectra of the investigated complexes of daunorubicin (DAU) and platinum nanoparticles (PtNPs) (A) in the wavenumber region 7000–4000 cm^{-1} and (B) insets at regions 6200–5387 cm^{-1} and 5300–4948 cm^{-1} . 5 nm PtNPs with DAU – blue, 30 nm PtNPs with DAU – orange, 50 nm PtNPs with DAU – red, 70 nm PtNPs with DAU – yellow, sodium citrate – pink, DAU alone – black, DAU solution in sodium citrate lyophilised – green. Arrows indicate the characteristic bands and interactions described in the text. All the samples were lyophilised prior to the experiment.

molecules can bind with PtNPs, forming aggregates of various sizes and shapes. Observed effects may be attributed to the changes in the water molecules arrangement around PtNPs during formation of their aggregates with DAU. Namely, the water molecules that form the hydration shells surrounding PtNPs become reorganized, and the system requires energy to break the hydrogen bonds linking these molecules, resulting in an endothermic outcome. However, this could also be attributed to a change in the molarity of the solvent (from 2 to 4 mM sodium citrate for 70 nm PtNPs). The results available across the literature vary in that matter – for ICR-191 interactions with PtNPs of 75 nm median size the enthalpy was calculated at roughly -4 kcal mol^{-1} ,¹⁰ whereas for DOX interactions with fullerene C_{60} the values were close to 0 kcal mol^{-1} , with slightly endothermic character.⁴⁰ This variability may be attributed to both the type of nanoparticles (metallic or organic) and the solvent in which they are dispersed. Nevertheless, since the results proved direct interactions, further thermal inspection was performed by utilizing differential scanning calorimetry (DSC). The DSC method can be used to characterize thermal transformations of PtNPs and provide insights into the state of

a drug in its complexes. Interactions between molecules can influence their chemical structure, hydrophilic properties, and association state, resulting in visible shifts in the thermograms.^{41,42} The results of the experiment are shown in Fig. 6.

DSC revealed that both 5 nm and 50 nm PtNPs-DAU complexes demonstrated glass transition (T_g) from the temperature 47.6 to 60 °C, a parameter important for the pharmaceutical industry and observed in nanodispersions or nanocomplexes^{43,44} (Fig. 6, part I). On the contrary, pure DAU did not. DSC curves also indicated the presence of water in nanoparticle samples after lyophilization (Fig. 6, part II). The peak at 184.13 °C corresponds to the melting endotherm of DAU ($T_m = 208\text{--}209$ °C). For DAU suspended in sodium citrate sample after lyophilization (Fig. 6, green curve), as well as for DAU complexed with PtNPs at the temperature near 183 °C only a broad area is seen but in the case of 30 nm DAU-PtNPs and 70 nm DAU-PtNPs there is a very small peak, meaning it is a crystalline residual of bulk. Part III of DSC curves of DAU-PtNPs indicates an exothermic process between the temperature range 220–260 °C, corresponding to the decomposition of complexes. Sodium citrate alone exhibited a melting peak at

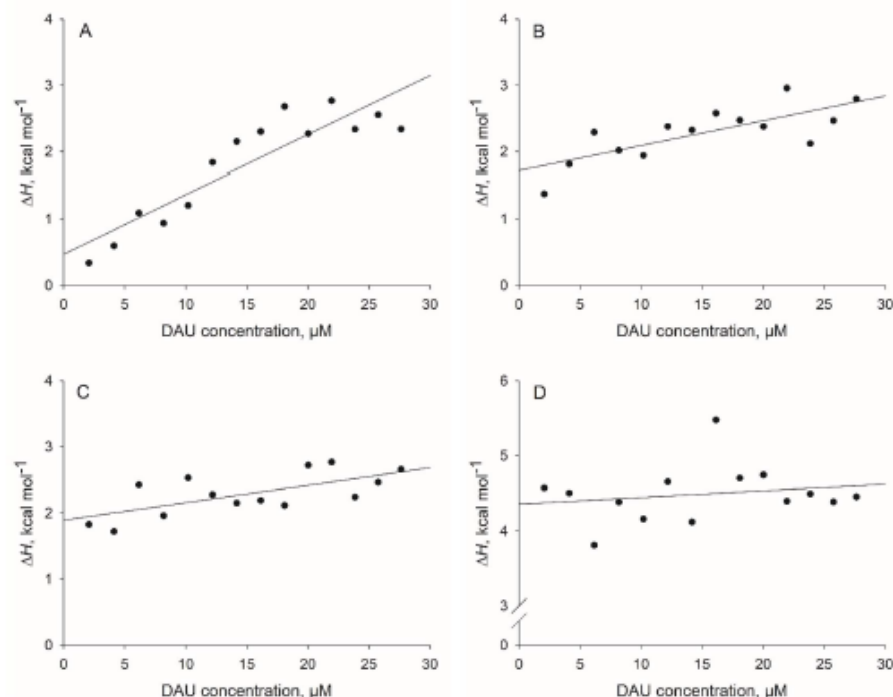


Fig. 5 Isothermal titration calorimetry analysis for the combination of daunorubicin (DAU) with platinum nanoparticles (PtNPs). (A) 5 nm PtNPs ($\Delta H = 0.47 \pm 0.21 \text{ kcal mol}^{-1}$), (B) 30 nm PtNPs ($\Delta H = 1.71 \pm 0.16 \text{ kcal mol}^{-1}$), (C) 50 nm PtNPs ($\Delta H = 1.89 \pm 0.14 \text{ kcal mol}^{-1}$), (D) 70 nm PtNPs ($\Delta H = 4.36 \pm 0.22 \text{ kcal mol}^{-1}$). DAU concentration range 0.002–0.029 mM, initial PtNPs concentration 0.05 mg mL^{-1} .

164 °C (Fig. 6, pink line) whereas each registered curve for PtNPs showed an exothermic peak at 245 °C (5 nm), 210 °C and 260 °C (30 nm), 230 °C (50 nm) and 250 °C (70 nm). Complexes prepared with 5 nm and 50 nm PtNPs indicate different physicochemical state and thermal properties. We observed an absence of a melting point peak for DAU-loaded nanoparticles at 180 °C for DAU-5 nm PtNPs sample which may be the result of the encapsulation process. A similar situation is known from literature.⁴⁵ Differences of DAU-50 nm PtNPs sample's curve at 193 °C may correspond to the change of energy in a complex, but not melting. DAU with 5 nm PtNPs is converted to the amorphous phase as well as the samples with 30 nm and 50 nm PtNPs. It is known that the size of nanoparticles forming complexes has a great effect on their activities.⁴⁶ In particular, small particles, such as 5 nm, demonstrate significant activity and stability, however, they have a problem of deactivation. Overall PtNPs influenced thermal properties of DAU in a size-dependent manner.

3.4 Kinetics of DAU release

It is reported that cancer cells have a more acidic pH compared to the healthy cells,^{46,47} and that pH targeting can be used for a controlled drug release, leading to the avoidance of harmful

effects on healthy tissues. To estimate if the release of daunorubicin can be altered by its interaction with diversely-sized platinum nanoparticles, we performed a release analysis *via* the membrane bag method in three distinct phosphate buffers of pH 5.4, 6.4, and 7.4. The results showed that whereas the drug alone could be released through the membrane pores to 100% in all the tested buffers, this decreased by roughly 30% with the addition of PtNPs in all the tested combinations and buffers (Fig. 7). The statistical analysis is available in SI (Tables S3–S6).

The most significant difference in the maximum released level was observed for 30 nm PtNPs at pH 5.4. On the contrary, 70 nm PtNPs exhibited the lowest degree of variation in terms of changing the diffusion level of DAU, irrespective of the buffer's pH. DAU itself showed a release to 100% in all the tested buffers within the first 12 h of the experiments. However, the addition of PtNPs leads to a decrease in the maximum diffusion, most notably in the case of 30 nm PtNPs. What is more, the mean release percentage was the lowest at pH 7.4 (75%), and the highest at pH 6.4 (85%). This observation is somewhat consistent with other studies, where the diffusion of anthracyclines was altered by various metallic nanoparticles, and resulted in a significantly higher drug release at lower pH.^{47–49} Interestingly,



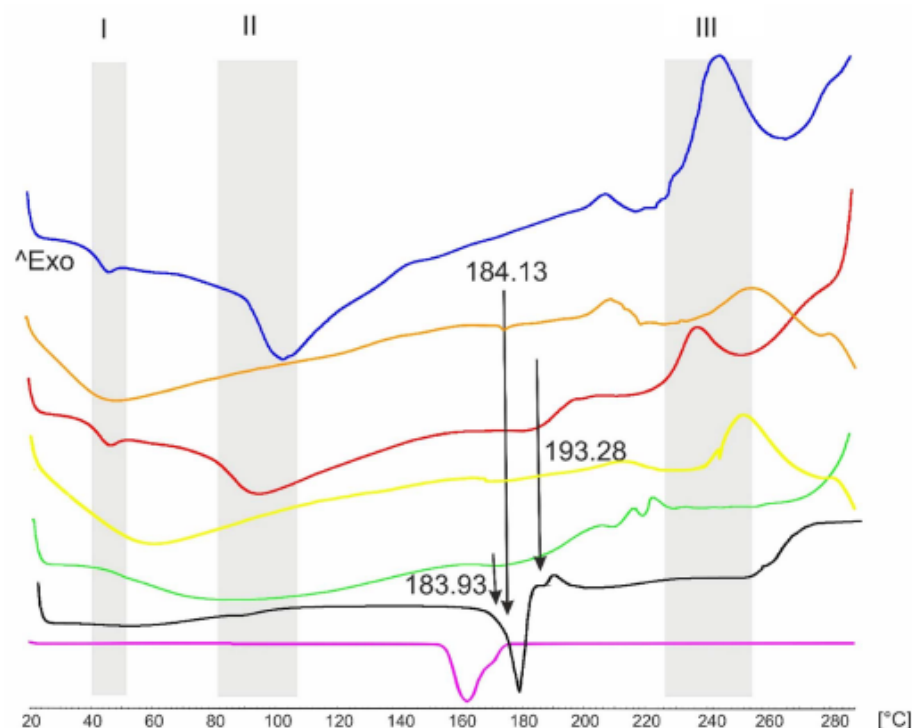


Fig. 6 DSC curves of daunorubicin (DAU) mixtures with platinum nanoparticles (PtNPs). 5 nm PtNPs with DAU – blue, 30 nm PtNPs with DAU – orange, 50 nm PtNPs with DAU – red, 70 nm PtNPs with DAU – yellow, DAU solution in sodium citrate lyophilised – green, DAU alone – black, sodium citrate – pink.

in all the buffers combination, 70 nm PtNPs with DAU yielded the highest drug diffusion in comparison to the remaining tested PtNPs sizes. This dissimilarity could be attributed to a smaller surface area to volume ratio, resulting in less possible interactions between the nanoparticles and the drug. Overall, the lowered release of DAU when combined with PtNPs is correlated with the results of DLS and NTA, where we observed the formation of PtNPs-DAU aggregates. Moreover, FTIR and NIR confirmed the interactions with most likely through formation of hydrogen, as well as electrostatic bonds between the nanoparticles and the drug. We conclude that combining PtNPs with DAU significantly alters anthracycline's release profiles irrespective of the pH.

Furthermore, we analysed the data utilizing the Korsmeyer-Peppas and Hopfenberg models, and calculated the maximum of the released substance (Q_{∞}), the exponent n , which is used to determine the transport mechanism, and the relaxation rate of this process (k_{KP} and k_r) – Table 3. The relaxation rate k_{KP} , based on the Korsmeyer-Peppas model, is related to Fick-type diffusion,⁵⁰ while the relaxation rate k_r comes from the Hopfenberg model.⁵¹

These models are commonly used to analyse drug release from polymer matrices. In the case of dialysis bags, the

diffusion process can typically be described by the Fickian equation. However, in this instance, DAU release proceeded similarly to the aforementioned case of polymer matrices. Based on these models, we established that the release exponents indicated the presence of an anomalous transport within all the experiments, with n values being in the range $0.5 < n < 1$. This implies that in all the samples, in addition to Fickian diffusion, there is an additional process influencing the release mechanism. Assuming that the DAU-nanoparticle system aggregates, and that this aggregation is mediated by hydrogen bonds, it may erode, similar to some polymer matrices, resulting in nonlinear DAU release.⁵² The similar release rate values obtained from the fitting result from the fact that, as previously reported, aggregates vary in size, and therefore the obtained result is an average. In literature, the results are incoherent in that matter, as the n exponent is sometimes reported to be below 0.5, suggesting a semi-fickian diffusion, other times it falls in the range $0.5 < n < 1$, similarly to our study.^{49,53–55} Nevertheless, it is visible that platinum nanoparticles alter the rate at which daunorubicin is released, however, the change seems to be unaffected by the pH of the buffer.

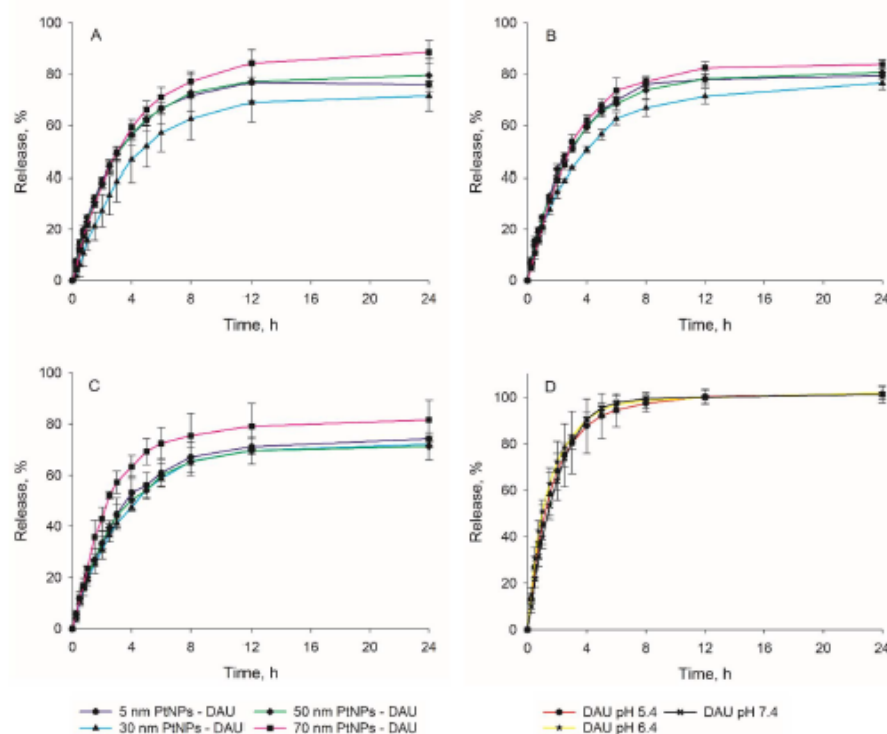


Fig. 7 Analysis of daunorubicin (DAU) release from DAU-platinum nanoparticles (PtNPs) complexes at various pH. (A) pH 5.4, (B) pH 6.4, (C) pH 7.4, (D) DAU alone.

Table 3 Korsmeyer–Peppas and Hopfenberg modelling analysis

	5 nm			30 nm			50 nm			70 nm			DAU alone		
pH	5.4	6.4	7.4	5.4	6.4	7.4	5.4	6.4	7.4	5.4	6.4	7.4	5.4	6.4	7.4
Q_{∞} (%)	76.5	79.1	75.1	73	76.7	72.7	79.3	80	69.5	83.8	86.1	82.5	99.3	99.7	103
k_r	0.35	0.34	0.31	0.24	0.28	0.28	0.32	0.34	0.3	0.27	0.3	0.33	0.58	0.66	0.5
k_{12p}	23.9	23.8	20.7	14	21.1	18.2	22.5	23.6	18.6	19.3	20	21.3	44.1	49.1	36
n	0.69	0.69	0.73	0.94	0.67	0.75	0.72	0.73	0.76	0.82	0.87	0.91	0.74	0.70	0.78
R^2	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

3.5 PtNPs influence DAU's biological activity

Taking into consideration the results of physicochemical analysis, we performed Ames mutagenicity tests on *Salmonella enterica* serovar Typhimurium TA98 strain to investigate the effects of PtNPs-DAU interactions on the DAU's mutagenic potential. 5 nm PtNPs significantly lowered the mutagenic effect in all tested concentrations (Fig. 8A). For the remaining sizes, we observed similar effects, excluding the lowest concentration of PtNPs (Fig. 8B–D). The optimal concentration of DAU was

chosen prior to the experiment (SI, Fig. S2). Importantly, PtNPs alone did not exhibit any mutagenic effects (SI, Fig. S3).

The Ames mutagenicity assay is a valuable tool for a moderately fast screening of the effects that interactions between two compounds have on the biological effects of one of them.⁴⁶ Our results show that with increasing concentrations of PtNPs the mutagenicity of DAU is lowered. Similar results were observed for PtNPs-DOX interactions,⁴⁴ as well as for PtNPs-ICR-191.⁴⁰ This could be attributed to aforementioned formation of PtNPs-

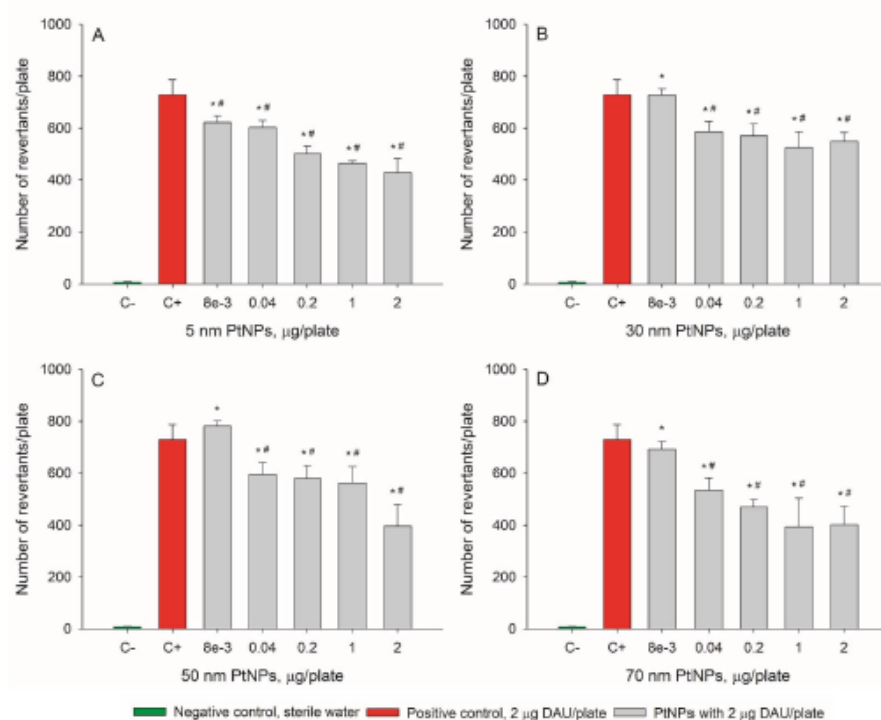


Fig. 8 Influence of platinum nanoparticles (PtNPs) on the mutagenicity of daunorubicin (DAU) in *Salmonella enterica* serovar Typhimurium TA98 strain. (A) 5 nm PtNPs with DAU, (B) 30 nm PtNPs with DAU, (C) 50 nm PtNPs with DAU, (D) 70 nm PtNPs with DAU. C- negative control (green, sterile water), C+ positive control (red, 2 µg DAU/plate). Results are reported as the average number of revertants per plate $\pm$ SD. * - significant difference from the negative control ($p < \alpha$; $\alpha = 0.05$); # - significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

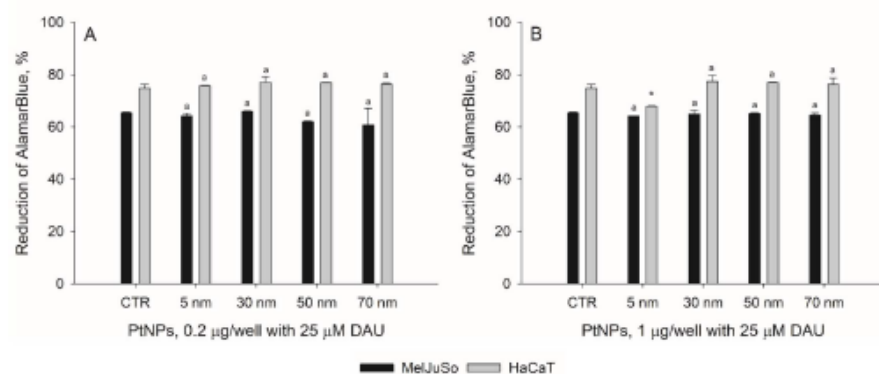


Fig. 9 Influence of platinum nanoparticles (PtNPs) on the cytotoxicity of daunorubicin (DAU) in MelJuSo and HaCaT cell lines. (A) 0.2 µg of PtNPs/well with 25 µM DAU, (B) 1 µg of PtNPs/well with 25 µM DAU. CTR - control (25 µM DAU), PtNPs-DAU mixtures in given concentrations with constant 25 µM DAU. MelJuSo cell line - black, HaCaT cell line - grey. Results are reported as the mean percentage difference between treated cells and untreated control $\pm$ SD. a - no significant difference from the control with DAU within a single cell line ($p < \alpha$; $\alpha = 0.05$), * - significant difference from the control with DAU within a single cell line ($p < \alpha$; $\alpha = 0.05$).

DAU aggregates, making the drug unavailable for the cells to incorporate. With the increasing concentration of PtNPs, the aggregates most likely become greater in size and bind more molecules of DAU, leaving it less available for the cells, thus resulting in lower mutagenicity.

Next, we analysed the influence of PtNPs on DAU's cytotoxicity on two eukaryotic cell lines: non-cancerous HaCaT and cancerous MelJuSo. Prior to testing various combinations, we examined PtNPs and observed they did not significantly alter the growth of neither cell lines (SI, Fig. S4). Based on the effects of PtNPs concentrations with Ames test, we examined their cytotoxicity at 0.2 and 1 μg per well with addition of 25 μM DAU (Fig. 9). None of the investigated combinations altered the cytotoxicity of the drug, apart from 5 nm at 1 μg per well in HaCaT cell line (Fig. 9B).

Despite various reports in the literature regarding PtNPs and their cytotoxic effects towards cancer cells, we did not observe any significant changes in cells viability in our tests. What is interesting, PtNPs significantly enhanced ICR-191 toxicity towards MelJuSo cells with no harmful effects towards healthy HaCaT cells.⁴⁰ Moreover, PtNPs significantly influenced cytotoxicity of DOX, one of 4 anthracyclines, with the MelJuSo cells being either killed in a similar or intensified manner, than with drug alone, while simultaneously enhancing the viability of HaCaT cells.⁴¹ The dissimilarity between the observed effects of these two anthracyclines and their combination with PtNPs may be attributed to the conformational change at C-14; DOX has an -H, while DAU has -OH group. It may seem a minor change, but it could alter the activity profile of the drug, as well as its binding capability (i.e. stability of formed aggregates), resulting in lesser effects towards these cell lines. It's worth noting that the observed effects can vary depending on chosen cell lines. Nevertheless, the results conclude that there are no significant effects of PtNPs-DAU interactions on the cytotoxicity of the drug in HaCaT and MelJuSo cell lines at the examined concentrations.

4 Conclusions

In summary, platinum nanoparticles (PtNPs) of distinct sizes 5, 30, 50 and 70 nm have been shown to interact with daunorubicin (DAU), leading to the formation of aggregates. Significant quenching of fluorescence indicated direct interaction between DAU and PtNPs of all sizes, while FTIR and NIR confirmed that providing precise data for exact bonds within the chemical structure of DAU. Calorimetric analyses using ITC and DSC techniques provided additional information on the interactions studied and showed that the interactions are of an endothermic nature, driven by entropy. Combining PtNPs with DAU results in altered DAU release kinetics, most notably by 30 nm PtNPs. The observed interactions reduce DAU's mutagenic capabilities in *Salmonella enterica* serovar Typhimurium TA98 strain, however, no significant effects were observed in neither cancerous MelJuSo nor non-cancerous HaCaT cell lines. Our study provides a thorough examination of PtNPs-DAU interactions and their influence on the drug's activity, and warrants further eukaryotic studies in regards to PtNPs role as potential activity modulators of classical anticancer drugs.

Author contributions

Marcin Zakrzewski: investigation, formal analysis, visualization, validation, writing – original draft. Patrycja Beldzińska: methodology, investigation, writing – review & editing. Felicja Gajdowska – investigation. Grzegorz Gołuski: writing – review & editing, methodology, visualization. Karolina Gackowska: investigation, writing – review & editing. Justyna Strankowska: investigation, writing – review & editing. Marzena Jamróiewicz: resources, investigation, writing – review & editing. Dariusz Wyrzykowski: resources, investigation, writing – review & editing. Katarzyna Grzyb: investigation, writing – review & editing. Danuta Gutowska-Owsiak: resources, writing – review & editing. Anna Synak: resources, investigation. Piotr Bojarski: resources. Jacek Piosik: conceptualization, project administration, resources, supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data of this study are available from the corresponding author upon request.

Supplementary information is available. See DOI: <https://doi.org/10.1039/d5ra02827b>.

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Table S1. Fourier Transform Infrared Spectroscopic (FTIR) characterization of daunorubicin (DAU) alone and in combination with platinum nanoparticles (PtNPs). The regions of significant change from DAU's band were marked in red.

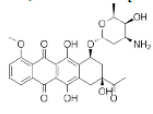
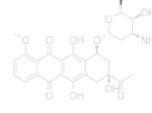
Chemical Bond	DAU, wavenumber [cm ⁻¹]	DAU, wavenumber [cm ⁻¹]	5 nm PtNPs-DAU, wavenumber [cm ⁻¹]	30 nm PtNPs-DAU, wavenumber [cm ⁻¹]	50 nm PtNPs-DAU, wavenumber [cm ⁻¹]	70 nm PtNPs-DAU, wavenumber [cm ⁻¹]
		Lyophilized from sodium citrate solution	Complex 1	Complex 2	Complex 3	Complex 4
N-H stretching primary amine	3543	3600 – 3400	3550 – 3400	3550 – 3400	3550 – 3400	3550 – 3400
O-H intermolecular bonded	3500 – 3290 3389	3386	3409	3427	3409	3429
C-H (CH ₂) stretching alkane	2978	2976	2979	2965	2971	2975
O-H stretching alcohol intramolecular bonded and CH (CH ₂) stretching alkane	2935, 2915, 2844	2937 – 2919, 2847	2931 – 2924, 2848	2926 – 2920, 2853	2937 – 2919, 2850	2931 – 2924, 2851
Asymmetric acid –C=O ketone	1711	1713	1713	1713	1683	1713
(benzoilo)-C=O amide	1617	1612	1612	1612	1623	1613
–C=C–	1579, 1444	1583	1583	1583	1583	1583
C-H (CH ₃)	1408	1415 – 1386 1400	1403 – 1380 1394	1403 – 1380 1398	1403 – 1380 1398	1403 – 1380 1401
O-H bending alcohol	1376, 1351	–, 1351	–, –	–, 1351	–, 1348	–, 1351
–C–O–C–	1285	1281	1282	1281	1281	1281
C–O stretching ester or –C–N– amine	1229 1209	1231 1209	1255 1209	1231 1209	1231 1209	1231 1209
O–H alcohol	1117	1117	1117	1117	1117	1115
C–O stretching	1088, 1070	1080, 1068	1078, –	1078, 1068	1078, 1065	1078, 1063
–C–O–C–	1033, 1010, 985	1034, 1010, 988	1036, 1012, 988	1036, 1012, 988	1036, 1007, 988	1036, 1011, 988
C=C bending alkene substituted	954, 940	954 – 943	944	951 – 943	951 – 943	943
strong C–H bending	916, 870, 842, 818	912, 872, 839, 818	912, 897, –, 839, –	912, 895, 872, 839, –	912, 897, 874, 839, 818	912, 897, 874, 839, 818
C–H bending	795, 763	791, 763	–, –	789, 763	789, 763	789, 763

Table S2. Near Infrared Spectroscopic (NIR) characterization of daunorubicin (DAU) alone and in combination with platinum nanoparticles (PtNPs). The regions of significant change from DAU's band were marked in red.

Chemical Bond	DAU, wavenumber [cm ⁻¹]	DAU, wavenumber [cm ⁻¹]	5 nm PtNPs-DAU, wavenumber [cm ⁻¹]	30 nm PtNPs-DAU, wavenumber [cm ⁻¹]	50 nm PtNPs-DAU, wavenumber [cm ⁻¹]	70 nm PtNPs-DAU, wavenumber [cm ⁻¹]
		Lyophilized from sodium citrate solution	Complex 1	Complex 2	Complex 3	Complex 4
O–H alcohol (RO–H), N–H amine	7000 – 6500	7000 – 6300	7000 – 6500	7000 – 6500	7000 – 6500	7000 – 5500
C–H alkene, =CH ₂ C–H (2ν), =CH ₂ , C–H aromatic C–H (aryl), C–H methyl C–H, C–H methyl C–H, OH associated as (ROHCH ₃), C–H methyl C–H, ether associated as (R–O–CH ₃)	6200 – 5387 5777	6300 – 5300	5536	6030 – 5600 5825, 5700	5536	–
C=O carbonyl	5339	–	5339	5358	5339	5358
O–H assigned to molecular water, N–H combination, amine	5300 – 4948 5176	5151	5168	5155	5184	5124
C=O and C–O stretching, N–H/C–N, C–H aromatic C–H (aryl)	4774 – 4571 4728, 4681, 4658	4850 – 4500	4721, 4681, 4653	4721, 4681, 4653	4715, 4681, 4647	4730, 4676, 4643
O–H/C–O, C–H starch (C–H and CH ₂), C–H (C–H bending)	4489 – 4310 4370, 4347	4355	4352	4377	4343	4358

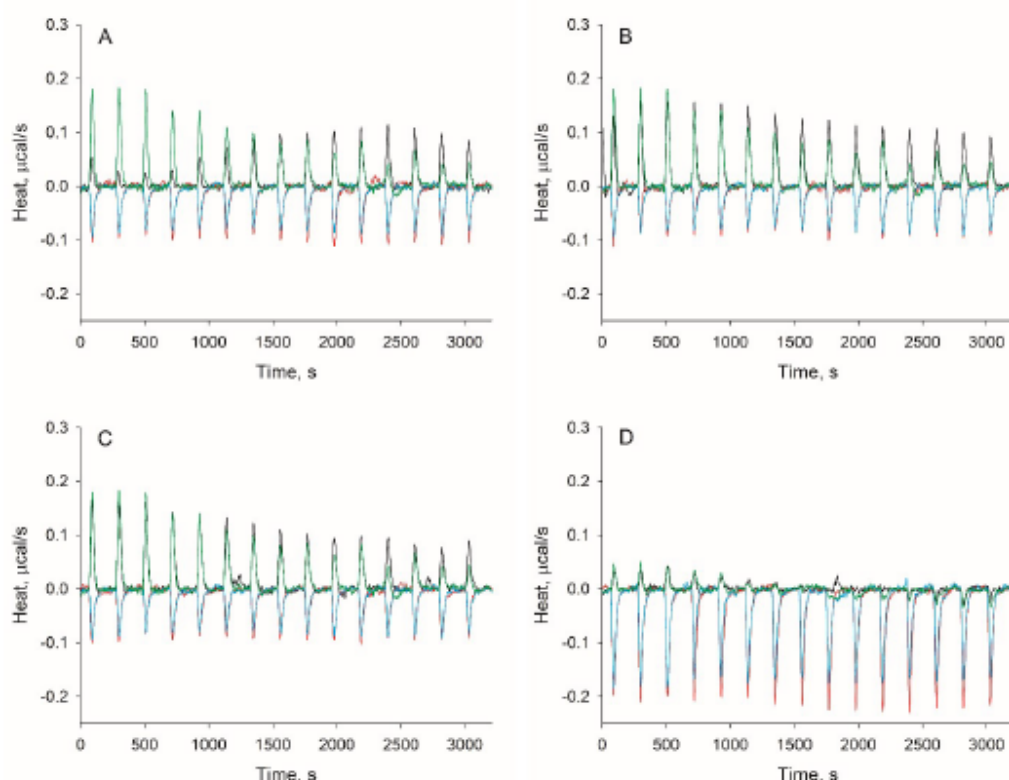


Figure S1. Thermograms showing microcalorimetric titrations of platinum nanoparticles (PtNPs) with daunorubicin (DAU). (A) – 5 nm PtNPs with DAU, (B) – 30 nm PtNPs with DAU, (C) – 50 nm PtNPs with DAU, (D) – 70 nm PtNPs with DAU; PtNPs interaction with DAU – black, water with PtNPs – red, water with appropriate sodium citrate solution – cyan, DAU with appropriate sodium citrate solution – green.

Modelling methods

The Korsmeyer-Peppas is an universal power-law model used when the specific transport mechanism of the active substance is unknown, expressed as: $Q = k_{KP}t^n$, and allows for the determination of the release rate constant of the active substance (k_{KP}) and the exponent n , which can be used to identify the release mechanism. The parameter k_{KP} accounts for the structure of the carrier, while the exponent n depends on the matrix geometry and the release mechanism. For thin polymer films, an exponent of $n = 0.5$ indicates Fickian diffusion or quasi-Fickian diffusion for $n < 0.5$, within the range of $0.5 < n < 1.0$ it suggests anomalous diffusion (anomalous transport), and $n = 1$ corresponds to zero-order release kinetics.

The Hopfenberg model is demonstrated as: $Q_t = Q_{\infty}(1 - \exp^{-k_r t})$, where k_r is the relaxation rate, and is typically used for polymer matrices releasing an active substance when swelling or erosion processes occur during the release, therefore the relaxation rate is related to the behaviour of the polymer chains, reorganization which affects the diffusion process of the active substance, usually by limiting it.

Table S3. Statistical analysis of differences between 5 – 70 nm platinum nanoparticles (PtNPs) release of daunorubicin (DAU) capability at pH 5.4. For the analysis, ANOVA was performed, followed by post-hoc Tukey's HSD test. The significance level for all the analyses was established at $\alpha=0.05$. X- significant difference from sample to sample ($p < \alpha$; $\alpha=0.05$).

Table S4. Statistical analysis of differences between 5 – 70 nm platinum nanoparticles (PtNPs) release of daunorubicin (DAU) capability at pH 6.4. For the analysis, ANOVA was performed, followed by post-hoc Tukey's HSD test. The significance level for all the analyses was established at $\alpha=0.05$. X- significant difference from sample to sample ($p < \alpha$; $\alpha=0.05$).

Table S5. Statistical analysis of differences between 5 – 70 nm platinum nanoparticles (PtNPs) release of daunorubicin (DAU) capability at pH 7.4. For the analysis, ANOVA was performed, followed by post-hoc Tukey's HSD test. The significance level for all the analyses was established at $\alpha=0.05$. X- significant difference from sample to sample ($p < \alpha$; $\alpha=0.05$).

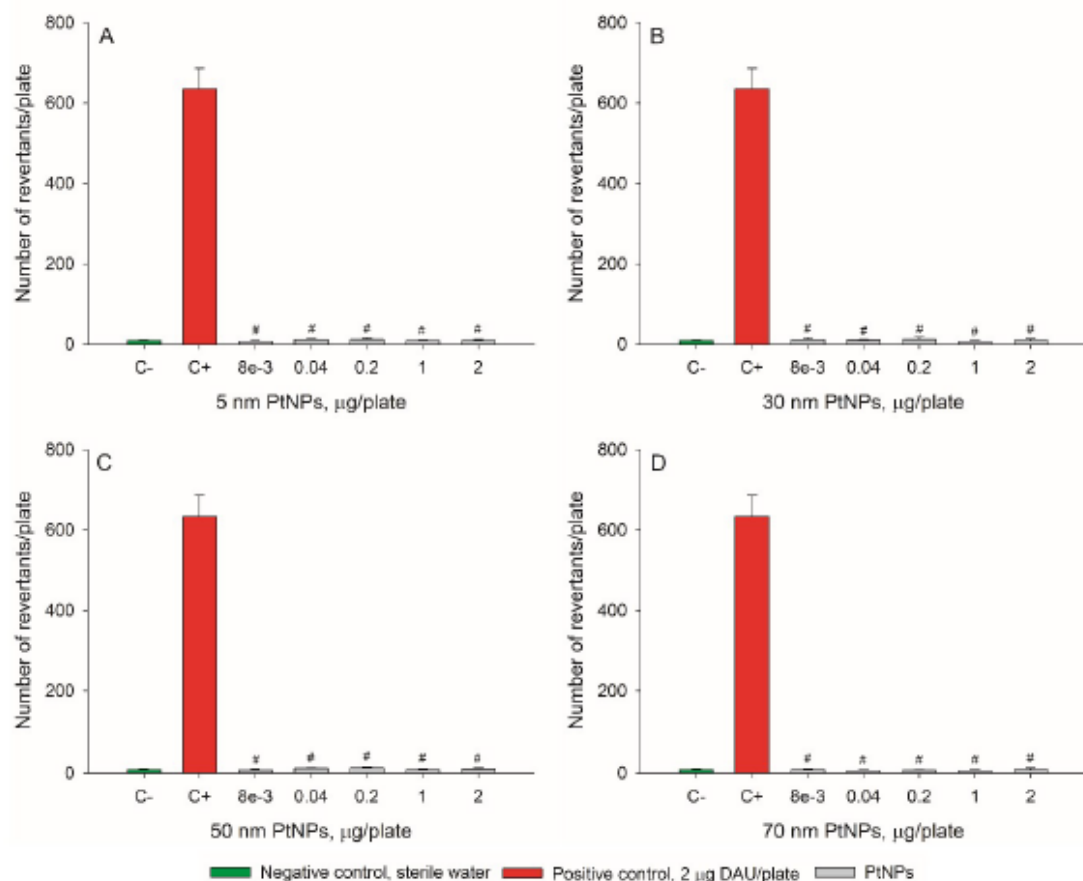


Figure S3. Mutagenicity of platinum nanoparticles (PtNPs) in *Salmonella enterica* serovar Typhimurium TA98 strain. (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. C- negative control (green, sterile water), C+ positive control (red, 2 µg DAU/plate), PtNPs (grey). Results are reported as the average number of revertants per plate $\pm$ SD. #- significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

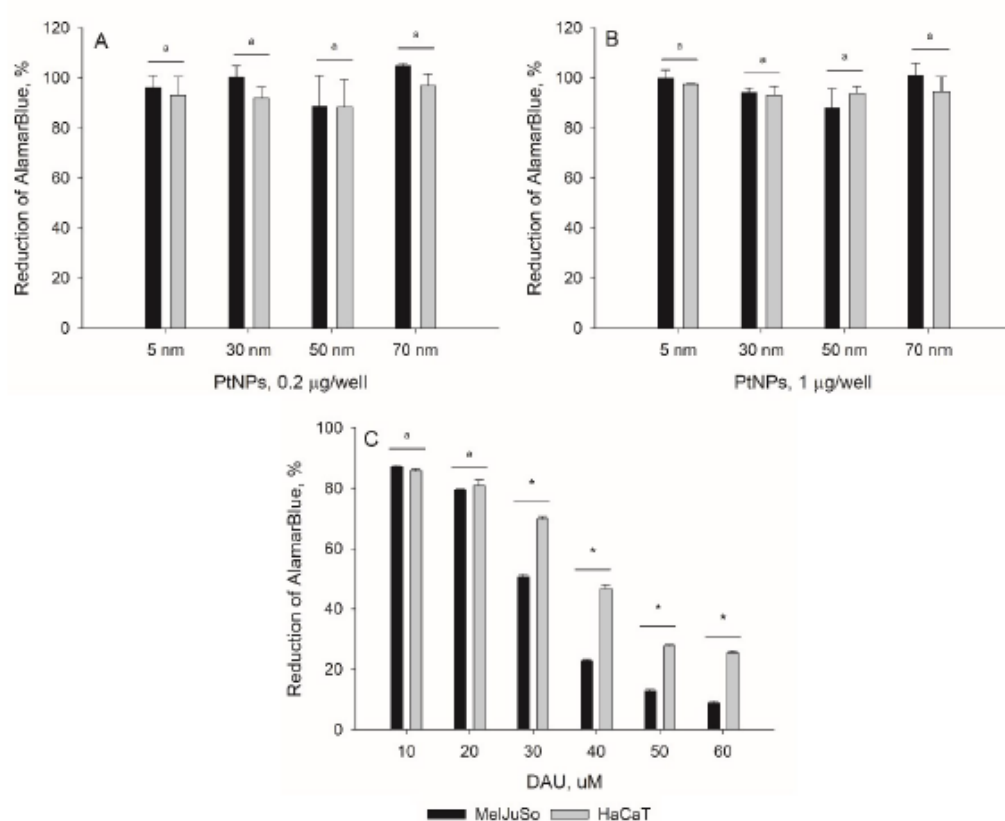


Figure S4. Cytotoxicity of platinum nanoparticles (PtNPs) in various concentrations in MelJuSo and HaCaT cell lines. (A) – 0.2 µg PtNPs/well, (B) – 1 µg PtNPs/well, (C) – DAU alone. Black – MelJuSo cell line, grey – HaCaT cell line. Results are reported as the mean percentage difference between treated cells and untreated control $\pm$ SD. * - statistically significant differences between tested cell lines ($p < \alpha$; $\alpha = 0.05$), a – no statistically significant differences between tested cell lines ($p < \alpha$; $\alpha = 0.05$).

2. Zakrzewski M., Bełdzińska P., Gackowska K., Yurchak A., Jamrógiewicz M., Wyrzykowski D., Bury K., Grzyb K., Gołński G., Piosik J. Platinum nanoparticles as modulators of idarubicin activity: a physicochemical and in vitro biological study. (Manuscript)

Platinum nanoparticles as modulators of idarubicin activity: a physicochemical and in vitro biological study

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Abstract

Cancer remains one of the leading causes of death worldwide. Although multiple treatment strategies have been developed, chemotherapy is still widely used. However, it is not indifferent to healthy tissue, leading to severe side effects, the major ones being bone marrow suppression and systemic toxicity. To overcome this problem, it has been proposed to modulate the activity of anticancer agents, using e.g. nanoparticles. In this study, we investigated whether platinum nanoparticles (PtNPs) of various sizes interact with idarubicin (IDA), an active pharmaceutical ingredient (API) used in medicine mainly in the treatment of acute leukaemia. Dynamic light scattering (DLS) demonstrated changes in the hydrodynamic diameter of PtNPs following IDA addition, which were further supported by atomic force microscopy (AFM) imaging. Moreover, we observed that PtNPs significantly quench IDA's fluorescence, suggesting their direct and close-proximity interactions, which were additionally shown by Fourier transform infrared (FTIR) and near infrared spectroscopies (NIR). Isothermal titration calorimetry (ITC) revealed that the interactions are endothermic, with the enthalpy ranging from 1.2 to 3.6 kcal/mol, and differential scanning calorimetry (DSC) demonstrated that the PtNPs-IDA combination alters the melting temperature of the API. Biological assays revealed that PtNPs of all tested sizes influenced IDA mutagenicity in *Salmonella enterica* serovar Typhimurium TA98 strain. Furthermore, PtNPs modulated the cytotoxicity of IDA towards SK-BR-3 and MCF-7 cell lines in a dose-dependent manner. Overall, these results demonstrate that PtNPs interact with IDA, altering its biological activity through slightly synergistic effects of the PtNPs-IDA combination against cancer cells, which is promising for further investigations.

Keywords

platinum nanoparticles, idarubicin, leukaemia, mutagenicity, cytotoxicity, MCF-7, SK-BR-3

1. Introduction

In 2022, cancer accounted for approximately 10 million deaths worldwide, ranking among the ten leading causes of mortality [1,2]. What is worse, its incidence is projected to increase by approximately 70% by 2050 [1]. Despite the development of various novel treatment regimens, chemotherapy remains the most widely applied [3,4]. However, despite its efficacy, it's not indifferent to healthy cells, leading to a multitude of side effects, the most severe being bone marrow suppression and increased risk of secondary malignancies [4]. Moreover, cancer cells can develop drug-resistance, yielding the therapy ineffective over time [3]. To address these limitations, current research focuses on modulating the activity of already existing anticancer agents by combining them with e.g. nanoparticles [5,6].

Nanomedicine, defined as the application of nanoscale materials for diagnostic and therapeutic purposes, has emerged rapidly within the last few decades [7]. In general, nanoparticles range from 1 to 100 nm in size, and can be fabricated using various physical, chemical or, lately, very common, biological methods [8,9]. Advances in the synthesis and characterisation techniques enabled precise control over the size, shape, and surface chemistry, thus broadening their potential for clinical applications, such as therapy, diagnostics, or drug delivery [10]. Among the wide range of nanomaterials, metallic nanoparticles have gained significant interest over the years, especially due to their unique characteristics, including a high surface-area-to-volume ratio and both optical and catalytic properties. In this group, platinum nanoparticles (PtNPs) have emerged considerably in the last decade in various biomedical fields, used as antibacterial and antifungal agents, as well as biosensors and contrast agents in bioimaging [11]. However, their most important feature is their anticancer potential. They have been shown to exert cytotoxicity towards various cancerous cell lines [12–14], while none or minor cytotoxicity towards healthy tissue was observed, indicating limited toxicity or possible protective effects [15,16]. The mechanism of PtNPs activity is broad, however, the main mode of action is established as reactive oxygen species (ROS) production [12,17]. Furthermore, PtNPs have been shown to accumulate strongly in tumours. The plausible reason for this phenomenon could be the enhanced permeability and retention (EPR) effect, caused by leaky blood vessels, leading to increased passive uptake of macromolecules [18]. Due to this, PtNPs have been applied as nanocarriers, resulting in more selective drug release at the tumour site [19–22]. What is more, PtNPs provide a

versatile platform for surface functionalization with diverse ligands, leading to broadened usage, thus making them an excellent tool for future modulations.

Idarubicin (IDA) is a well-known active pharmaceutical ingredient (API) used in anticancer therapy. It belongs to the anthracycline class of antibiotics and is a semisynthetic derivative of daunorubicin (DAU), with a methoxy group changed for hydrogen at the R1 position [23]. This seemingly small substitution yields IDA more lipophilic, improving membrane penetration [24,25]. Similar to other anthracyclines, IDA acts through intercalation to DNA, inhibition of topoisomerase II activity, and generation of free radicals [26,27]. Therefore, it is primarily utilised in the treatment of acute myeloid and lymphoblastic leukaemia, also in combination with cytarabine (CYT) [25], resulting in overall better efficacy than the DAU-CYT combination [28]. Unfortunately, use of this chemotherapy can result in acute side effects, such as myelosuppression or cardiotoxicity [26,29]. However, due to its efficacy, it is still widely used worldwide.

In view of this fact and taking into consideration that PtNPs have demonstrated a multitude of anticancer effects on their own and in combination with cytotoxic agents, herein we decided to verify if spherical PtNPs of distinct sizes 5, 30, 50 and 70 nm can alter the activity of IDA. Hence, we employed various physicochemical techniques, starting with size measurements, through spectral scans, ending with thermal inspection of PtNPs-IDA combinations. Next, we analysed the influence of PtNPs on the biological activity of IDA using Ames mutagenicity and cytotoxicity assays on prokaryotic and eukaryotic cells, respectively.

2. Experimental

2.1 Materials

Platinum nanoparticles (PtNPs) at concentration 0.05 mg/mL and 1 mg/mL were purchased from nanoComposix (San Diego, California, USA). The nanoparticles were dispersed in 2 mM sodium citrate solution for 5, 30 and 50 nm PtNPs and in 4 mM sodium citrate solution for 70 nm PtNPs. Idarubicin hydrochloride (IDA) was purchased from Molnova (Ann Arbor, Indiana, USA). The stock solutions were prepared by dissolving IDA in deionised water, and the desired concentrations were validated via spectroscopic methods with the molar extinction coefficient of $\epsilon_{526.5} = 3.64 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ [30].

The bacterial strain of *Salmonella enterica* serovar Typhimurium TA98 was purchased from Xenometrix (Allschwil, Switzerland). Nutrient Agar, Nutrient Broth, Glucose, Ampicillin, L-Histidine and Biotin and necessary salts were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) according to the Ames *Salmonella*/microsome mutagenicity assay (Mortelmans & Zeiger, 2000). SK-BR-3 and MCF-7 cell lines were received from the Laboratory of Molecular Enzymology and Oncology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk. Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS), 4 mM L-glutamine, glucose and antibiotic-antimycotic solution were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA).

2.2 Methods

2.2.1 Dynamic Light Scattering (DLS)

The hydrodynamic diameter of the PtNPs and the change in registered sizes upon addition of IDA were evaluated in polystyrene cuvettes at 25 °C utilising Zetasizer Nano S from Malvern Panalytical (Malvern, Great Britain). The initial concentration of PtNPs was 2.38 µg/mL, and the concentration range of IDA was 5 µM – 50 µM.

2.2.2 Atomic Force Microscopy (AFM)

Atomic force microscopy visualisation of the PtNPs alone and their mixtures with IDA was performed at 23 °C in air, in PeakForce Tapping mode, using BioScope Resolve AFM (Bruker, Bremen, Germany). PtNPs (0.05 mg/mL) were mixed with 0.187 µM IDA

at room temperature. Subsequently, 100 mM cysteamine was deposited onto a freshly cleaned mica surface for 5 min, and afterwards the solution was aspirated. Then samples were added on mica and incubated for 5 min at room temperature, followed by washing with deionised water and drying with streams of nitrogen gas prior to AFM imaging. The ScanAsyst-Fluid+ probe (Bruker) was used for PtNPs alone and PtNPs-IDA mixtures imaging (resonant frequency $f_0 = 150$ kHz; spring constant $k = 0.7$ N/m). Images were registered at 512×512 pixels with a PeakForce Tapping frequency of 1 kHz and amplitude of 150 nm. A height sensor signal was used to display the image using NanoScope Analysis v1.9.

2.2.3 Fluorescence spectroscopy

The fluorescence measurements were performed in quartz cuvettes at a temperature of 25 ± 0.1 °C using Jasco FP-8500 (Jasco, Easton, Maryland, USA) with a Peltier thermostat. All the measurements were performed in 2 mM sodium citrate for 5, 30 and 50 nm PtNPs and in 4 mM sodium citrate for 70 nm PtNPs. In each sample the initial concentration of IDA was 37.2 μ M followed by titration with PtNPs in the concentration range 0 – 10.62 mg/mL or analogous volumes of appropriate sodium citrate solution (negative control).

2.2.4 Fourier Transform Infrared Spectroscopy (FTIR) and Near Infrared Spectroscopy (NIR)

FTIR and NIR spectra of complexes formed between IDA and PtNPs were recorded on Jasco-4700 instrument (IR: 4000–400 cm^{-1} NIR: 8200–4000 cm^{-1}) with 32 scans, 4 cm^{-1} resolution (Jasco Company, Tokyo, Japan). All prepared samples were lyophilised prior to the procedure. The NIR transmission technique was applied using dry samples of IDA, PtNPs, and sodium citrate obtained in the process of lyophilisation. The materials were compressed with potassium bromide to form a disc. All spectra were recorded as a background and analysis of spectra was performed using Spectra Analysis software (Jasco Company, Tokyo, Japan).

2.2.5 Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry measurements were performed at 25 °C on an AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The volumes of the sample and reference cells were 1.4491 mL. The ITC experiment consisted of 12 injections of 10.02 μ L each of the IDA solution, with the first injection being only

2 μL (as is typical to establish a stable baseline and account for diffusion across the syringe tip). The concentration range of IDA was 0.002 - 0.029 mM, and it was titrated into the sample cell containing PtNPs at an initial concentration of 0.05 mg/mL. Background titrations were performed by injecting IDA into an appropriate sodium citrate solution and appropriate sodium citrate solution to PtNPs. The results of the background titrations were subtracted from each experimental result to account for the heat of dilution. Each injection lasted 20 s, and the injection spacing was set to 210 seconds. To reach a homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm.

2.2.6 Differential Scanning Calorimetry (DSC)

The thermal behaviour of the samples was measured by Differential Scanning Calorimetry (DSC), STAR-1 System, Mettler Toledo (Greifensee, Switzerland), which was calibrated with indium. The analysis was carried out in aluminum crucibles 40 μL , at a temperature range from 20 $^{\circ}\text{C}$ to 300 $^{\circ}\text{C}$. For performing the DSC analysis, 1–5 mg of the samples was weighed. DSC curves were recorded and analysed by the STAR-1 System from Mettler Toledo combined with the intercooler system. Samples were heated at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ and a nitrogen flow rate 60 mL/min. All tests were standardised to each sample size.

2.2.7 Ames mutagenicity assay

The influence of PtNPs on the mutagenic activity of IDA was assessed using Ames mutagenicity plate assay with *Salmonella enterica* serovar Typhimurium TA98 strain, according to the procedure described by Woziwodzka et al.[32]. A mixture containing 100 μL of the overnight bacteria culture, 50 μL of 3% NaCl, and 100 μL of the tested solution (or sterile distilled water as a negative control) was incubated for 4 h in darkness at 37 $^{\circ}\text{C}$ and 220 rpm. Afterwards, the mixture was centrifuged at 11840 g, the bacterial pellet was washed with 0.75% NaCl and resuspended in 300 μL of 0.75% NaCl solution containing 0.1 μM histidine and 0.1 μM biotin. The bacterial suspension was spread on a glucose minimal agar plate and incubated at 37 $^{\circ}\text{C}$ in the dark. After 48 h incubation, the number of revertant colonies per plate was calculated. All experiments were performed in triplicate.

2.2.8 Cell culture and cytotoxicity assay

The SK-BR-3 and MCF-7 cell lines were cultivated in DMEM containing 4500 mg/L glucose, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin. Both cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. For the alamarBlue reduction assays, the cells were seeded on a 96-well plate (2 x 10⁴ cells/well and 4 x 10⁴ cells/well for SK-BR-3 and MCF-7 cell lines, respectively) and incubated overnight in a humidified atmosphere at 37 °C and with 5% CO₂. Next, the cells were washed thrice with appropriate medium devoid of FBS. Subsequently, PtNPs alone, at concentrations 0.2, 1 and 5 µg/mL, and in combination with IDA (20 – 50 µM for SK-BR-3 and 10 – 40 µM for MCF-7) were added to cell cultures (90 µL/well), each sample in three replicates and incubated for 20 h. After that, 10 µL of alamarBlue (BioRad) was added to each well, and the plates were further incubated for 4 h in conditions described above. The absorbance was measured at 570 nm and 600 nm, and the percentage of alamarBlue reduction was calculated as a difference between treated and untreated cells, according to the protocol provided by the manufacturer:

$$diff(\%) = \frac{(O_2 \times A_1) - (O_1 \times A_2)}{(O_2 \times P_1) - (O_1 \times P_2)}$$

diff – difference between treated and control cells; O₁ – ε₅₇₀ of oxidized alamarBlue (80586 M⁻¹cm⁻¹); O₂ – ε₆₀₀ of oxidized alamarBlue (117216 M⁻¹cm⁻¹); A₁ – absorbance of the test wells at 570 nm; A₂ – absorbance of test wells at 600 nm; P₁ – absorbance of control wells at 570 nm; P₂ – absorbance of control wells at 600 nm.

2.2.9 Computer analysis

All the graphs were prepared in SigmaPlot 14.5 from Systat Software Inc. (San Jose, California, USA). All statistical analyses were performed in Statistica 14.0 from TIBCO Software Inc. (Palo Alto, California, USA). For the Ames mutagenicity test and the cytotoxicity assays one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's HSD test was applied. The significance level for all the analyses was established at α = 0.05.

3. Results and discussion

Metallic nanoparticles present a broad spectrum of properties, making them a useful tool for biomedical applications. There have been many studies on the efficacy of gold [33], silver [34], or iron nanoparticles [35], with the results showing significant antimicrobial and often also anticancer effects. Moreover, these nanoparticles have also been widely employed in both bioimaging and drug delivery systems (DDS) [36,37]. Despite the potential improvement in the medicines' efficacy, in this study we focused on analysing if platinum nanoparticles, sized between 5 and 70 nm, interact with idarubicin, and what is the outcome of these associations on the biological activity of IDA.

3.1 PtNPs aggregate with IDA

Firstly, we employed dynamic light scattering (DLS) to assess potential interactions between PtNPs and IDA (**Figure 1**). 5 nm PtNPs presented a polydisperse population, with the major fraction being $\sim 23 \pm 1.3$ nm in size, however, the addition of 5 μ M of IDA resulted in a noticeable change in the registered hydrodynamic diameter. With 50 μ M total, the population became monodisperse as evidenced by the decrease of the polydispersity index (PDI) from 0.5 to 0.24, and the mean size changed to 539.7 ± 16 nm. In the case of 30, 50 and 70 nm PtNPs, we observed a significant change in registered diameters only in combination with 50 μ M of IDA, while all the PDIs remained below 0.3. The precise data are available in the supplementary information (**Table S1**). These findings indicate the formation of PtNPs-IDA heteroaggregates. It is also possible that the mere presence of IDA in the mixture may also induce the creation of PtNPs homoaggregates of various sizes, which would indicate a high complexity of the aggregation process, however, this speculation still requires confirmation.

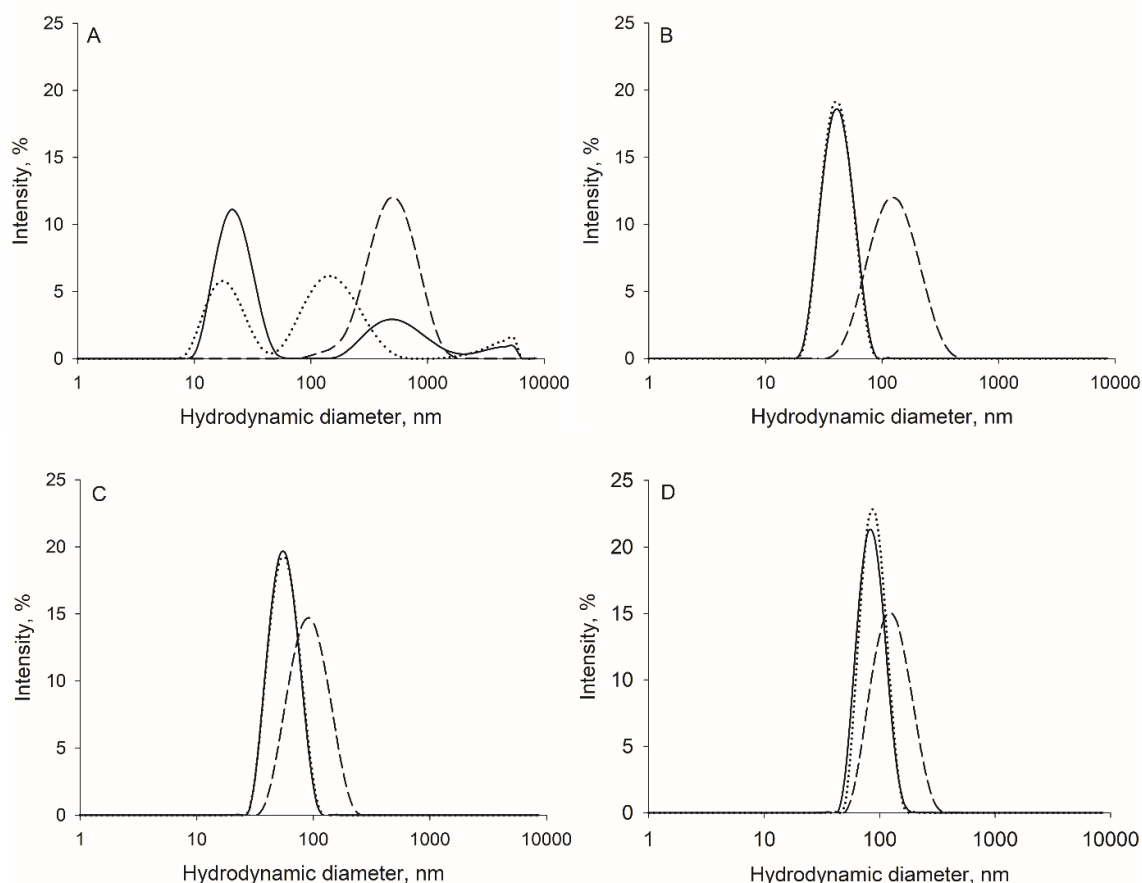


Figure 1. Aggregation pattern of platinum nanoparticles (PtNPs) with idarubicin (IDA). (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs. Solid line – nanoparticles alone, dotted line – nanoparticles with the addition of 5 μ M IDA, dashed line – nanoparticles with the addition of 50 μ M IDA.

Furthermore, we evaluated the formation of PtNPs-IDA aggregates using atomic force microscopy (AFM) (**Figure 2**). All tested PtNPs sizes were visualised as single dots or very small clusters. With the addition of IDA, we observed formation of aggregates in all the samples, and the mean registered size increased 225, 46, 21 and 20-fold for 5 nm, 30 nm, 50 nm and 70 nm PtNPs, respectively (**Table S2**). The observed aggregates were of various sizes and shapes, thus once more reminding of the possibility that IDA induces the formation of PtNP-IDA heteroaggregates, as well as PtNPs homoaggregates.

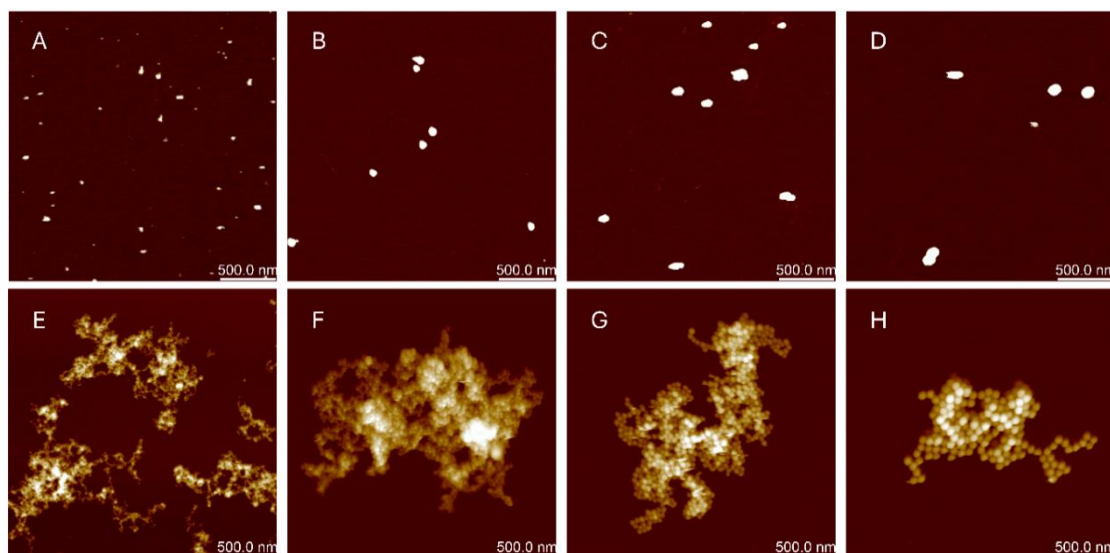


Figure 2. Atomic force microscopy (AFM) pictures of platinum nanoparticles (PtNPs) alone and in combination with idarubicin (IDA). (A – D) 5, 30, 50 and 70 nm PtNPs, respectively; (E – H) 5 nm, 30 nm, 50 nm and 70 nm PtNPs with IDA, respectively.

Overall, the AFM and DLS are consistent with our previous studies, where PtNPs were found to aggregate with doxorubicin (DOX) [38], epirubicin (EPI) [39] and daunorubicin (DAU) [40], as well as with other studies on PtNPs-DOX [41] and AuNPs-DOX [42].

3.2 PtNPs interact with IDA

To further establish the nature of interactions between PtNPs and IDA, we utilised IDAs' fluorescence capabilities and performed fluorescence spectroscopy (**Figure 3**). All tested sizes of PtNPs significantly quenched the fluorescence of IDA. On the contrary, titration with identical volumes of appropriate sodium citrate solution didn't show corresponding effects. The full fluorescence spectra are available in the supplementary information (**Figure S1**).

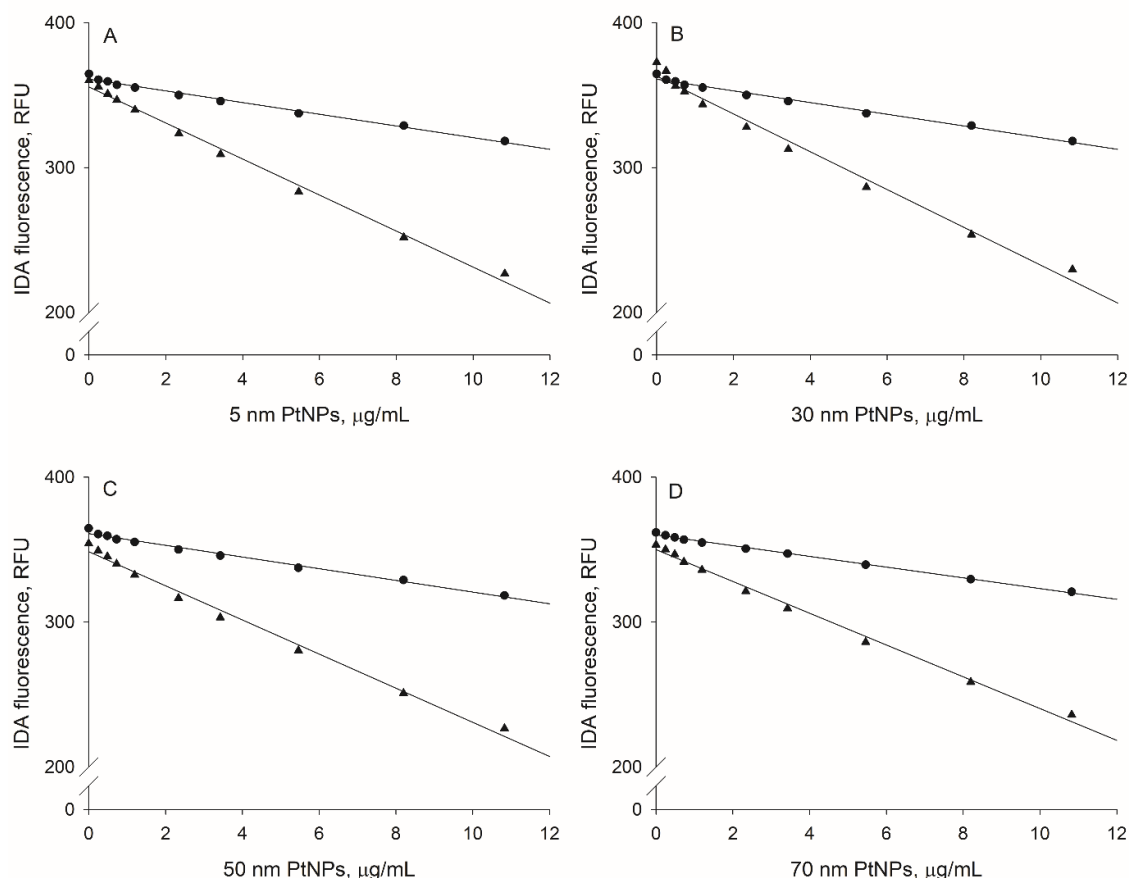


Figure 3. Platinum nanoparticles (PtNPs) influence on idarubicin's (IDA) fluorescence at 545 nm. (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs. Dots – negative control (IDA with sodium citrate), triangles – IDA with PtNPs.

Metallic nanoparticles are known for, among others, metal enhanced fluorescence (MEF) effect, which can increase the fluorescence signal of a fluorophore if the two are at an optimal distance. However, at distances lower than 5 nm, the metallic nanoparticles can quench the fluorescence, presumably by the surface plasmon resonance (SPR) effect [43,44]. The observed quenching therefore indicates close-proximity interactions between IDA molecules and the PtNPs surface. Analogous effects were observed for PtNPs-DOX [38,45], PtNPs-EPI [39] and PtNPs-DAU [40] analyses, as well as in other studies on gold, silver and iron nanoparticles [46–48].

Next, we performed Fourier transform infrared spectroscopy (FTIR) to further evaluate the interactions between PtNPs and IDA (**Figure 4**). The analysis allowed to presume that IDA may interact with PtNPs primarily through the R-C=O (ketone) moiety and CH_n groups. In all analysed spectra, weak absorption features were observed in the 2500-2600 cm⁻¹ region. These bands have been previously attributed to CH_n groups [49], or to

hydrogen-linked bond stretching of alcohols, phenols, carbohydrates, and carboxylic acids. They are commonly interpreted as overtone and combination bands involving deformation modes of CH₃ and CH₂ groups located in the 1400-1300 cm⁻¹ range (1402 and 1371 cm⁻¹ for nanosystems, and 1402 and 1375 cm⁻¹ for free amorphous IDA). Although such weak features are present in many hydrocarbons, their precise energy positions are strongly influenced by the local bonding environment. Furthermore, additional bands were detected at 2707, 2663, 2611 and 2536 cm⁻¹, whereas in the amorphous IDA only a band at 2542 cm⁻¹ was registered. The band at 2546 cm⁻¹ can be attributed to the anthraquinone ring system with a tetracyclic aromatic structure, characteristic for anthracyclines, arising from methyl groups substituted at different positions on the double ring. The feature at 2707 cm⁻¹ in the crystalline IDA may be associated with CH₃ overtone or combination bands, similarly to those observed at 2790, 2663 and 2611 cm⁻¹. Of that, the reduction in band intensity in the amorphous state of IDA, accompanied by a shift from 2542 to 2546 cm⁻¹ in the samples with PtNPs may indicate modifications within the substituted anthraquinone system and suggest the presence of possible intermolecular interactions between IDA and PtNPs [50].

The absorption band corresponding to the C=O stretching vibration of IDA was observed in the 1750-1550 cm⁻¹ region. To enhance band resolution, a Savitzky–Golay second-derivative treatment was applied, as the use of second derivatives of the spectra can be a fruitful approach for the purpose of revealing unresolved bands, particularly in the context of complex spectra [51,52]. The processed spectra revealed distinct differences in vibrational energies, with components identified at 1714, 1622, and 1586 cm⁻¹. The second-derivative analysis also demonstrated variations in the -C=C- stretching region, with bands observed at 1562 and 1531 cm⁻¹, particularly pronounced for samples containing 50 and 70 nm nanoparticles. Additional spectral changes were detected near 1493 cm⁻¹, where shifts to 1493 and 1483 cm⁻¹ were observed, typically associated with aromatic -C=C- vibrations. All observed alterations are presented in the supplementary information (**Table S3**).

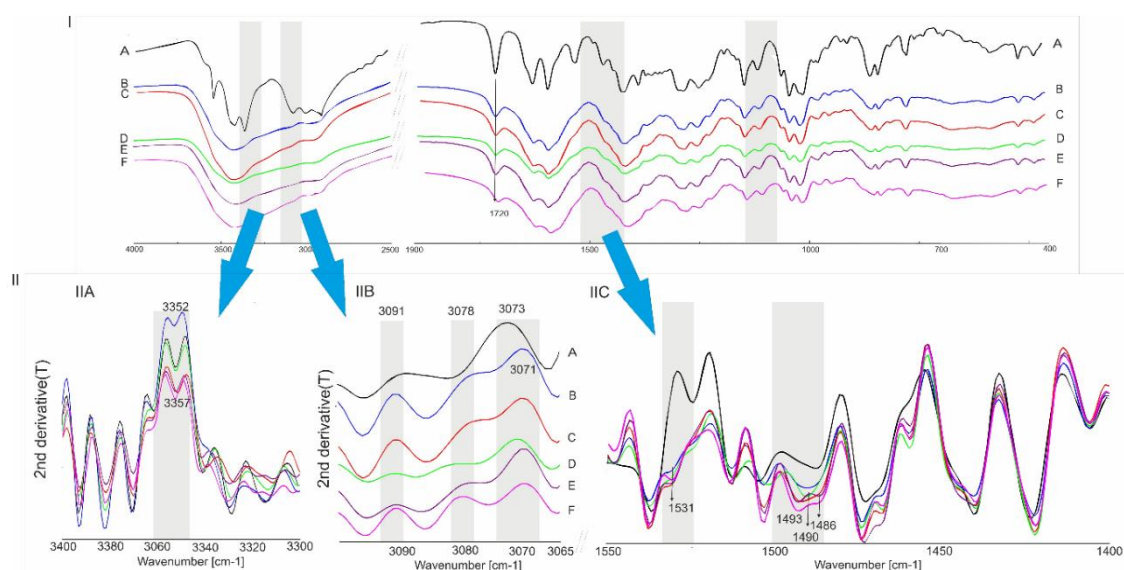


Figure 4. Fourier transform infrared spectroscopy (FTIR) spectra of the investigated complexes of idarubicin (IDA) and platinum nanoparticles (PtNPs) I) at regions 4000-2500 cm^{-1} and 1900-400 cm^{-1} , and II) their second derivatives. (A) black – IDA, (B) blue – IDA lyophilised from sodium citrate, (C) red – 5 nm PtNPs with IDA, (D) green – 30 nm PtNPs with IDA, (E) violet – 50 nm PtNPs with IDA, (F) pink – 70 nm PtNPs with IDA.

In addition to FTIR, we performed near-infrared spectroscopy (NIR) (**Figure 5**). Herein, we observed tentative interactions majorly in the region of O-H assigned to molecular water and N-H combination from the amine group at 5300-4900 cm^{-1} range. Another difference was detected in the intensity of the band corresponding to C-O and C-H vibrations (including aromatic C-H and CH_2 groups), with the highest intensities observed for samples containing 50 and 70 nm PtNPs, particularly at 4358 cm^{-1} and 5149 cm^{-1} . The detailed data is available in the supplementary information (**Table S4**).

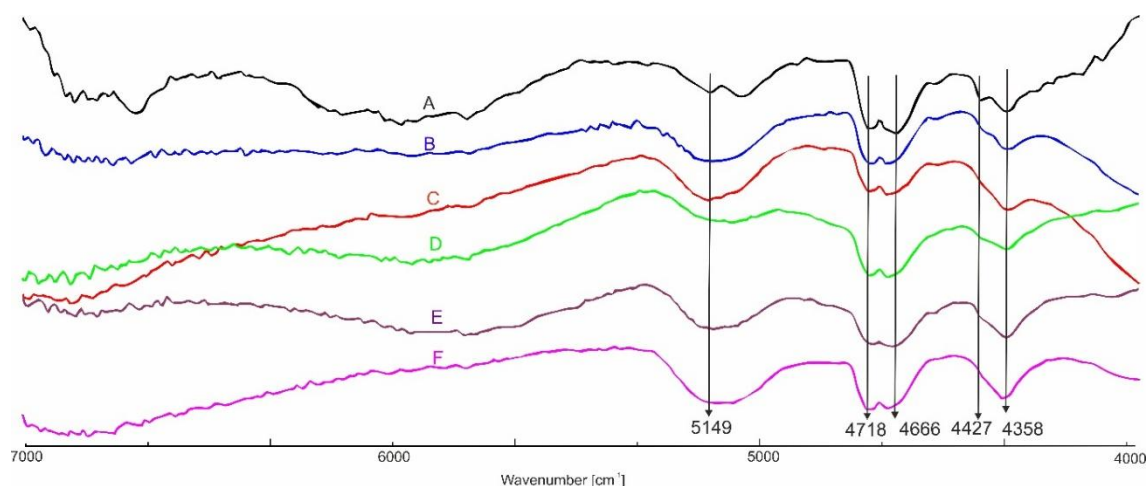


Figure 5. Near Infrared Spectroscopy (NIR) spectra of the investigated complexes of idarubicin (IDA) and platinum nanoparticles (PtNPs) in the wavenumber region 7000 - 4000 cm^{-1} . (A) black – IDA, (B) blue – IDA lyophilised from sodium citrate, (C) red - 5 nm PtNPs with IDA, (D) green - 30 nm PtNPs with IDA, (E) violet - 50 nm PtNPs with IDA, (F) pink - 70 nm PtNPs with IDA.

Overall, these findings indicate some signs of possible interactions between PtNPs and IDA, thus providing additional important recognition of the aggregation and fluorescence quenching results. In literature, it was also reported that PtNPs interact with other compounds through N-H groups [53] and O-H groups [21,41], most likely via hydrogen bonding, as well as through alterations in carbonyl bonds [52]. Herein, spectral changes indicating these mechanisms were also registered, thus further confirming the data from previous studies.

3.3 PtNPs-IDA interactions are endothermic

In the next step, we investigated the thermodynamic characteristics of the observed PtNPs-IDA complexes formation. Isothermal titration calorimetry (ITC) revealed that the binding process is endothermic (**Figure 6**), with the enthalpy ranging from ~ 1 to almost 4 kcal/mol. Interestingly, we did not observe a notable change in the case of sizes 5 to 50 nm, however, 70 nm PtNPs yielded the highest registered total heat. The raw thermal changes due to interactions between PtNPs and IDA are available in supplementary information (**Figure S2**). Overall, it could be suggested that, based on the positive enthalpy values, the formation of the complexes might be entropy-driven processes. In our previous studies, we observed that the enthalpy values increased with the diameter of

platinum nanoparticles when combined with other anthracyclines. However, in the case of DOX and EPI, the values registered for 5-50 nm PtNPs were negative, exhibiting an exothermic reaction [38,39]. Furthermore, negative enthalpy values were also observed for PtNPs in combination with acridine mutagen ICR-191 [54], as well as for naked AgNPs [55]. This inconsistency in the thermal behaviour between metallic nanoparticles and various compounds suggests that when characterising specific interactions of metallic nanoparticles with a chosen drug, one should not extrapolate the results towards other systems, even those that appear closely related.

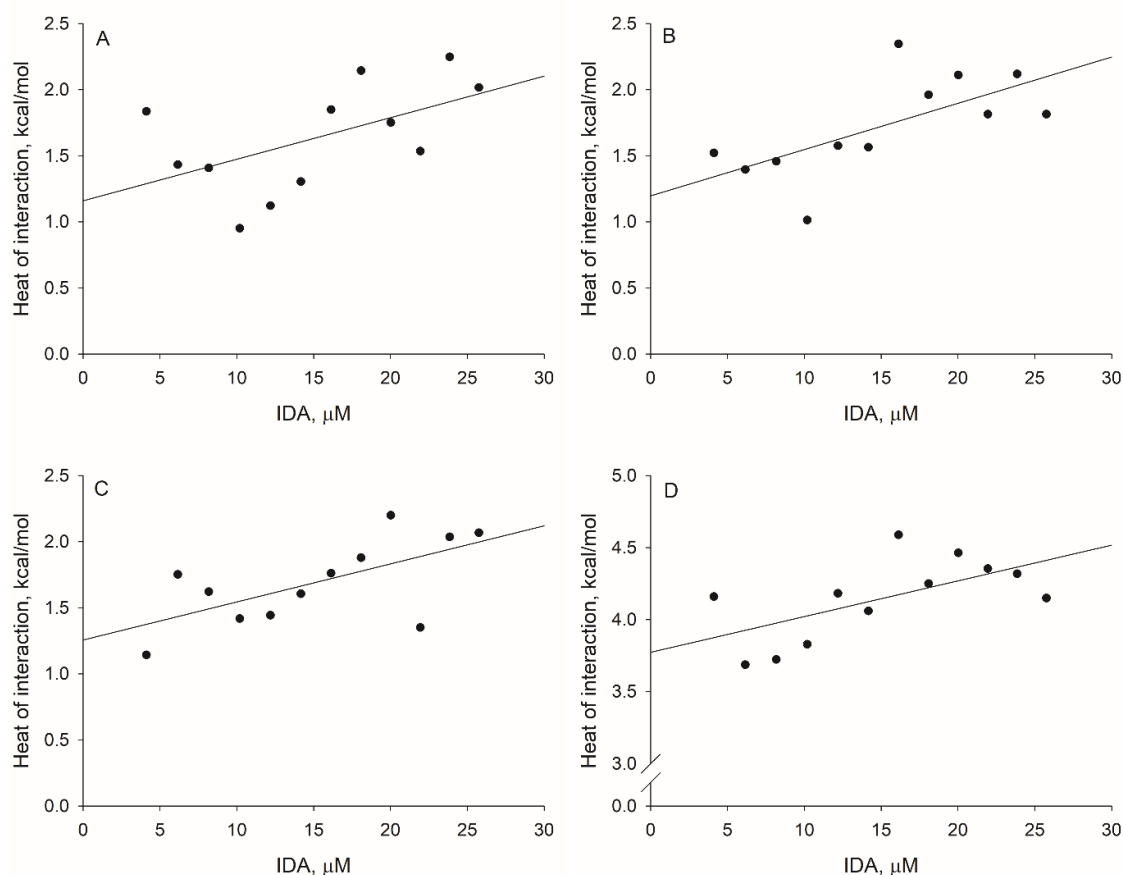


Figure 6. Isothermal titration calorimetry analysis for platinum nanoparticles (PtNPs) interactions with idarubicin (IDA). (A) 5 nm PtNPs ($\Delta H = 1.16 \pm 0.25$ kcal/mol), (B) 30 nm PtNPs ($\Delta H = 1.2 \pm 0.21$ kcal/mol), (C) 50 nm PtNPs ($\Delta H = 1.26 \pm 0.18$ kcal/mol), (D) 70 nm PtNPs ($\Delta H = 3.77 \pm 0.1$ kcal/mol). IDA concentration range 2 – 29 μM , initial PtNPs concentration 0.05 mg/mL.

Molecular interactions between IDA and PtNPs may influence the chemical structure and association state of the compound, which can manifest as shifts in endothermic and exothermic transitions [56,57]. Therefore, we decided to utilise differential scanning

calorimetry (DSC) to establish how PtNPs affect the melting point of IDA (**Figure 7**). From DSC thermograms, it can be seen that in the range 93-113 °C an endothermic peak arises, which can be attributed to moisture loss or possible polymorphic transitions at lower temperatures. The free IDA is characterised by a melting peak at T=197 °C. However, when IDA was prepared as a lyophilised sample from sodium citrate solution, this peak shifted to T=185 °C. When IDA was combined with PtNPs, the melting point shifted significantly only in the case of 70 nm PtNPs, with the changes in the remaining PtNPs sizes negligible. The exact data can be found in the supplementary information (**Table S5**).

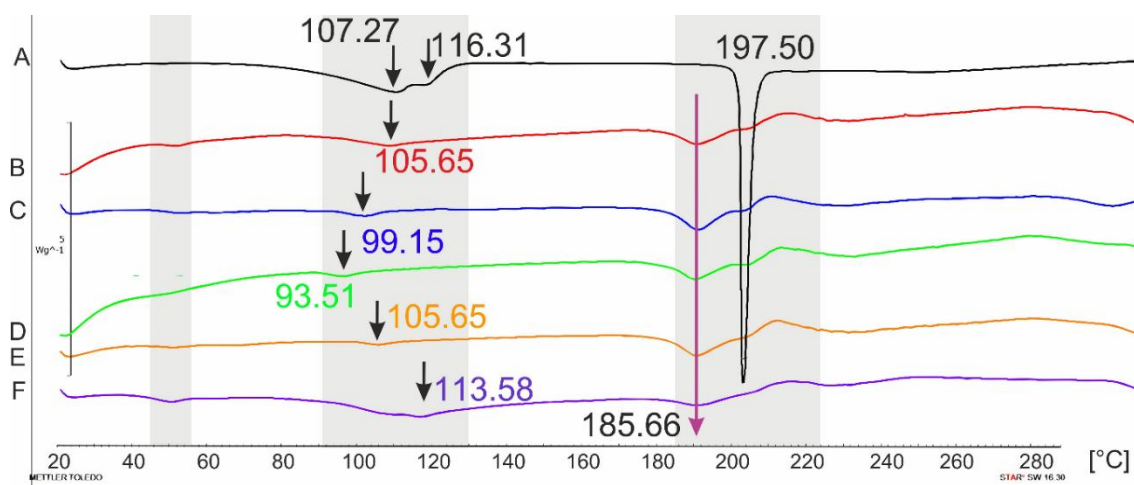


Figure 7. Differential scanning calorimetry (DSC) curves of platinum nanoparticles (PtNPs) and idarubicin (IDA). (A) black – IDA, (B) red – IDA lyophilized from sodium citrate, (C) blue – 5 nm PtNPs with IDA, (D) green – 30 nm PtNPs with IDA, (E) orange – 50 nm PtNPs with IDA, (F) violet – 70 nm PtNPs with IDA.

Overall, the IDA–PtNP complexes demonstrate similar thermal profiles; however, the system containing 70 nm nanoparticles shows transitions at slightly higher temperatures, indicating a more pronounced alteration of the thermal properties compared to IDA without nanoparticles. A possible explanation to observed variation in thermal properties could be that with the increase of nanoparticle size from 5 to 70 nm, the surface-area-to-volume ratio decreases 14 times, which results in a reduced surface curvature, thus enabling stronger or more extended adsorption of IDA onto the nanoparticle surface [58,59].

3.4 PtNPs influence IDA's biological activity

Having shown the existence of PtNPs-IDA interactions, we decided to investigate their influence on the biological activity of IDA. Firstly, we employed the Ames mutagenicity plate assay to see if the addition of PtNPs changes the mutagenicity of IDA towards *Salmonella enterica* serovar Typhimurium TA98 strain (**Figure 8**). The observed results show a significant decrease of IDAs mutagenicity upon addition of all tested concentrations of PtNPs, however, as the concentration of PtNPs increased, the mutagenicity gradually returned to the baseline of the positive control (IDA alone). Importantly, PtNPs alone did not induce any mutagenic effect on their own (**Figure S3**).

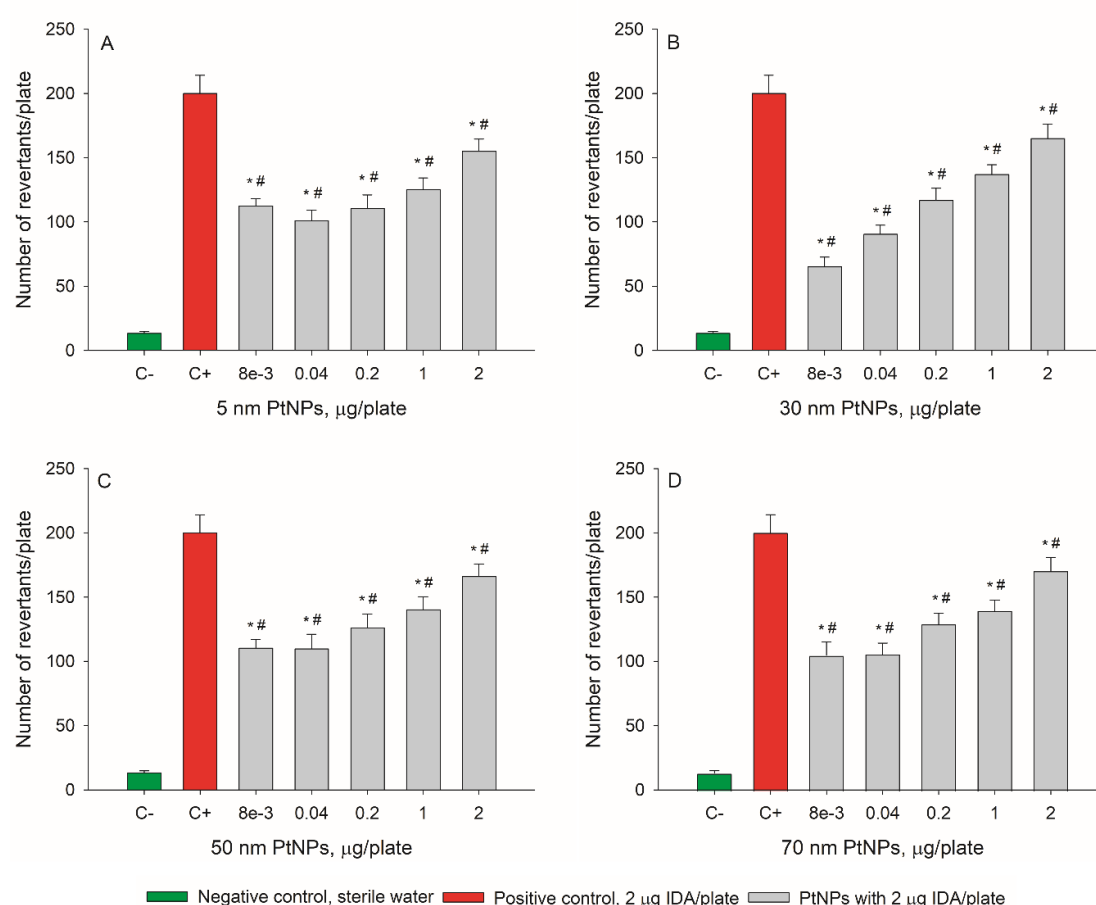


Figure 8. Influence of platinum nanoparticles (PtNPs) on mutagenicity of idarubicin (IDA) in *Salmonella enterica* serovar Typhimurium TA98 strain. (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs. C- negative control (sterile water), C+ positive control (2 µg IDA/plate). Results are reported as the average number of revertants per plate $\pm$ SD. *- significant difference from the negative control, # - significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

The observed effect stands in contrast to the results obtained for PtNPs interactions with DOX [38], EPI [39] or DAU [40], where the increase in the concentration of PtNPs resulted in lowering the mutagenicity of the API. This phenomenon can be attributed to numerous mechanisms. First of all, in contrast to the aforementioned anthracyclines, IDA lacks the methoxy group at the R1 position, thus making it the most lipophilic of all these analogues [23]. This feature promotes more efficient penetration of cell membranes by IDA and results in more efficient ROS generation due to higher intracellular accumulation. Furthermore, among the consequences of increased lipophilicity is an increase in the affinity towards hydrophobic surfaces, one that PtNPs present. Taking this into consideration, we hypothesise that at lower concentrations of PtNPs, the API-to-nanoparticle ratio is high. As a result, PtNPs become heavily coated with IDA molecules, which reduces the drug's bioavailability and leads to a lower number of revertant colonies. However, with a rise in PtNPs concentration, the total surface area increases, accompanied by a lower API-to-nanoparticle ratio, which may result in an adsorption-desorption equilibrium change [60]. Higher concentration of PtNPs may result in an increased homoaggregation of the nanoparticles, as well as the ratio of PtNPs and IDA molecules in heteroaggregates. As a consequence, in the lowest PtNPs concentration, the concentration of free, biologically active form of IDA increases, leading to the restoration of IDA mutagenic activity, as evidenced by the increase of revertant colonies count. However, as the concentration of PtNPs increases, the size of PtNPs aggregates becomes greater, which in turn may reduce PtNPs availability for bacteria, thus, preventing them from exhibiting their protective effects. Nevertheless, a fraction of IDA may remain aggregated or clathrated with the PtNPs, therefore, its mutagenic activity, even in the highest PtNPs concentration, remains below the level of the positive control.

Finally, we decided to test the influence of PtNPs on the cytotoxicity of IDA on two breast cancer cell lines, SK-BR-3 and MCF-7, differing in the expressed receptors, with SK-BR-3 being more aggressive (HER2+)[61] (**Figure 9**). In the case of the former, we found that at 20 and 30 μM of IDA, the majority of PtNPs sizes increase the cytotoxicity of the API, irrespective of the administered concentration. However, at 40 and 50 μM of IDA, we either observed no significant effects or a decrease in the PtNPs-IDA toxicity toward the cells' viability. In the case of MCF-7 cells, 0.2 μg /well of PtNPs increased the cytotoxicity of all concentrations of IDA, excluding 70 nm PtNPs (**Figure 9**, B). However, at 1 μg /well, the effects were less pronounced, and with 5 μg /well, we observed

similar effects as for SK-BR-3; with the increase in IDA's concentration, the viability of the cells increased (**Figure 9, D, F**). Importantly, almost all PtNPs exhibit anticancer activity of their own towards both cell lines in a dose-dependent manner (**Figure S4**).

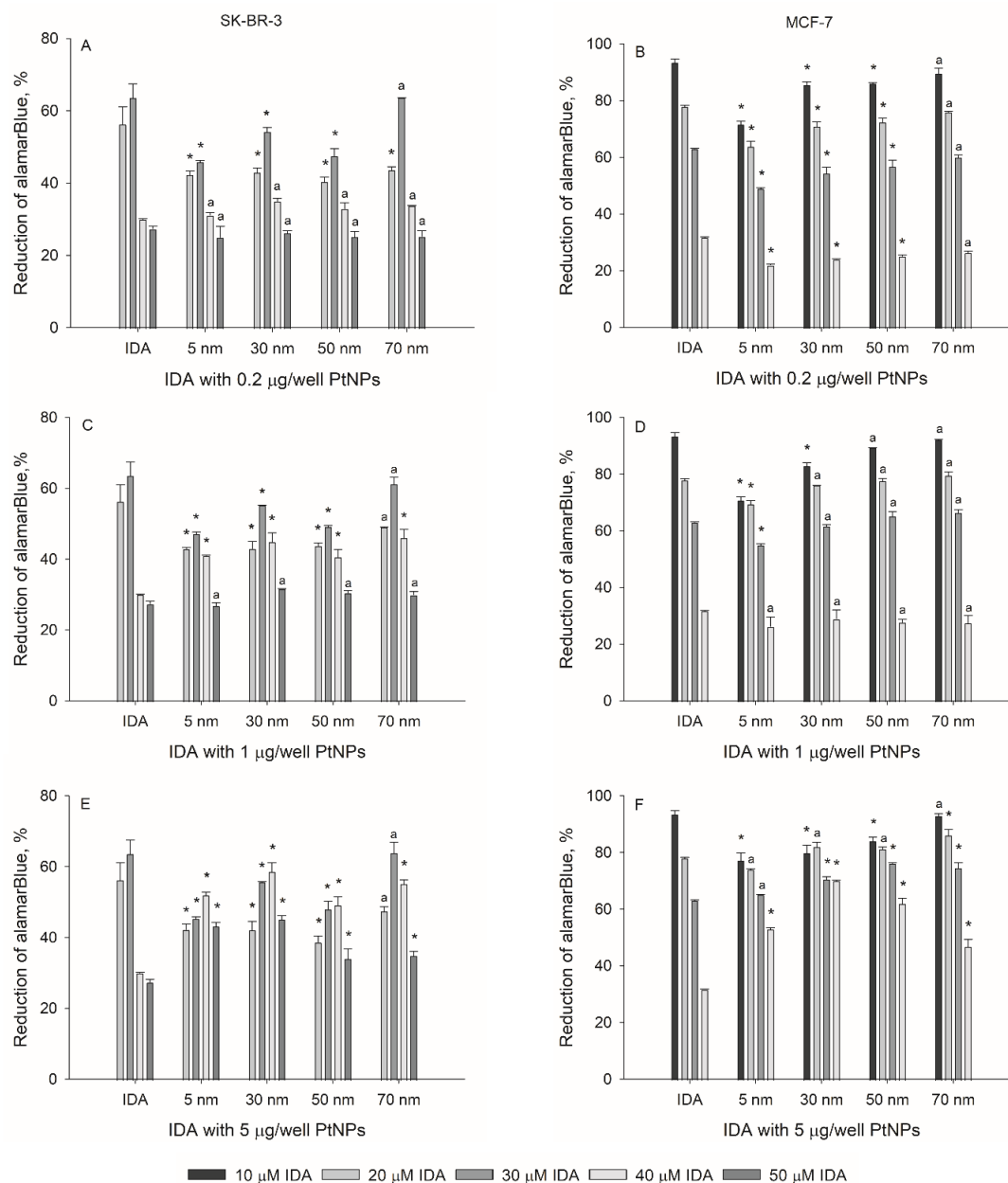


Figure 9. Influence of platinum nanoparticles (PtNPs) on cytotoxicity of idarubicin (IDA) on SK-BR-3 and MCF-7 cell lines. (A, C, E) influence of 5 – 70 nm PtNPs on IDA's cytotoxicity at 0.2, 1 and 5 μg/well on SK-BR-3 cell line; (B, D, F) influence of 5 – 70 nm PtNPs on IDA's cytotoxicity at 0.2, 1 and 5 μg/well on MCF-7 cell line. Results are reported as the mean reduction of alamarBlue in regard to the cells treated with the same concentration of IDA alone ± standard deviation (SD). a – no significant difference, * - significant difference ($p < \alpha$, $\alpha = 0.05$).

The findings are intriguing because as mentioned before, platinum nanoparticles alone were toxic to the cancer cells, yet at lower concentrations of the API in each cell line we tended to observe an increased toxicity, but with the increase in the dosage of both, the effects began to diminish, and we observed an increased viability of cells, especially when combined with 5 $\mu\text{g}/\text{well}$ PtNPs. While studies on IDA alone have demonstrated its efficacy on both of our selected cell lines [62,63], there is limited data available on the combination of IDA with metallic nanoparticles. In our previous studies on anthracyclines and platinum nanoparticles, we observed that PtNPs at similar tested sizes and concentrations had a protective effect towards the non-cancerous HaCaT cell line, and an improved cytotoxicity towards the cancerous MelJuSo cell line at the highest concentration of each nanoparticle [38]. Contrasting to that, with daunorubicin, we did not register major changes in the cytotoxicity of PtNPs-DAU combinations [40], yet with epirubicin, we have shown that at 5 $\mu\text{g}/\text{well}$ all the PtNPs increase the toxicity of the compound towards the MelJuSo cell line, at the same time exhibiting protective effects on HaCaT cells [39]. However, IDA did not yield the same results, and there are several possible explanations for this discrepancy. Firstly, as mentioned in the Ames test discussion, it is worth reminding that of these anthracyclines, IDA is the most lipophilic, therefore its interactions with PtNPs may present a different profile than what we have seen before with other antibiotics from this group. Secondly, PtNPs can act as both pro-oxidant and antioxidant agents [64,65]. When administered alone, they exhibited anticancer activity, and when combined with minor concentrations of IDA they increased its efficacy, most likely by enhancing the redox cycling. However, as the concentration of IDA and PtNPs rose, the effects started to diminish, and the viability of cells gradually increased. This could be partly explained by an increase in aggregation, as evidenced by DLS and AFM. Our earlier speculations about the possible induction of PtNPs homoaggregation by IDA molecules may also be partially useful to elucidate such protective effects against cells. Such complexes are likely to be of greater size than the capacity of membrane pores, thus not allowing these complexes to enter the intracellular environment reducing the PtNPs intracellular concentration and, in consequence, cytotoxic potential. One can imagine that such induced homoaggregates and their conglomerates may also clathrate IDA molecules, radically decreasing their concentration in free, biologically active form. Therefore, the concentration of IDA inside cancer cells will be significantly reduced, which corresponds to decreases in the treatment efficacy, leading to observed cancer cells' increased survivability in greater PtNPs concentrations.

The proposed mechanism tends to explain the seemingly contradictory results of the Ames test and cytotoxicity assays. The complexity of the interaction pattern, dependent on the dosage of the two, shows that the combination of PtNPs and IDA may be useful, yet there is a thin line between synergy and antagonism. In terms of differences in results between PtNPs and other anthracyclines, it is worth noting that DOX and EPI were tested on HaCaT and MelJuSo cells, thus the mechanisms of damage repair and the susceptibility towards specific API treatments can vary in comparison to breast cells.

Overall, the results of our study indicate that platinum nanoparticles interact with idarubicin, an anticancer antibiotic drug. What is more, the interaction can alter the biological activity of this API, both in its mutagenicity towards bacteria and in the cytotoxicity against SK-BR-3 and MCF-7 breast cancer cell lines. However, the improvement of the cytotoxicity was visible only under certain circumstances, and thus the model requires further biological analysis, with more emphasis on the *in vivo* studies for possible translational results. Nevertheless, more effect was observed on the SK-BR-3 cell line than on the MCF-7, which can be attributed to the higher sensitivity of this cell line, most likely due to the overexpression of the HER2 receptor. Lastly, the increase in concentration of PtNPs did not always result in greater cytotoxicity, thus reminding us that “The more the better” is not always correct.

5. Conclusions

Summing up, platinum nanoparticles (PtNPs), of distinct sizes 5, 30, 50 and 70 nm, have been shown to interact with idarubicin (IDA). We observed the formation of PtNPs-IDA aggregates providing significant insight on the short interactions distance and involvement of the amine groups in the bonds formation. Moreover our results indicate endothermic nature of interactions, while intensity of the changes is most pronounced in case of 70 nm PtNPs. Furthermore, all PtNPs sizes altered the mutagenic potential of IDA in a dose-dependent manner. Cytotoxicity analyses on cancerous MCF-7 and SK-BR-3 cell lines revealed that PtNPs alone exhibit significant cytotoxicity in majority, however, when combined with IDA, they improved its efficacy only at lower concentrations of the API, whereas as the concentration increased, the viability of the cells rose, especially with 5 µg/well of PtNPs. While the study was performed on two cell lines, a broadened range of *in vitro*, with further *in vivo* studies is needed for better overall conclusions of the

impact of PtNPs on IDAs cytotoxicity. Nevertheless, these results show that PtNPs interact with IDA and influence the activity of the API, making it more efficient at particular concentrations.

6. Acknowledgements

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7. Data availability

Data will be available upon request.

8. Declaration of competing interest

None.

9. Author contribution statement

M.Z.: Project administration, Investigation, Methodology, Visualization, Formal analysis, Writing – original draft, review & editing. P.B.: Investigation, Writing – review & editing. K.G.: Investigation. A.Y.: Investigation. M.J.: Investigation, Visualization, Writing – review & editing. D.W.: Investigation, Writing – review & editing. K.B.: Investigation. K.G.: Resources, Writing – review & editing. G.G.: Validation, Writing – review & editing J.P.: Conceptualization, Resources, Supervision, Writing – review & editing

All the authors have approved the final manuscript.

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Supplementary materials

Table S1. Characteristics of platinum nanoparticles (PtNPs) provided by the manufacturer.

Parameter	5 nm PtNPs	30 nm PtNPs	50 nm PtNPs	70 nm PtNPs
Hydrodynamic diameter, nm	Not reported	39	65	78
Zeta potential, mV	Not reported	-44	-55	-48
Platinum purity, %	99.99	99.99	99.99	99.99
Particle surface	Bare (Citrate)	Bare (Citrate)	Bare (Citrate)	Bare (Citrate)

Table S2. Data for DLS analysis of platinum nanoparticles (PtNPs) alone and in combination with idarubicin (IDA). SD – standard deviation, Pdl – polydispersity index.

Sample		Pdl $\pm$ SD	Hydrodynamic diameter, nm $\pm$ SD	Area, % $\pm$ SD
5 nm PtNPs			22.96 $\pm$ 1.31	67.03 $\pm$ 3.78
	alone	0.5 $\pm$ 0.06	615.9 $\pm$ 73.4	27.7 $\pm$ 1.91
			3897.5 $\pm$ 284.96	5.23 $\pm$ 4.82
			171.7 $\pm$ 19.9	55.93 $\pm$ 2.13
	+ 5 μ M IDA	0.74 $\pm$ 0.01	19.62 $\pm$ 1.7	36.7 $\pm$ 1.45
			4074 $\pm$ 539.75	7.33 $\pm$ 2.76
30 nm PtNPs	+ 50 μ M IDA	0.24 $\pm$ 0.03	539.73 $\pm$ 16.05	100
	alone	0.07 $\pm$ 0.01	42.93 $\pm$ 0.36	100
	+ 5 μ M IDA	0.07 $\pm$ 0.02	42.58 $\pm$ 0.43	100
50 nm PtNPs	+ 50 μ M IDA	0.15 $\pm$ 0.01	138.1 $\pm$ 34.45	100
	alone	0.05 $\pm$ 0.01	57.31 $\pm$ 1.35	100
	+ 5 μ M IDA	0.06 $\pm$ 0.01	57.8 $\pm$ 0.44	100
70 nm PtNPs	+ 50 μ M IDA	0.13 $\pm$ 0.01	96.76 $\pm$ 0.56	100
	alone	0.06 $\pm$ 0.03	87.4 $\pm$ 3.04	100
	+ 5 μ M IDA	0.02 $\pm$ 0.01	90.24 $\pm$ 3.05	100
	+ 50 μ M IDA	0.11 $\pm$ 0.02	133.97 $\pm$ 4.6	100

Table S3. Data for the AFM analysis of platinum nanoparticles (PtNPs) alone and in combination with idarubicin (IDA). SD – standard deviation.

	5 nm PtNPs		30 nm PtNPs		50 nm PtNPS		70 nm PtNPs	
	Alone	With IDA	Alone	With IDA	Alone	With IDA	Alone	With IDA
Mean size (nm)	5.4	1217.6	41.8	1927.8	66.6	1435.2	80.8	1600.3
SD	0.9	165.9	10.8	481.2	28.3	680.6	15.6	187.8

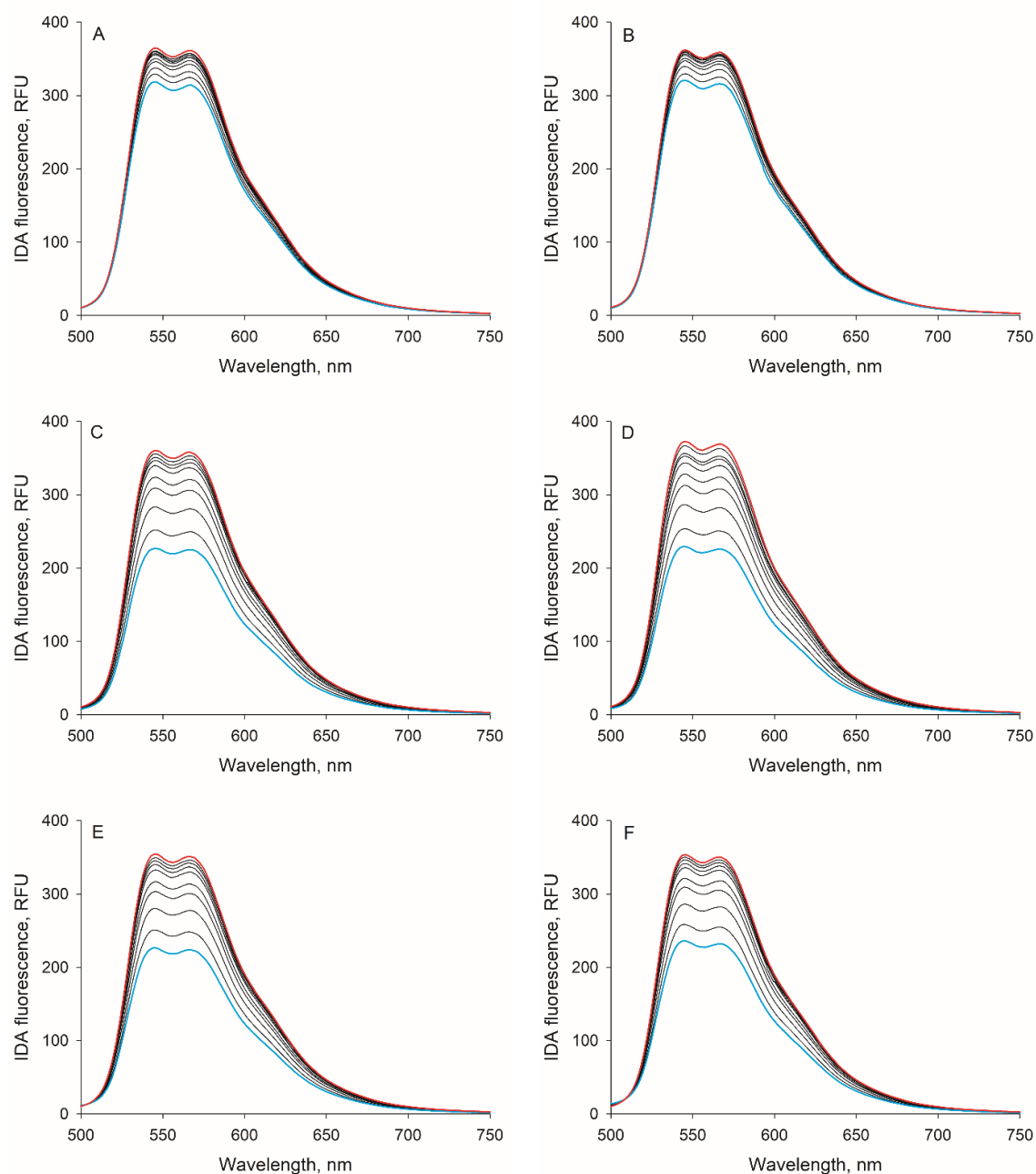


Figure S1. Changes in the fluorescence of idarubicin (IDA) upon titration with various sizes of platinum nanoparticles (PtNPs) or appropriate sodium citrate solution (buffer). (A, B) – 2 mM or 4 mM sodium citrate, respectively, (C – F) – 5, 30, 50 and 70 nm PtNPs, respectively. Red line – IDA alone, blue – IDA with the highest titrated volume of PtNPs or buffer. IDA concentration range: 37.2 – 29.3 μM , PtNPs concentration range 0 – 10.62 $\mu\text{g/mL}$.

Table S4. Fourier transform infrared spectroscopic (FTIR) characterization of idarubicin (IDA) alone and in combination with various sizes of platinum nanoparticles (PtNPs). The regions of significant changes in IDA's band were marked in red.

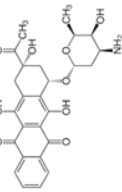
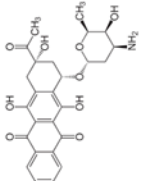
Chemical Bond	IDA 	IDA			
		5 nm PtNPs with IDA	30 nm PtNPs with IDA	50 nm PtNPs with IDA	70 nm PtNPs with IDA
		Complex 1	Complex 2	Complex 3	Complex 4
		Lyophilized from sodium citrate solution			
O-H intermolecular bonded	3578	-	-	-	-
N-H stretching primary amine	3535	3600 – 3400	3535	3535	3535
O-H intermolecular bonded	3436, 3410, 3352	3417, 3350, 3436, 3410, 3357	3436, 3410, 3357	3436, 3410, 3357	3436, 3410, 3352
C-H (CH ₂) stretching alkane	3072	3066	3066	3066	3066
O-H stretching alcohol intramolecular bonded	2992, 2946, 2924, 2904	2979, 2924	2979, 2924	2979, 2924	2979, 2924
hydrogen-bonded dimers of ketone group	2707, 2663, 2611, 2546	2542	2546	2546	2546
Asymmetric acid ketone -C=O	1713	1713	1713	1713	1713
(benzoilo)-C=O amide	1623	1621	1621	1621	1621
-C=C-	1587, 1521	1587	1587, 1562	1587, 1562	1587, 1562
C-H (CH ₃)	1454, 1431, 1352, 1338, 1324	1454, 1349, 1321	1403 – 1380	1403 – 1380	1403 – 1380
O-H bending alcohol	1408, 1371	1402, 1375	1394	1398	1401
-C-O-C-	1263	1264	1402, 1371	1402, 1371	1402, 1371
C-O stretching ester or	1225, 1205,	1231, 1204	1231, 1204	1231, 1204	1231, 1204
-C-N- amine	1165, 1147, 1133	1165, 1133	1165, 1133	1165, 1133	1165, 1133
O-H alcohol	1117	1117	1117	1117	1117
C-O stretching	1086	1082	1082	1080	1078, 1063
-C-O-C-	1028, 1009, 977	1030, 1010, 986	1030, 1010, 986	1030, 1010, 986	1030, 1010, 986
C=C bending alkene substituted	950, 941	941	941	941	941
strong C-H bending	918, 886, 872, 853, 816,	916, 888, -872, 815	916, 888, -, 872, 815	916, 888, -, 872, 815	916, 888, -, 872, 815
C=C bending	798, 769, 729, 710	797, 767, 731	797, 767, 731	797, 767, 731	797, 767, 731

Table S5. Near infrared spectroscopic (NIR) characterization of idarubicin (IDA) and its complexes with various sizes of platinum nanoparticles (PtNPs). The regions of significant changes in IDA' s band were marked in red.

Chemical Bond	IDA 	IDA				
		Lyophilized from sodium citrate solution	5 nm PtNPs with IDA	30 nm PtNPs with IDA	50 nm PtNPs with IDA	70 nm PtNPs with IDA
O–H alcohol (RO–H), N–H amine	6200 – 5500	6160 – 5340	6300 – 5300	6300 – 5300	6300 – 5300	–
O–H assigned to molecular water,	5300 – 4900	5300 – 4900	5300 – 4900	5300 – 4900	5300 – 4900	5300 – 4900
N–H combination, amine C=O and C–O stretching, N–H/C–N, C–H aromatic C–H (aryl)	5149	5149	5092	5144	5146	5136
O–H/C–O, C–H starch (.C–H and CH ₂), C–H (.C–H bending)	4718, 4666	4718, 4666	4718, 4666	4718, 4666	4718, 4666	4718, 4666
	4358	4358	4358	4358	4358	4358

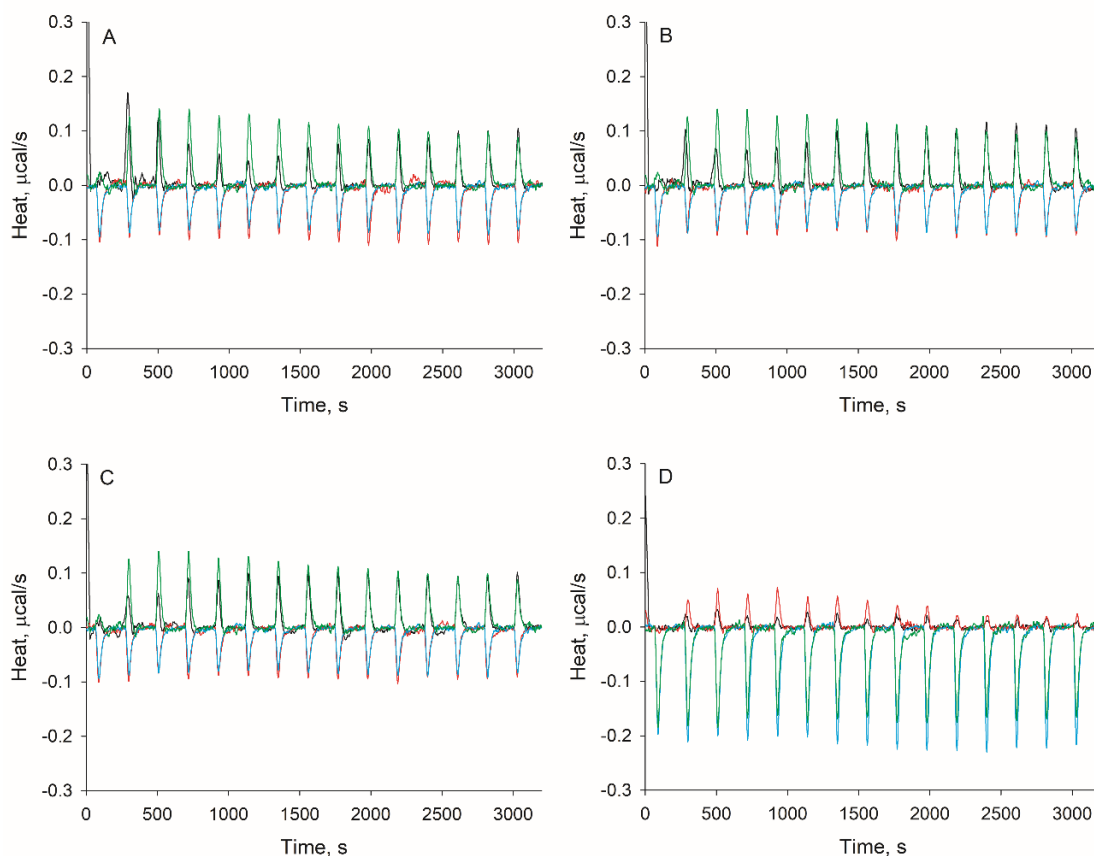


Figure S2. Thermograms showing microcalorimetric titrations of platinum nanoparticles (PtNPs) with idarubicin (IDA). (A) 5 nm PtNPs, (B) 30 nm PtNPs, (C) 50 nm PtNPs, (D) 70 nm PtNPs. Black – PtNPs with IDA, red – water with PtNPs, cyan – water with appropriate sodium citrate solution, green – IDA with appropriate sodium citrate solution.

Table S6. DSC data for idarubicin (IDA) samples combined with platinum nanoparticles (PtNPs) of different sizes. Numbers marked in red are of significant change with respect to lyophilised IDA.

	IDA				
	lyophilised from sodium citrate solution	IDA with 5 nm PtNPs	IDA with 30 nm PtNPs	IDA with 50 nm PtNPs	IDA with 70 nm PtNPs
Water loss T [°C]	105.65	99.15	93.51	102.80	113.58
ΔH [J/g]	-17.88	-4.86	-3.01	-3.35	-33.37
T_m [°C]	185.66	185.95	185.00	185.43	186.29
ΔH [J/g]	-15.47	-19.48	-18.39	-20.59	-12.91
T_d [°C]	208.40	204.70	207.05	206.87	208.70
ΔH [J/g]	20.26	17.77	21.67	24.89	12.36

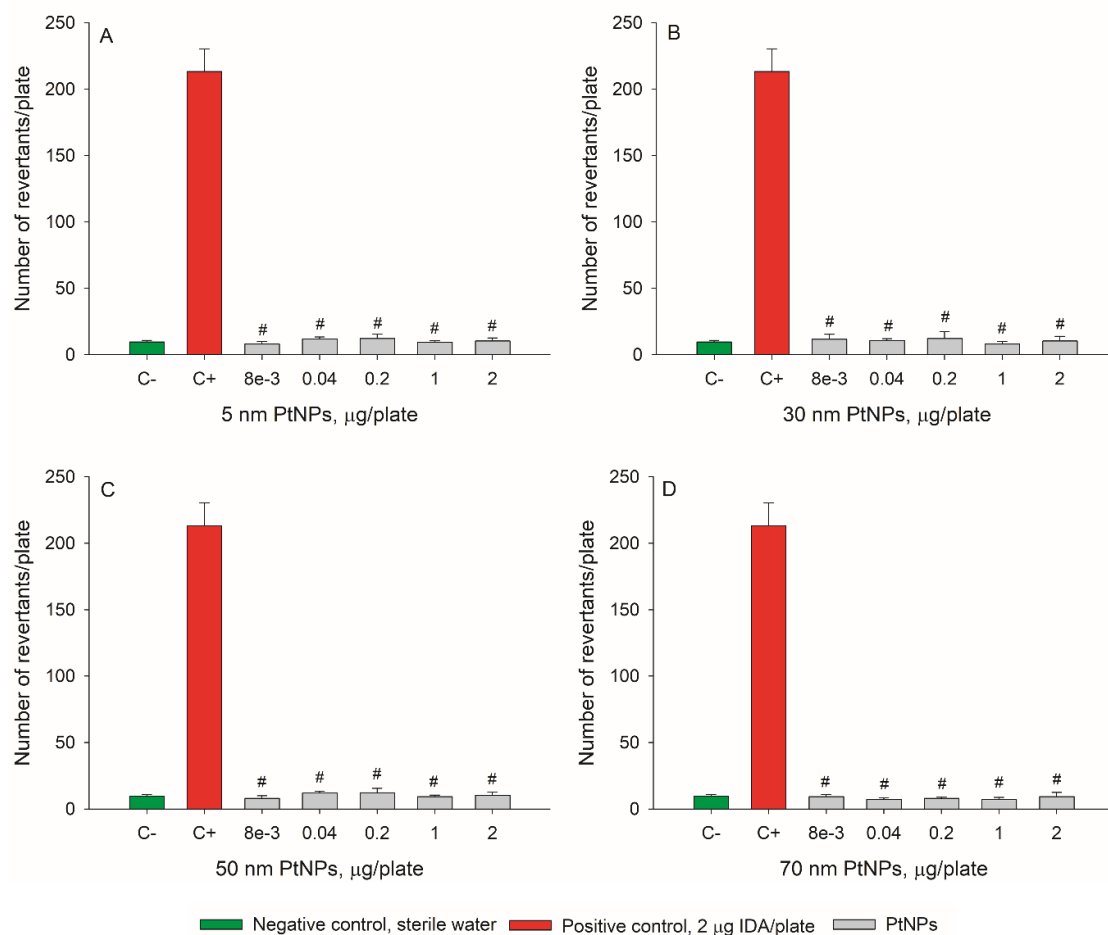


Figure S3. Mutagenicity of platinum nanoparticles (PtNPs) in *Salmonella enterica* serovar Typhimurium TA98 strain. (A – D) – 5, 30, 50 and 70 nm PtNPs, respectively. C- negative control (sterile water), C+ positive control (2 µg IDA/plate). Results are reported as the average number of revertants per plate $\pm$ SD. #- significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

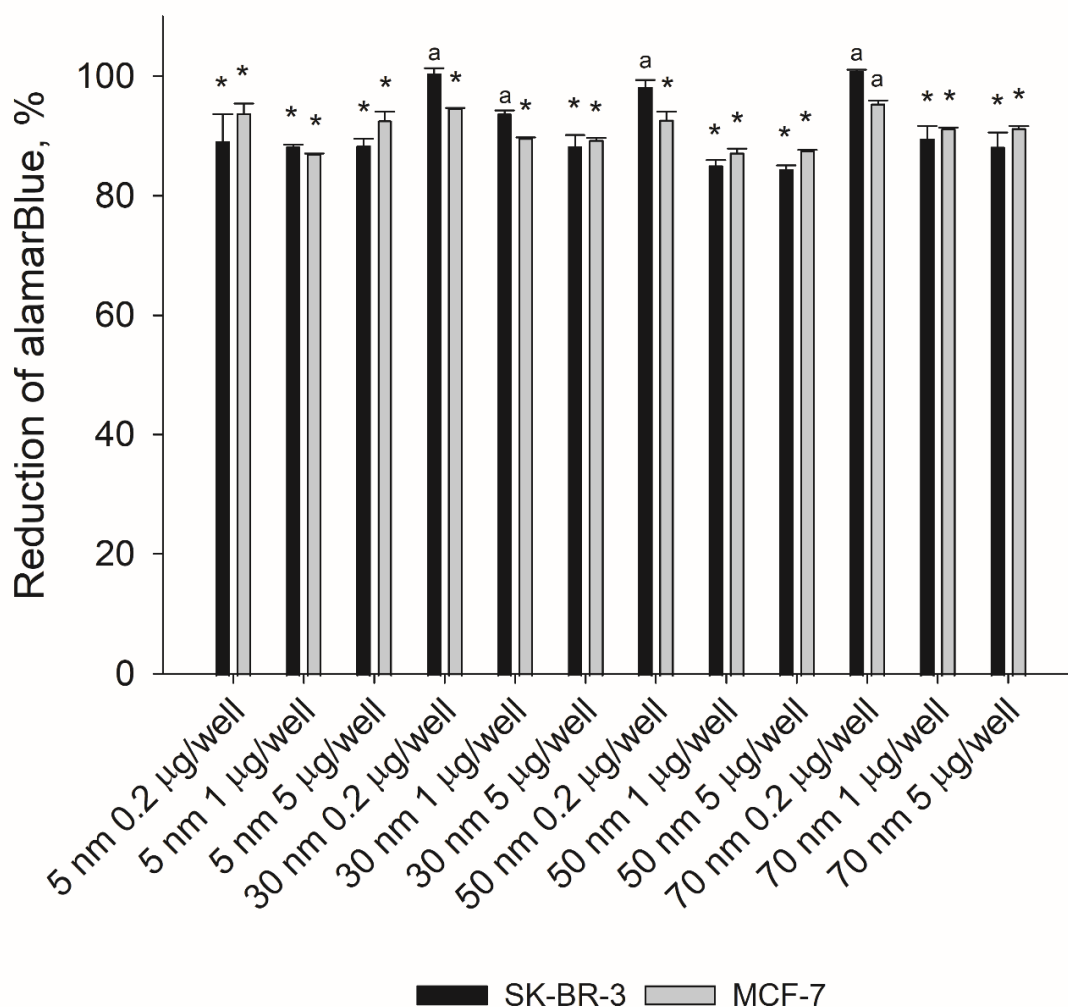


Figure S4. Anticancer activity of platinum nanoparticles (PtNPs) on SK-BR-3 and MCF-7 cell lines. Results are reported as the mean reduction of alamarBlue in regard to the untreated cells (negative control, 100% viability) $\pm$ standard deviation (SD). a – no significant difference, * - significant difference ($p < \alpha$, $\alpha = 0.05$).

3. Zakrzewski M., Kalisz I., Bełdzińska P., Grzyb K., Bury K., Jamróiewicz M., Wyrzykowski D., Gołński G., Piosik J. Comparative analysis of platinum nanoparticles with mitomycin C and its activated metabolite.

Comparative analysis of platinum nanoparticles interactions with mitomycin C and its activated metabolite

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Abstract

As of 2026, cancer remains one of the leading causes of death worldwide. In spite of substantial medical advancements, chemotherapy continues to be among the most commonly applied treatment strategies. However, conventional chemotherapeutic agents are not selective towards cancer cells and frequently induce severe side effects, including myelosuppression, gastrointestinal toxicity, and cardiotoxicity. Therefore, increasing attention has been directed towards the modulation of existing anticancer drugs through combination with metallic nanoparticles. Herein, we verified if platinum nanoparticles (PtNPs) of defined sizes 5, 30, 50, and 70 nm interact with mitomycin C (MMC) and one of its metabolites, 2,7-diaminomitosene (DAM). DLS and AFM analyses revealed differing aggregation patterns, with MMC exhibiting aggregation primarily under dry conditions. Further analyses utilizing FTIR and NIR spectroscopies suggested that PtNPs interact with MMC predominantly through weak non-covalent interactions. Thermal inspection utilizing ITC and DSC showed that both these APIs interact in an endothermic manner, though the intact MMC might be interpreted as negligible. Furthermore, Ames mutagenicity assay on *Salmonella enterica* serovar Typhimurium TA102 revealed dose-dependent reduction of mutagenic potential by PtNPs, with more pronounced effects observed for DAM. Ultimately, the cytotoxicity analyses on MCF-7 and SK-BR-3 breast cancer cell lines revealed that PtNPs increase the efficacy of MMC in a dose-dependent manner, while exerting the opposite effect in combination with DAM. Collectively, these findings suggest that PtNPs might be a useful tool in regard to modifying the bioactivity of mitomycin C, and could serve as a basis for future nanoparticle-assisted drug delivery systems.

Keywords

platinum nanoparticles, mitomycin C, mitosenes, anticancer activity, cytotoxicity, modulation

1. Introduction

Cancer remains one of the leading causes of death worldwide, posing a major challenge to global health. Despite extensive research, its complex pathogenesis and heterogeneity make effective treatment difficult to achieve. Novel therapeutic regimes have emerged, including targeted and immune therapies, however, chemotherapy remains the most widely used to this day. Despite being an effective anticancer approach, usage of it is often associated with severe side effects, such as cardiotoxicity or bone marrow suppression. The persistence of high mortality rates underscores the urgent need to search for novel and more efficient therapeutic approaches. Current research focuses on modulating the activity of anticancer agents utilising nanoscience in many ways, including nanoparticles.

Nanoparticles have been in the scientific community's sphere of interest since the early 60's. Classical definition renders them as particles ranging in size from 1 to 100 nm. They can be of various shapes, with the most common being the spherical ones. Furthermore, they can be made of different materials, and as such, they are divided into carbon-based, organic, and inorganic nanoparticles. Within the last group, metallic nanoparticles have gained significant interest over the years. Gold, silver and iron oxide nanoparticles have been examined thoroughly, with lots of research proving both their antibacterial and anticancer properties. Within the group of nanomaterials, platinum nanoparticles (PtNPs) remain relatively understudied compared to other metals from this group [1]. According to the newest knowledge in that matter, they have been shown to be a potent antibacterial agent [2]. More importantly, they also possess anticancer properties on their own, but their exact mechanism has not yet been established; literature strongly suggests the main mode of action to be reactive oxygen species generation, but there are indications of their interference with PI3K and MAPK signalling pathways [3,4]. What is more, when tested on both healthy and cancerous cell lines, they did not present significant cytotoxicity towards the healthy cells [5]. Furthermore, their potential is harnessed as a drug delivery system (DDS), and a few studies have already shown promising results with DOX [6]. Due to differences in size, shape, and composition, they exhibit diverse properties related to surface modifications, optical properties, and cell entry capabilities. These features make them an excellent tool for a customizable and controllable modulator of a drug's activity.

Mitomycin C (MMC) is an antibiotic initially isolated from *Streptomyces caespitosus*, and quickly enough, it has presented itself as a prosperous anticancer agent [7]. It is typically used in the treatment of anal, bladder, and breast carcinomas, however, it can also be applied to other gastrointestinal cancers [8]. As for classification, this active pharmaceutical ingredient (API) belongs to the alkylating agents group and works by cross-linking the complementary DNA strands, with high affinity towards the CpG sequences [9]. Furthermore, it suppresses RNA and protein synthesis when administered at higher concentrations, leading to antibacterial properties. Nevertheless, as with many chemotherapeutics, the usage of this compound leads to numerous side effects, some of them being as adverse as bone marrow toxicity and a high risk of myelosuppression upon prolonged application. Furthermore, it can lead to haemolytic uremic syndrome (HUS), impairing the kidney function and posing a major threat to the overall health of the patient [10]. Nevertheless, MMC remains a potent anticancer antibiotic with significant cytotoxic activity through DNA crosslinking. According to the manufacturer, MMC exhibits a relatively high solubility in organic solvents such as DMSO, reaching up to 15 mg/mL, whereas in aqueous solutions it solubility decreases substantially to approximately 0.5 – 1 mg/mL [11]. Nowadays, many APIs are combined with HCl to improve their solubility, however MMC has long been known to undergo degradation at pH below 6 [12,13]. This process involves a series of structural rearrangements, including the opening of the aziridine ring, ultimately leading to the formation of mitosenes, among which 2,7-diaminomitosene (2,7-DAM) is considered one of the major products [14]. Despite the degradation process, DAM remains biologically active and can alkylate DNA, despite its structural differences with MMC [14,15]. Since such degradation can also occur under in vitro conditions [16], both MMC dispersed in DMSO and MMC dispersed in water of pH 5 were examined in the present study, with a focus on comparison of their physicochemical interactions and biological activity after combination with platinum nanoparticles of diverse sizes 5, 30, 50, and 70 nm.

2. Materials and Methods

2.1 Materials

Platinum nanoparticles (PtNPs) at concentrations of 0.05 mg/mL and 1 mg/mL were purchased from nanoComposix (San Diego, California, USA). The nanoparticles were dispersed in 2 mM trisodium citrate for 5, 30, and 50 nm PtNPs and in 4 mM trisodium citrate for 70 nm PtNPs. Mitomycin C (MMC) was obtained from STI (Poznań, Poland). The stock solutions were prepared by dissolving mitomycin C in the desired solvent, and the concentration of each sample was calculated using Lambert-Beer's Law based on the spectrophotometer analysis.

The bacterial strain of *Salmonella enterica* serovar Typhimurium TA102 was purchased from Xenometrix (Allschwil, Switzerland). Nutrient Agar, Nutrient Broth, Glucose, Ampicillin, L-Histidine, Biotin, and Vogel-Bonner salts were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) according to the Ames Salmonella/microsome mutagenicity assay [17].

SK-BR-3 and MCF-7 cell lines were received from the Laboratory of Molecular Enzymology and Oncology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk. Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS), 4 mM L-glutamine, glucose and antibiotic-antimycotic solution were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA).

2.2 Methods

2.2.1. Preparation of MMC and mitosenes solutions

In this study, we have prepared three distinct MMC solutions: in DMSO, in sodium citrate, and in water with pH 5. After weighing the desired amount of MMC powder, it was suspended in either of these solvents, and the final concentration was determined using a spectrophotometer in the UV-Vis region. For DMSO and sodium citrate solutions, the molar extinction coefficient of $\epsilon_{360} = 2.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ was used [18], whereas for water with a pH of 5, we used a molar extinction coefficient of $\epsilon_{310} = 1.03 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ [16].

2.2.2. Dynamic Light Scattering (DLS)

The hydrodynamic diameter of the PtNPs and the change in registered sizes upon addition of either MMC dissolved in DMSO or DAM were evaluated in polystyrene cuvettes at 25 °C utilising Zetasizer Nano S from Malvern Panalytical (Malvern, Great Britain). The initial concentration of PtNPs was 2.38 µg/mL, and the concentration range of MMC was 6 µM – 30 µM.

2.2.3 Atomic Force Microscopy

Atomic force microscopy visualisation of the PtNPs alone, with MMC dissolved in DMSO, and with DAM, was performed at 23 °C in air, in PeakForce Tapping mode, using BioScope Resolve AFM (Bruker, Bremen, Germany). PtNPs (0.05 mg/mL) were mixed with either 0.3 µM MMC or 0.3 µM DAM at room temperature. Subsequently, 100 mM cysteamine was deposited onto a freshly cleaned mica surface for 5 min, and afterwards the solution was aspirated. Then, samples were added on mica and incubated for 5 min at room temperature, followed by washing with deionised water and drying with streams of nitrogen gas prior to AFM imaging. The ScanAsyst-Fluid+ probe (Bruker) was used for PtNPs alone and PtNPs-MMC mixtures imaging (resonant frequency f_0 = 150 kHz; spring constant k = 0.7 N/m). Images were registered at 512×512 pixels with a PeakForce Tapping frequency of 1 kHz and amplitude of 150 nm. A height sensor signal was used to display the image using NanoScope Analysis v1.9.

2.2.4 Fourier Transform Infrared Spectroscopy (FTIR) and Near Infrared Spectroscopy (NIR)

FTIR and NIR spectra of complexes formed between lyophilised MMC (dissolved in sodium citrate) and PtNPs were recorded on Jasco-4700 instrument (IR: 4000-400 cm^{-1} , NIR: 8200-4000 cm^{-1}) with 32 scans, 4 cm^{-1} resolution (Jasco Company, Tokyo, Japan). All prepared samples were lyophilised prior to the procedure. The NIR Transmission technique was applied using dry salt KBr and dry samples of MMC, PtNPs, and sodium citrate obtained in the process of lyophilisation. The materials were compressed with potassium bromide to form a disc. All spectra were recorded as a background, and analysis of spectra was performed using Spectra Analysis software (Jasco Company, Tokyo, Japan).

2.2.5 Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry measurements were performed at 25 °C on AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA). The volumes of the sample and reference cells were 1.4491 mL. The experiment consisted of 12 injections of 10.02 µL with a 2 µL for the first injection only of either MMC dissolved in sodium citrate or DAM solution (concentration range 2 – 29 µM) into the sample cell containing PtNPs (initial concentration 0.05 mg/mL). Background titrations were performed by injecting MMC into the appropriate sodium citrate solution and the appropriate sodium citrate solution to PtNPs. The results of the background titrations were subtracted from each experimental result to account for the heat of dilution. Each injection lasted 20 s. To reach a homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm.

2.2.6 Differential Scanning Calorimetry (DSC)

The thermal behaviour of the lyophilised MMC (dissolved in sodium citrate) mixtures with PtNPs was measured by Differential Scanning Calorimetry (DSC), STAR-1 System, Mettler Toledo (Switzerland), which was calibrated with indium. The analysis was carried out in aluminum crucibles, 40 µl at a temperature range from 20 °C to 300 °C. For performing the DSC analysis, 1-5 mg of the samples were weighed. DSC curves were recorded and analysed by the STAR-1 System (Mettler Toledo, Switzerland) combined with the intercooler system. Samples were heated at a rate of 10°C/min and a nitrogen flow rate of 60 mL/min. All tests were standardised to each sample size.

2.2.7 Ames mutagenicity assay

The influence of PtNPs on the mutagenic activity of either MMC dissolved in DMSO or DAM was assessed using the Ames mutagenicity plate assay with *Salmonella enterica* serovar Typhimurium TA102 strain, according to the procedure described by Woziwodzka et al. [19]. A mixture containing 100 µL of the overnight bacteria culture, 50 µL of 3% NaCl, and 100 µL of the tested solution (or sterile distilled water as a negative control) was incubated for 4 h in darkness at 37 °C and 220 rpm. Afterwards, the mixture was centrifuged at 11840 g, the bacterial pellet was washed with 0.75% NaCl, and resuspended in 300 µL of 0.75% NaCl solution containing 0.1 µM histidine and 0.1 µM biotin. The bacterial suspension was spread on a glucose minimal agar plate and

incubated at 37 °C in the dark. After 48 h incubation, the number of revertant colonies per plate was calculated. All experiments were performed in triplicate.

2.2.8 Cell culture and cytotoxicity assay

The SK-BR-3 and MCF-7 cell lines were cultivated in DMEM containing 4500 mg/L glucose, supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. Both cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. For the alamarBlue reduction assays, the cells were seeded on a 96-well plate (2 x 10⁴ cells/well and 4 x 10⁴ cells/well for SK-BR-3 and MCF-7 cell lines, respectively) and incubated overnight in a humidified atmosphere at 37 °C and with 5% CO₂. Next, the cells were washed thrice with appropriate medium devoid of FBS. Subsequently, PtNPs alone, at concentrations 0.04, 0.2 and 1 µg/well, and in combination with MMC dissolved in DMSO (5, 30, 40 and 50 µM for SK-BR-3; 5, 30, 60 and 90 µM for MCF-7) or DAM (5, 30, 60 and 90 µM for both cell lines) were added to cell cultures (90 µL/well), each sample in three replicates and incubated for 20 h. After that, 10 µL of alamarBlue (BioRad) was added to each well, and the plates were further incubated for 4 h in conditions described above. The absorbance was measured at 570 nm and 600 nm, and the percentage of alamarBlue reduction was calculated as a difference between treated and untreated cells, according to the protocol provided by the manufacturer:

$$diff(\%) = \frac{(O_2 \times A_1) - (O_1 \times A_2)}{(O_2 \times P_1) - (O_1 \times P_2)}$$

diff – difference between treated and control cells; O₁ – ε₅₇₀ of oxidized alamarBlue (80586 M⁻¹cm⁻¹); O₂ – ε₆₀₀ of oxidized alamarBlue (117216 M⁻¹cm⁻¹); A₁ – absorbance of the test wells at 570 nm; A₂ – absorbance of test wells at 600 nm; P₁ – absorbance of control wells at 570 nm; P₂ – absorbance of control wells at 600 nm.

2.2.9 Computer analysis

All of the graphs were prepared in SigmaPlot 14.5 from Systat Software Inc. (San Jose, California, USA). All statistical analyses were performed in Statistica 14.0 from TIBCO Software Inc. (Palo Alto, California, USA). For the Ames mutagenicity test and cytotoxicity assay, one-way variance analysis (ANOVA) followed by the post-hoc Tukey's HSD test was applied. The significance level for all the analyses was established at α = 0.05.

Results and discussion

3.1 Characterisation of MMC in different solvents

All the solutions of mitomycin C (MMC) were tested spectrophotometrically in order to calculate their concentrations (**Figure S1**). MMC dispersed in DMSO exhibited a major peak at 360 nm, which corresponds well with the literature [20]. When dispersed in acidic conditions, the peak at 360 nm disappeared, and new ones appeared: around 250, 310, and a broad peak in the range 500 – 600 nm. Despite the fact that the degradation may result in different degradation products, the observed peaks correspond to 2,7-diaminomitosene, which is reported as the major degradation metabolite of MMC under acidic conditions [15]. Therefore, we decided to abbreviate the degradation products as 2,7-diaminomitosenes (DAM).

3.2 Characterisation of PtNPs interactions with MMC and DAM

To preliminarily see if platinum nanoparticles (PtNPs) interact with mitomycin C (MMC), we performed dynamic light scattering (DLS) analysis (**Figure 1**). 5 nm PtNPs presented a polydisperse population, divided majorly into two groups; bigger one around the declared size, and a fraction of aggregated ones around 100 nm. The rest of the tested PtNPs presented a hydrodynamic diameter close to the one declared by the manufacturer. Upon addition of either 6 or 30 μ M of MMC dispersed in DMSO, no significant change was observed in either of the samples, which was also visible in the raw data (**Table S1**). Importantly, addition of identical volumes of DMSO did not alter the registered hydrodynamic radius, neither visually (**Figure S2**) nor in raw data (**Table S2**).

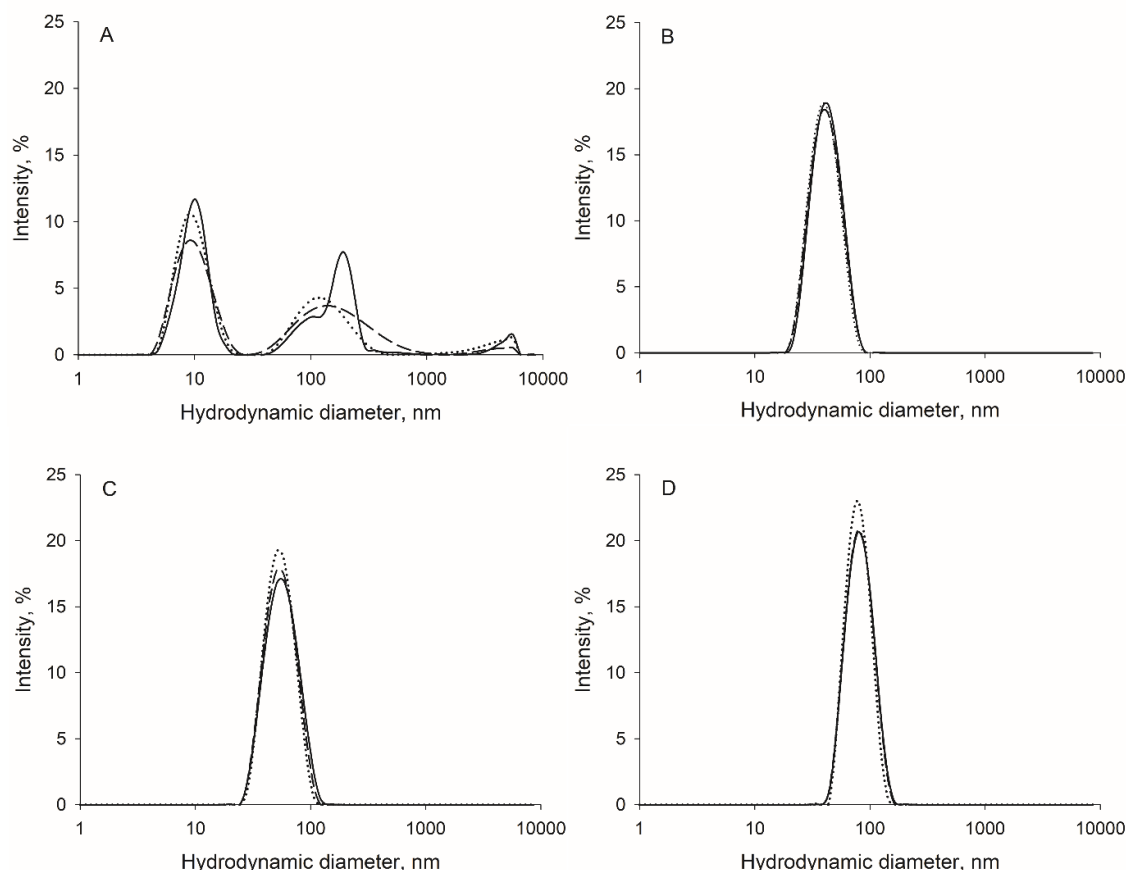


Figure 1. Hydrodynamic diameters of platinum nanoparticles (PtNPs) alone and in combination with mitomycin C (MMC). (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. Solid line – nanoparticles alone, dotted line – with 6 μ M of MMC, dashed line – with 30 μ M of MMC.

Next, we performed a similar analysis with mitomycin's degradation products, mitosenes (**Figure 2**). Interestingly, we observed a significant change in size, which was more pronounced with the increase in DAMs concentration. Importantly, the addition of the highest concentration of DAM resulted in lowering the polydispersity index and a uniform size of aggregates, which was notable for 5 nm PtNPs (**Table S3**). The differences between MMC and DAM could likely be explained by the structural differences; through the reduction process, the MMC's aziridine ring opens, exposing additional amine and carbonyl groups along with hydroxyl groups, all of which may contribute to the formation of weak, non-covalent, and most likely hydrogen-bonds with PtNPs [15,21,22].

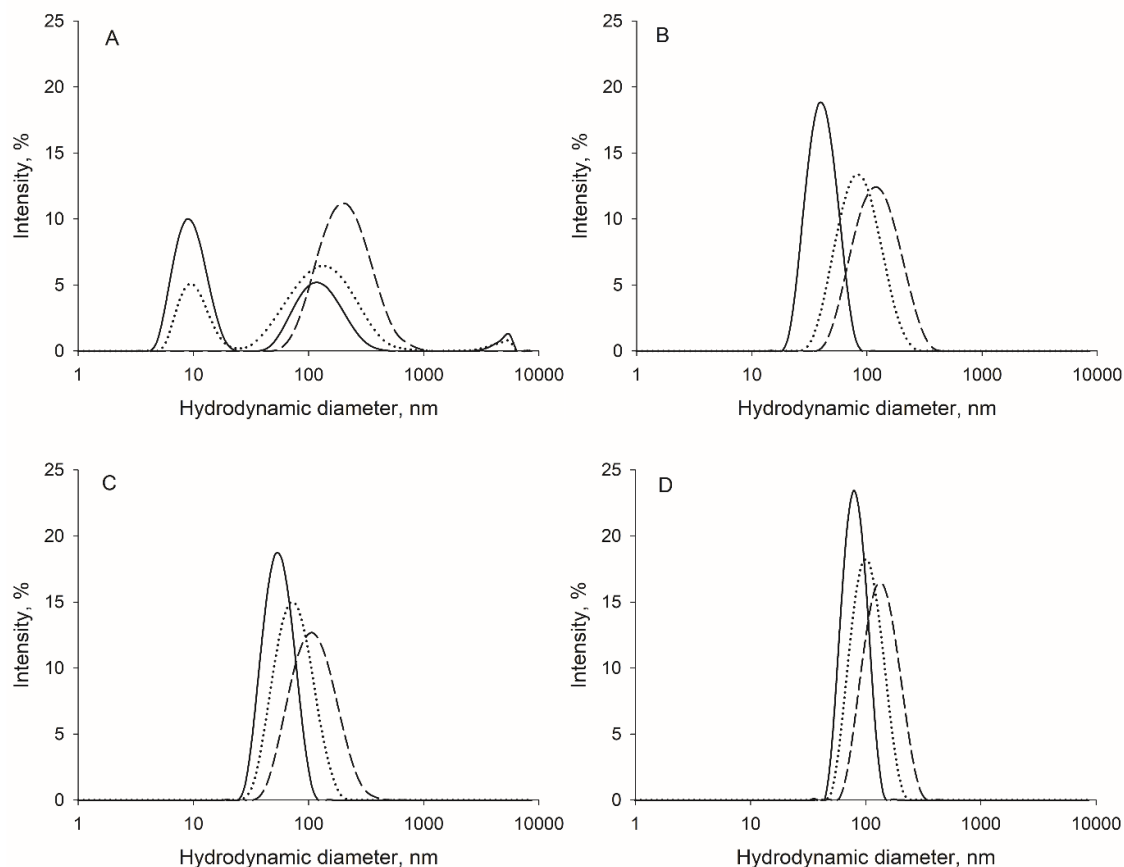


Figure 2. Hydrodynamic diameters of platinum nanoparticles (PtNPs) alone and in combination with mitosenes (DAM). (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. Solid line – nanoparticles alone, dotted line – with 6 μ M of DAM, dashed line – with 30 μ M of DAM.

To further investigate these discrepancies, we decided to visually inspect the changes upon combining PtNPs with either MMC or DAM using Atomic Force Microscopy (AFM) (**Figure 3**).

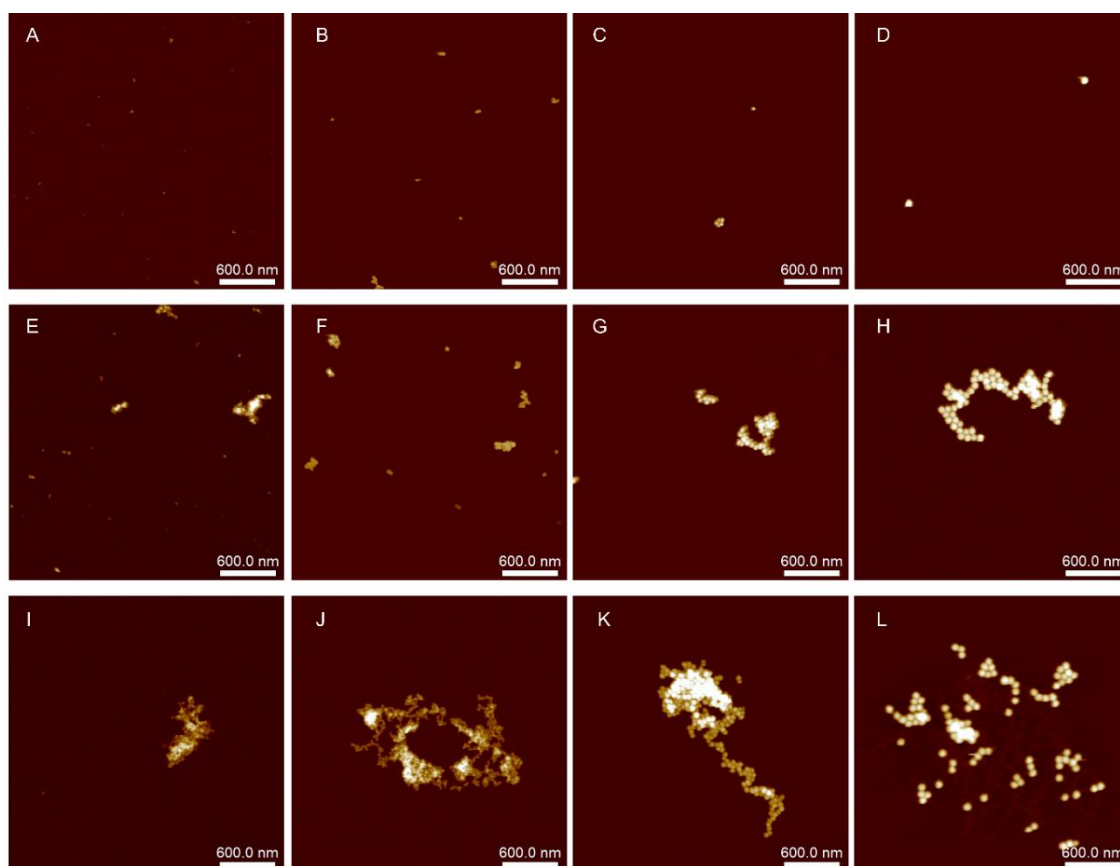


Figure 3. Atomic Force Microscopy (AFM) pictures of platinum nanoparticles (PtNPs) alone and in combination with either mitomycin C (MMC) or mitosenes (DAM). (A – D) – 5, 30, 50 and 70 nm PtNPs, respectively; (E – H) – 5, 30, 50 and 70 nm PtNPs with MMC, respectively; (I – L) – 5, 30, 50 and 70 nm PtNPs with DAM, respectively.

The imaging revealed that the PtNPs alone were mostly visualised as single dots (**Figure 3, A - D**), with diameters consistent with the manufacturer's datasheet (**Table S4**). When combined with MMC dispersed in DMSO, we registered slight aggregation (**Figure 3, E-H**), however, the addition of DAM resulted in a more pronounced aggregate formation (**Figure 3, I-L**), as evidenced by the computed data in **Table S4**. Similar trends were previously reported for PtNPs in combination with doxorubicin (DOX) [23] and epirubicin (EPI) [24]. However, these results stand in contrast to the ones obtained by DLS, as we did not register any significant size changes following the addition of MMC to PtNPs. A plausible explanation to this discrepancy is that these methods differ significantly; DLS measures the hydrodynamic diameter of spheres in a colloidal environment, whereas AFM typically requires sample drying prior to analysis. Consequently, the aggregation observed in AFM images of PtNPs-MMC samples may be

attributed to surface deposition or local concentration effects upon drying, rather than aggregation in the liquid phase. Nevertheless, these inconsistencies do not exclude the possibility that AFM captures hypothetical weak interactions between PtNPs and MMC that remain undetectable under hydrated conditions.

3.3 Spectroscopic analysis of MMC

Infrared spectroscopy (IR) is widely used for chemical identification, as well as for the evaluation of intermolecular interactions [25,26], therefore, to gain deeper insight into the interactions occurring between mitomycin and platinum nanoparticles, Fourier Transform Infrared Spectroscopy (FTIR) was performed (**Figure 4**).

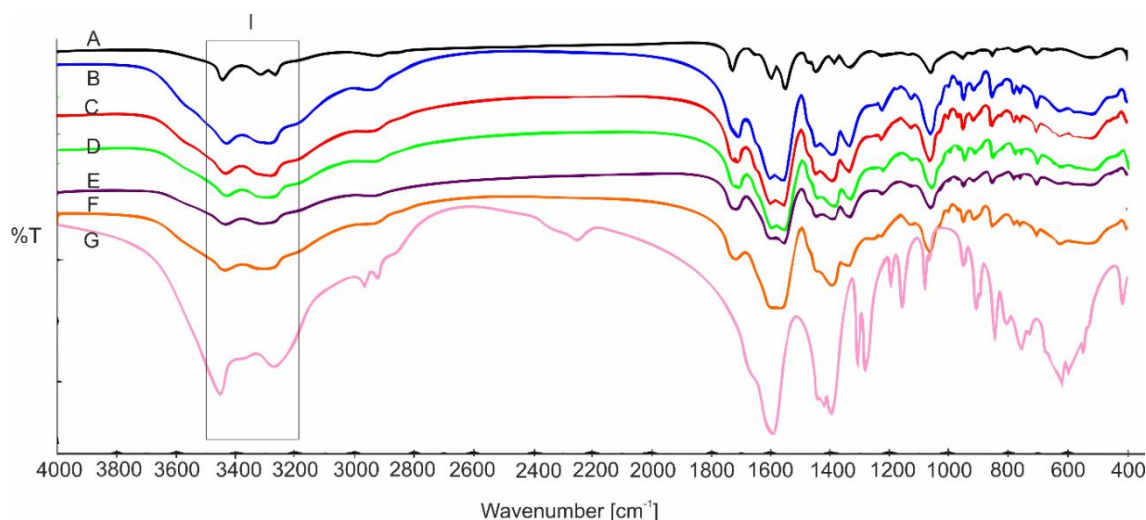


Figure 4. Fourier Transform Infrared Spectroscopy (FTIR) spectra of the investigated complexes of mitomycin C (MMC) and platinum nanoparticles (PtNPs) in the region 4000 – 400 cm⁻¹. (A) – MMC, (B) – MMC lyophilised from sodium citrate buffer, (C) – MMC with 5 nm PtNPs, (D) – MMC with 30 nm PtNPs, (E) – MMC with 50 nm PtNPs, (F) – MMC with 70 nm PtNPs, (G) – sodium citrate buffer.

The spectra of all complexes are very similar to that of the parent compound, indicating that the overall chemical structure of MMC remains preserved after interaction with platinum nanoparticles. A broad absorption band observed at 3427 and 3277 cm⁻¹ is attributed to N-H stretching vibrations, indicating the presence of hydrogen-bonding interactions. The band at 2938 cm⁻¹ corresponds to aliphatic C-H stretching vibrations, confirming the presence of methyl and methylene groups. To visualise the changes in a more distinct manner, we decided to apply 1st and 2nd derivatives (**Figure 5**).

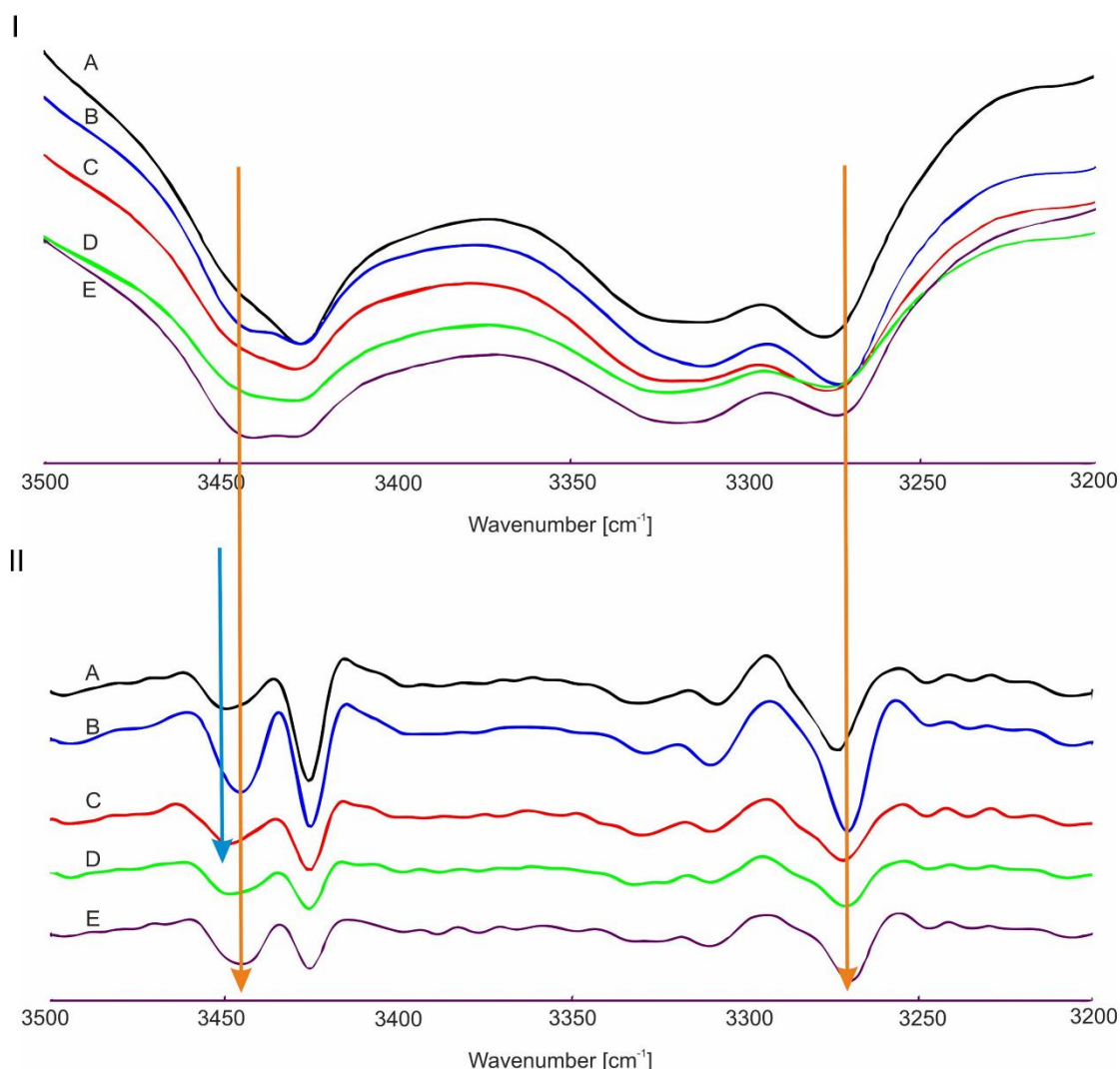


Figure 5. Fourier Transform Infrared Spectroscopy (FTIR) 1st (I) and 2nd (II) derivatives of the investigated mitomycin C (MMC) complexes with platinum nanoparticles (PtNPs) in the region 3500 – 3200 cm⁻¹. (A) – MMC lyophilised from sodium citrate buffer, (B) – MMC with 5 nm PtNPs, (C) – MMC with 30 nm PtNPs, (D) – MMC with 50 nm PtNPs, (E) – MMC with 70 nm PtNPs.

In the case of complexes containing 30 nm and 50 nm platinum nanoparticles, the MMC band at approximately 3443 cm⁻¹ becomes deformed (doubled) and partially split, which may be associated with interactions occurring in the N-H stretching region. Similar observations have been reported previously. In a study that investigated the interactions of mitomycin with nanoparticles formed via micellar self-assembly driven by PEG, an enhanced anticancer efficacy against bladder cancer was demonstrated, accompanied by observable shifts in FTIR bands [27]. Small shifts were observed in the N-H stretching

region ($3429 \rightarrow 3421 \text{ cm}^{-1}$), as well as in the amide-related region, including N-H bending vibrations overlapping with amide-like modes ($1548 \rightarrow 1529 \text{ cm}^{-1}$ and $1250 \rightarrow 1243 \text{ cm}^{-1}$). Importantly, no significant changes were observed for the band corresponding to the C=O stretching vibration of the quinone moiety, located at 1711 cm^{-1} , which is a key structural feature of mitomycin.

In another study, the authors investigated the interactions of MMC with nanoparticles based on PLA (polylactide), including PLA and PLA-MMC-SPC systems (containing phosphatidylcholine) [28]. Changes were observed in the FTIR spectra in the $3300\text{-}3250 \text{ cm}^{-1}$ region (N-H stretching), where the bands merged into a single broad peak, as well as the disappearance of the band at $\sim 1231 \text{ cm}^{-1}$. Notably, even the absence of distinct peak shifts suggests the presence of weak physical (non-covalent) interactions between MMC and the nanoparticle matrix. The authors did not mention any shifts at the bands at 1445 and 1396 cm^{-1} , which correspond to CH_2 and CH_3 bending vibrations, or at 1335 cm^{-1} , which is assigned to C-N stretching vibrations. Also, the fingerprint region, the absorption at 1226 cm^{-1} attributed to C-O stretching vibrations of ether or methoxy groups, the bands at 1126 and 1064 cm^{-1} associated with C-N and C-O-C stretching vibrations, characteristic of heterocyclic and ether functionalities, are stable and not influenced by interactions. Finally, it can be concluded that the dominant interaction mechanism is hydrogen bonding rather than covalent bond formation [27]. Overall, the FTIR analysis indicates that the interaction between MMC and platinum nanoparticles is mainly based on weak intermolecular forces, particularly hydrogen bonding, rather than on the formation of covalent bonds [27].

Additionally, we performed Near Infrared Spectroscopy (NIR) to further inspect the possible intermolecular changes involving O-H, N-H, and C-H groups (**Figure 6**).

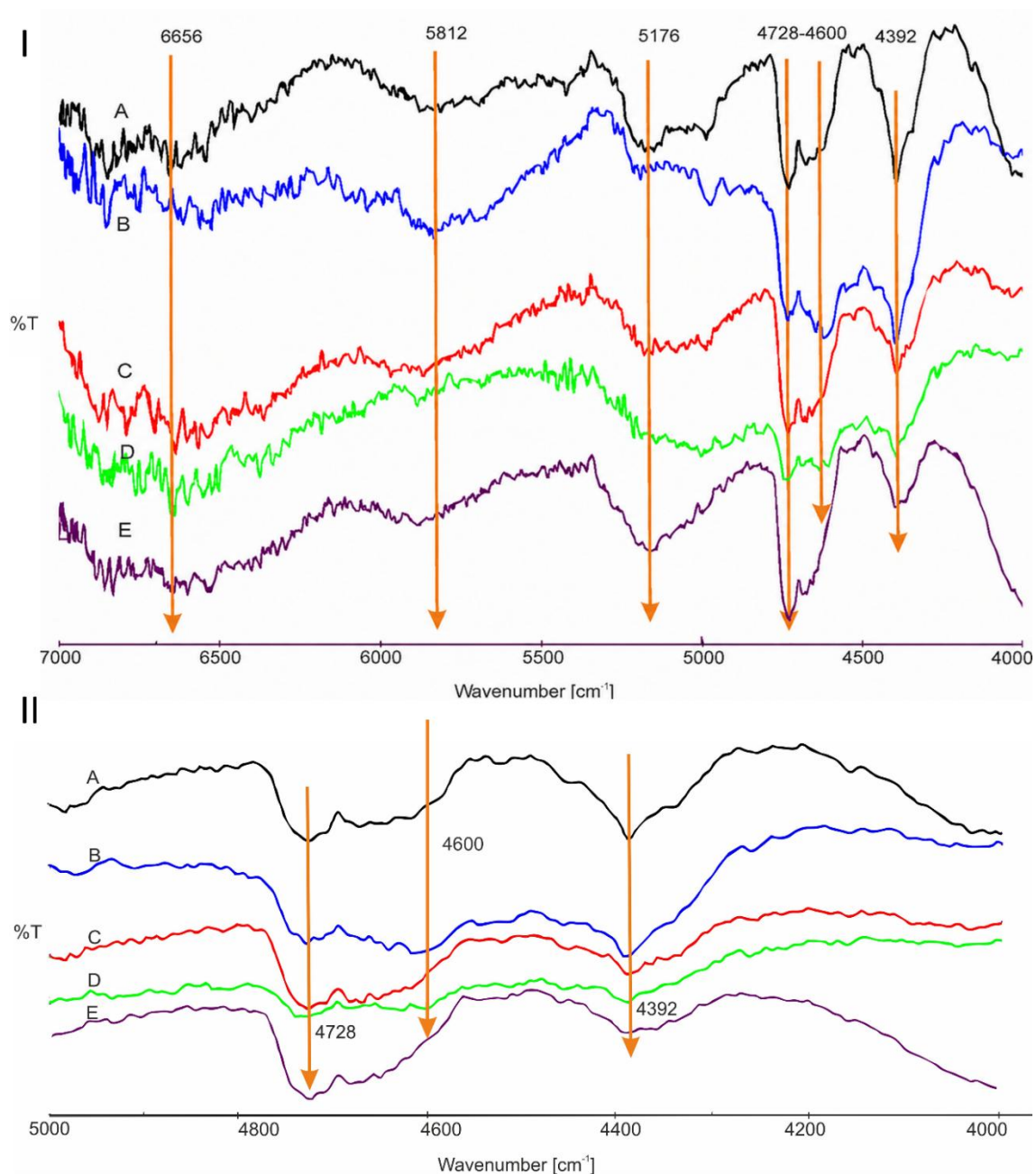


Figure 6. Near Infrared Spectroscopy (NIR) of the investigated mitomycin C (MMC) complexes with platinum nanoparticles (PtNPs) in the region 7000 – 4000 cm^{-1} (I), and a close up 5000 – 4000 cm^{-1} (II). (A) – MMC lyophilised from sodium citrate buffer, (B) – MMC with 5 nm PtNPs, (C) – MMC with 30 nm PtNPs, (D) – MMC with 50 nm PtNPs, (E) – MMC with 70 nm PtNPs.

The spectra exhibit several broad absorption bands corresponding to overtone and combination vibrations of functional groups such as N-H and C-H. Specifically, the bands

at 6846 and 6656 cm^{-1} are assigned to the first overtones of N-H stretching vibrations. Their presence indicates the involvement of hydroxyl and amine groups in hydrogen-bonding interactions. The spectral band observed at 5812 cm^{-1} corresponds to the first overtone of C-H stretching vibrations. In turn, the absorption at 5176 cm^{-1} is attributed to N-H combination modes, making this region particularly sensitive to intermolecular interactions. The band at 4278 cm^{-1} is associated with combination vibrations involving C-H stretching and deformation modes, reflecting the presence of methyl, methylene, and aromatic groups. Notably, this band becomes weaker and less intense in the complex containing 50 nm PtNPs. Furthermore, the lowest energy band at 4392 cm^{-1} is assigned to complex combination vibrations of C-H and N-H groups, including contributions from aromatic ring modes. An interesting effect is observed in this region, as the intensity of this band decreases in the complexes containing 50 and 70 nm PtNPs. The observed reduction in intensity of the bands at 4728 and 4392 cm^{-1} may indicate changes in the local molecular environment of MMC upon interaction with larger PtNPs. These spectral variations suggest the involvement of weak intermolecular interactions, particularly hydrogen bonding and changes in vibrational coupling, rather than the formation of strong covalent bonds.

Overall, the NIR results are consistent with the FTIR analysis and support the conclusion that MMC interacts with PtNPs mainly through non-covalent interactions. In literature, similar observations were previously reported for the PtNPs-EPI combination [24], where the interactions were likewise attributed predominantly to weak non-covalent. Besides hydrogen bonding, PtNPs may also interact with MMC through van der Waals forces, electrostatic interactions, or coordination-like interactions through amino groups. However, based on the spectroscopic results of our study, hydrogen bonds appear to be the dominant interaction mechanism.

3.4 Thermal behaviour of MMC and DAM with PtNPs

Moreover, we decided to inspect the thermal changes resulting from the interaction between MMC, DAM, and PtNPs. Since DMSO can give a strong exothermic heat of dilution upon contact with water, which is the solvent of PtNPs, we decided to disperse MMC in sodium citrate at 0.3 μM to remain its solubility and minimise the possible heat of dilution (**Figure 7**). Herein, in all cases, we observed a slightly exothermic reaction,

with the highest amount of energy released by interactions of 70 nm PtNPs. However, the overall enthalpy changes were close to 0, so one could assume the interactions are non-existent, especially taking into consideration the DLS results.

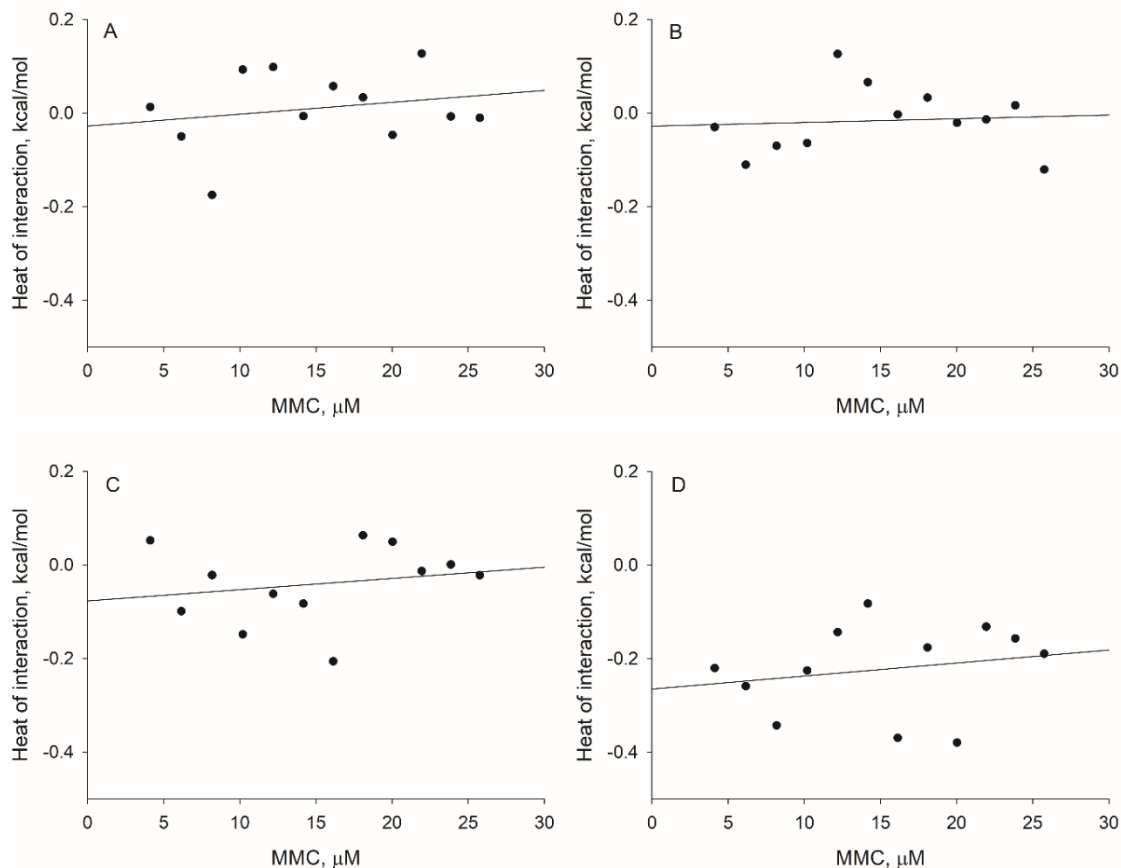


Figure 7. Isothermal titration calorimetry analysis for platinum nanoparticles (PtNPs) interactions with mitomycin C (MMC) dispersed in sodium citrate buffer. (A) – 5 nm PtNPs ($\Delta H = -0.03 \pm 0.06$ kcal/mol), (B) – 30 nm PtNPs ($\Delta H = -0.03 \pm 0.05$ kcal/mol), (C) – 50 nm PtNPs ($\Delta H = -0.08 \pm 0.06$ kcal/mol), (D) – 70 nm PtNPs ($\Delta H = -0.27 \pm 0.07$ kcal/mol).

To follow up, we prepared a sample of DAM with identical molarity as MMC and performed a similar analysis (**Figure 8**). This time we observed that all the tested sizes interact in an endothermic manner, with the enthalpy constantly rising alongside the increase of PtNPs size from 0.36 kcal/mol (5 nm) to 2.65 kcal/mol (70 nm). The thermograms for both MMC and DAM analyses are available in the supplementary data (**Figure S3**).

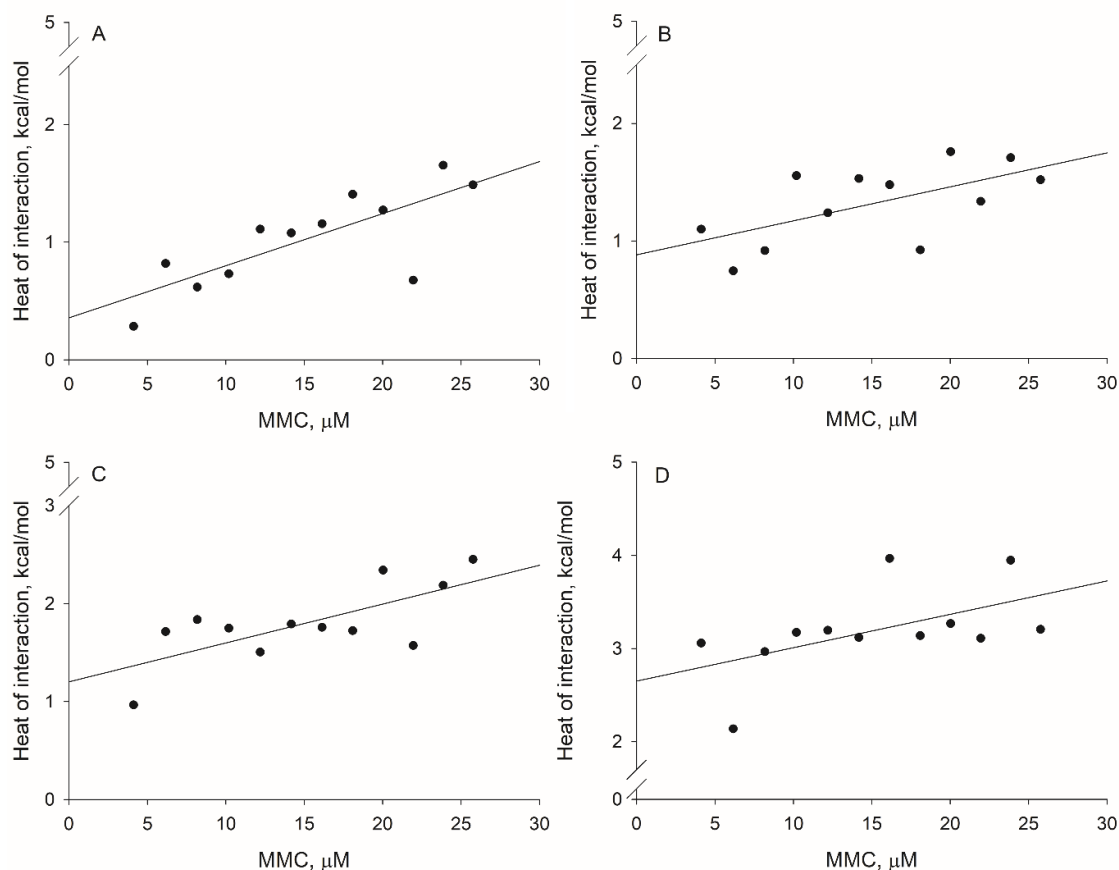


Figure 8. Isothermal titration calorimetry analysis for platinum nanoparticles (PtNPs) interactions with mitosenes (DAM). (A) – 5 nm PtNPs ($\Delta H = 0.36 \pm 0.19$ kcal/mol), (B) – 30 nm PtNPs ($\Delta H = 0.88 \pm 0.19$ kcal/mol), (C) – 50 nm PtNPs ($\Delta H = 1.2 \pm 0.2$ kcal/mol), (D) – 70 nm PtNPs ($\Delta H = 2.65 \pm 0.29$ kcal/mol).

The discrepancy between the interaction enthalpy of PtNPs-MMC and PtNPs-DAM conjugates may be linked with the results of DLS, AFM, and spectroscopic analyses. In DLS, we did not register any changes in size with MMC; on the contrary, DAM yielded a significant aggregate formation. In AFM, we observed some clusters, but it is worth reminding that these two methods differ in the environment; DLS inspects the aggregation in their natural, colloidal state, whereas AFM examines samples under dry conditions, where, due to evaporation of the solvent, nanoparticles and molecules of API may be forced to cluster and adsorb. Nevertheless, one cannot exclude possible interactions as our spectroscopic results suggest formation of weak, non-covalent bonds between PtNPs and MMC dispersed in sodium citrate.

Additionally, we performed differential scanning calorimetry (DSC) to further inspect the thermal changes that interactions between PtNPs and MMC inflict on the API (**Figure 9**).

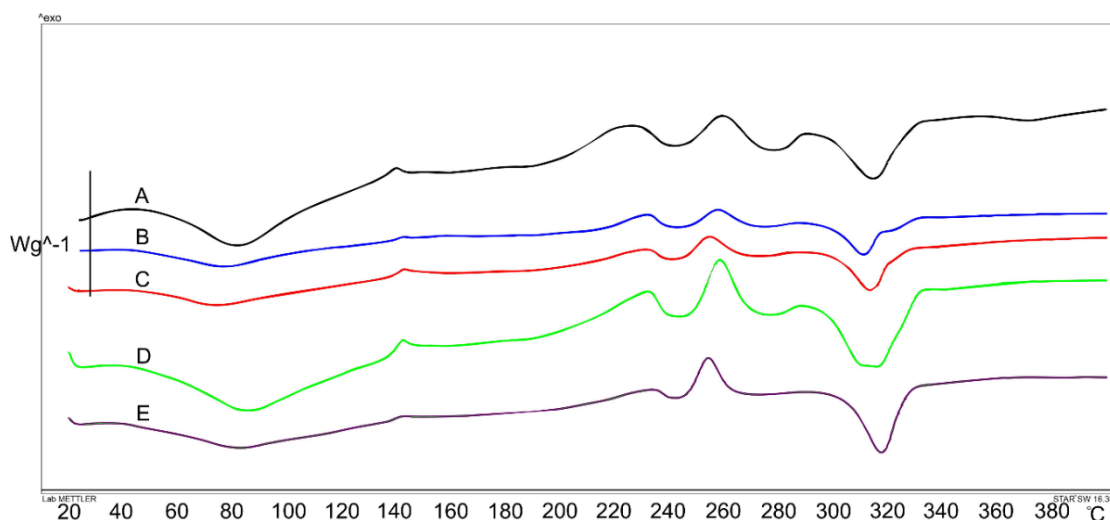


Figure 9. Differential Scanning Calorimetry (DSC) curves of platinum nanoparticles (PtNPs) and mitomycin C (MMC) dispersed in sodium citrate buffer. (A) – MMC lyophilised form sodium citrate, (B) – MMC with 5 nm PtNPs, (C) – MMC with 30 nm PtNPs, (D) MMC with 50 nm PtNPs, (E) – MMC with 70 nm PtNPs.

The DSC thermogram of pure MMC is characterised by the absence of a sharp melting endotherm typically associated with highly crystalline compounds. Instead, MMC exhibits broad thermal events in the range of approximately 140-180 °C, followed by a dominant transition between 180 and 260 °C, which is attributed to thermal decomposition. This behaviour indicates that mitomycin undergoes degradation prior to melting [28–30]. Minor endothermic effects observed at lower temperatures (below 120 °C) are commonly associated with the loss of adsorbed or bound moisture [28]. In the present study, the DSC profiles of MMC-PtNPs complexes retain the general thermal behaviour characteristic of MMC, although noticeable differences in peak shape and intensity are observed in comparison with the pure compound. The broadening and partial suppression of thermal transitions may indicate interactions between MMC and PtNPs, leading to changes in the local molecular organisation of the drug. However, the absence of entirely new thermal events suggests that no new covalent structures are formed during complexation.

Overall, the DSC analysis supports the spectroscopic findings obtained from FTIR and NIR measurements, indicating that MMC interacts with PtNPs mainly through weak intermolecular forces, such as hydrogen bonding and physical adsorption, rather than through strong chemical bond formation, which is consistent with our previous studies on PtNPs interactions with anthracyclines [24,31].

3.5 Biological influence of PtNPs on MMC and DAM

In our final step of study on PtNPs-MMC interactions, we decided to verify if PtNPs influence the activity of MMC. First, we employed the Ames mutagenicity assay on *Salmonella enterica* Typhimurium TA102 strain (**Figure 10**).

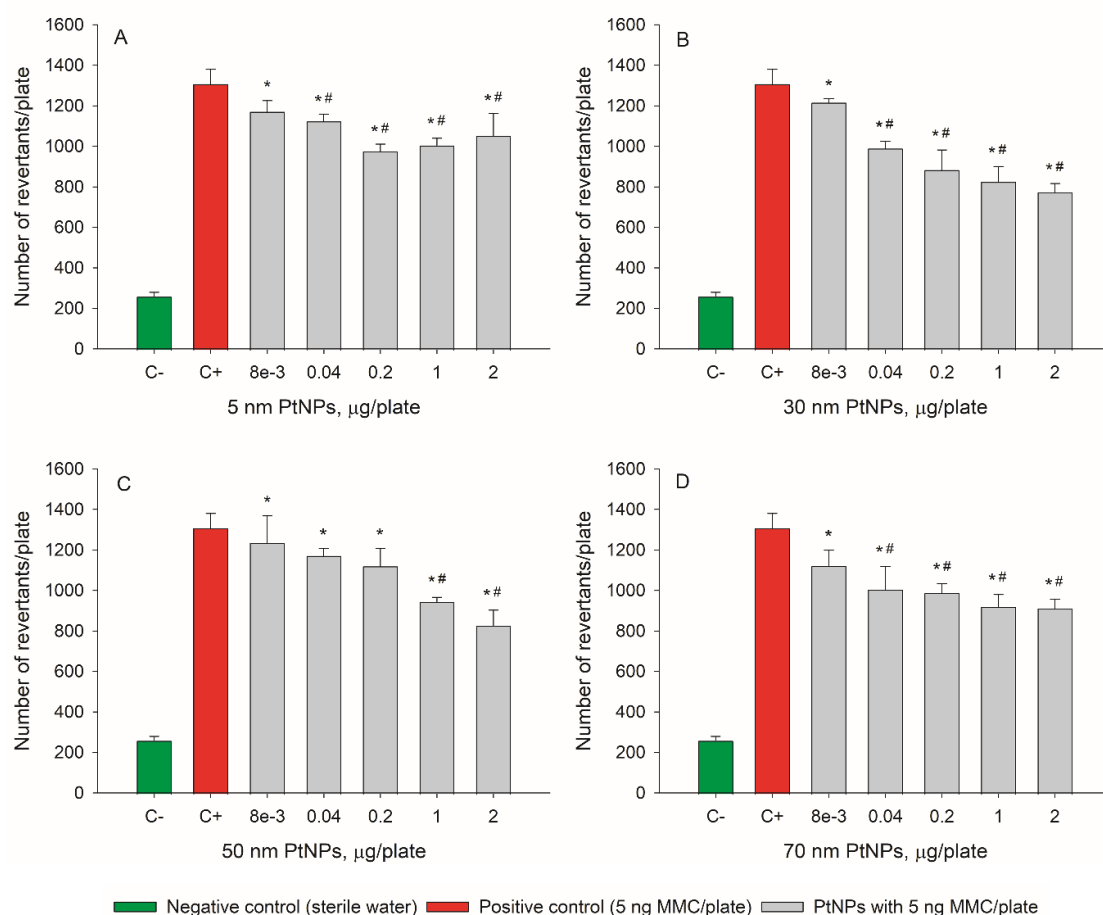


Figure 10. Influence of platinum nanoparticles (PtNPs) on the mutagenicity of mitomycin C (MMC) in *Salmonella enterica* serovar Typhimurium TA102 strain. (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. C- negative control (sterile water), C+ positive control (5 ng MMC/plate). Results are reported as the average number of revertants per plate $\pm$ SD. *- significant difference from the negative control, # - significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

MMC exhibited strong mutagenic activity in a dose-dependent manner, yielding approximately fivefold more revertant colonies compared to the negative control representing spontaneous mutations (**Figure S4**). Despite the ambiguous results of aggregation analyses in PtNPs-MMC complexes, the addition of PtNPs to MMC led to a

partial yet noticeable reduction in MMC-induced mutagenicity. This effect was most pronounced for 30 and 50 nm PtNPs, particularly at higher nanoparticle concentrations, suggesting that PtNPs may partially decrease the biological availability of the drug. Interestingly, the effects were dose-dependent. Importantly, nanoparticles were neither cytotoxic nor mutagenic on the bacterial cells (**Figure S5**). Since we compared both MMC and its metabolites, in our next step, we performed a similar analysis using DAM (**Figure 11**).

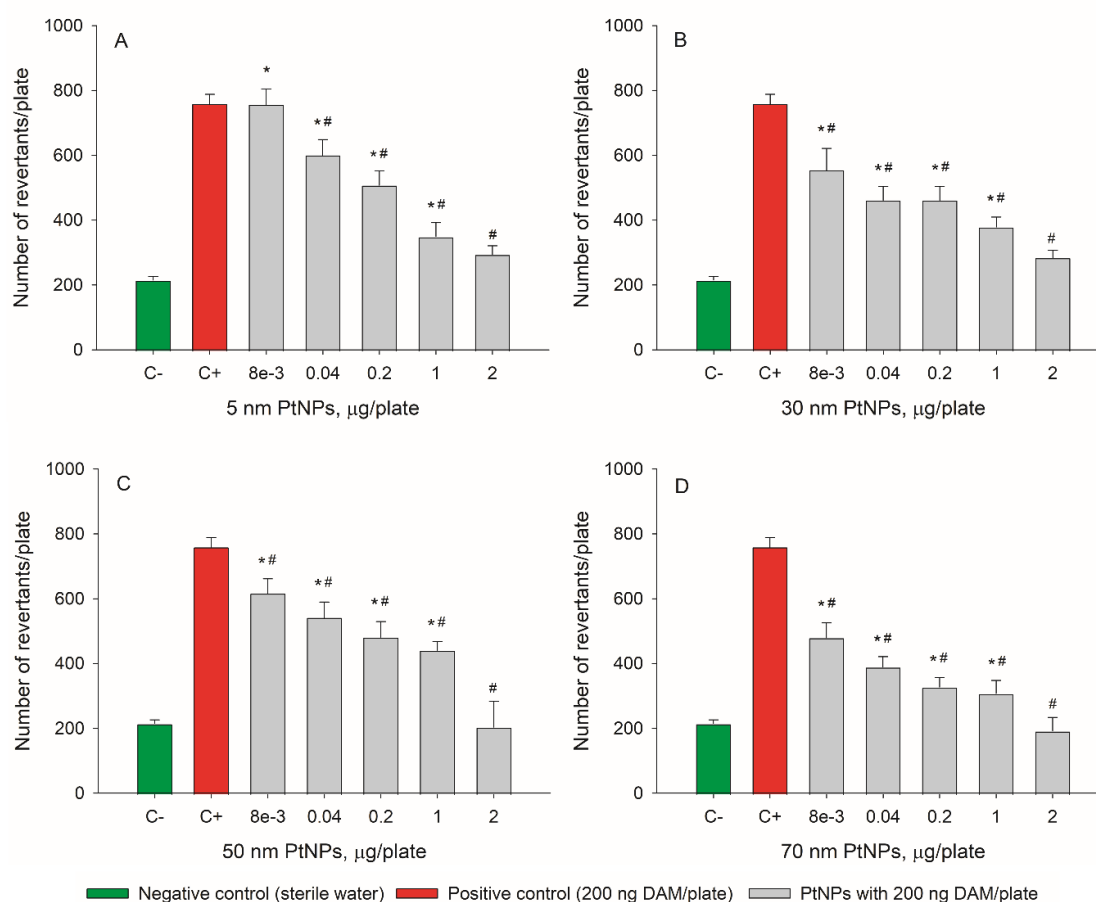


Figure 11. Influence of platinum nanoparticles (PtNPs) on the mutagenicity of diaminomitosenes (DAM) in *Salmonella enterica* serovar Typhimurium TA102 strain. (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. C- negative control (sterile water), C+ positive control (200 ng DAM/plate). Results are reported as the average number of revertants per plate $\pm$ SD. *- significant difference from the negative control, # - significant difference from the positive control ($p < \alpha$; $\alpha = 0.05$).

DAM alone was a less potent mutagen than MMC, with roughly fourfold more revertant colonies than the negative control (**Figure S4**). Following the addition of PtNPs, we observed a gradual dose-dependent decrease in the observed mutagenicity of the API, with the combinations containing the highest PtNPs concentrations reaching levels comparable to the negative control across all tested nanoparticle sizes, and this effect was significant especially for 50 and 70 nm PtNPs. These results suggest that platinum nanoparticles interact with DAM comparatively stronger than with MMC, and we suspect this effect is likely through adsorption and/or aggregation, which in turn limits the drug's bioavailability to bacterial cells, and consequently reduces the overall mutagenicity.

Overall, the Ames assay results appear somewhat contradictory, as the reduction in MMC mutagenicity does not fully correspond with the ambiguous aggregation patterns observed in DLS and AFM analyses. In contrast, combinations of PtNPs with DAM demonstrated a distinct decrease in the count of revertant colonies in tandem with the concentration of PtNPs; the higher the concentration of PtNPs the lower the revertant colony count. The most plausible explanation is that mitosenes form aggregates with PtNPs, and that increasing PtNPs concentrations promote the formation of larger PtNPs-DAM clusters, ultimately resulting in negligible mutagenic activity at the highest nanoparticle concentrations. Conversely, for MMC, the interactions detected by FTIR and NIR spectroscopies were not strongly marked, with equivocal conclusions of the aggregation studies. Based on our previous results, we propose that PtNPs interact with MMC primarily through weak intermolecular interactions, which may partially affect the drug's biological availability without inducing extensive aggregation. In our previous studies involving PtNPs combined with daunorubicin (DAU) [31], doxorubicin (DOX) [23], epirubicin (EPI) [24] and acridine mutagen ICR-191 [32] more pronounced aggregation phenomena and modulation effects were observed, suggesting that the PtNPs-MMC interactions are comparatively weaker.

Finally, we investigated the influence of PtNPs on the activity of MMC and DAM in eukaryotic cells *in vitro*. For this purpose, we have selected two breast cancer cell lines differing in the profile of expressed receptors, namely the luminal-A MCF-7 cell line and the HER2-positive SK-BR-3 cell line. Mitomycin alone had a slightly different toxicity profile between the two models, showing greater efficacy towards SK-BR-3 cells, although the general trend of increased cytotoxicity with increasing drug concentration was maintained (**Figure 12**).

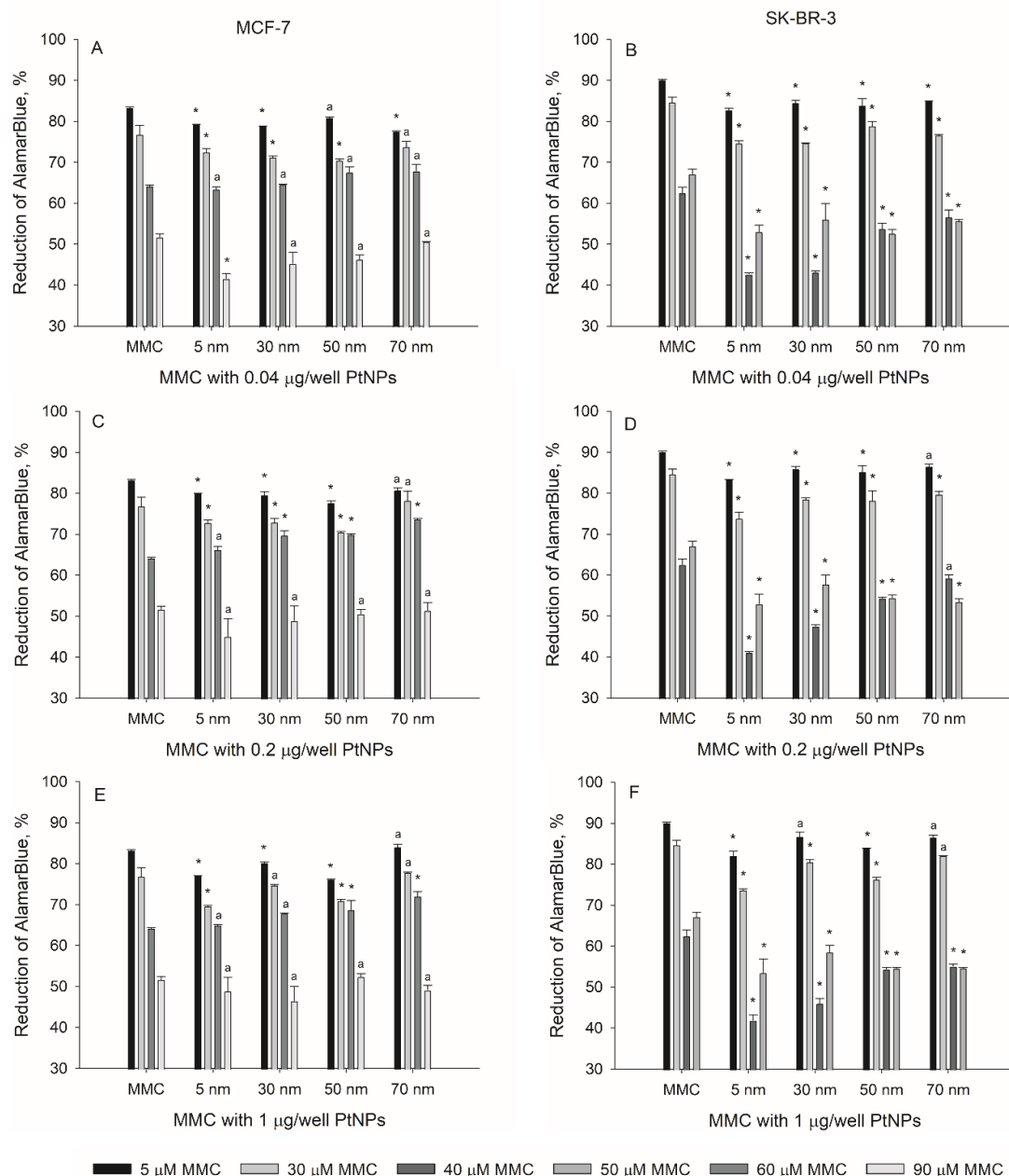


Figure 12. Influence of platinum nanoparticles (PtNPs) on the cytotoxicity of mitomycin C (MMC) dispersed in DMSO on MCF-7 and SK-BR-3 cell lines. Diverse MMC concentrations combined with 0.04, 0.2, and 1 μg/well PtNPs against MCF-7 and SK-BR-3 cell lines (panels A, C, E, and panels B, D, F, respectively). Results are reported as the mean reduction of alamarBlue in regard to the cells treated with the same concentration of mitomycin alone ± standard deviation (SD). a – no significant difference, * – significant difference ($p < \alpha$, $\alpha = 0.05$).

When 5 and 30 μM MMC were combined with PtNPs in the MCF-7 cell line, we observed a statistically significant, though moderate increase in the cytotoxicity of the API in almost all the tested nanoparticle concentrations. However, with 60 μM , the effect was either diminished or, on the contrary, more viability was observed. Ultimately, at 90 μM MMC, we did not register substantial changes in cytotoxicity following the PtNPs addition.

In the SK-BR-3 cell line, all tested combinations of MMC and PtNPs resulted in a significant enhancement of cytotoxicity, with minor fluctuations depending on the nanoparticle size. Namely, 5 nm PtNPs frequently induced the greatest decrease in metabolic activity at higher MMC concentrations, particularly at PtNPs concentrations of 0.2 and 1 $\mu\text{g}/\text{well}$. At the same time, 50 nm and 70 nm PtNPs also demonstrated increased cytotoxic effects under selected conditions, although the response pattern was less uniform than that observed in MCF-7 cells. Interestingly, nanoparticles alone exhibited a notable cytotoxicity level at concentrations of 0.2 and 1 $\mu\text{g}/\text{well}$, but were mostly found not significantly toxic at levels of 0.04 $\mu\text{g}/\text{well}$ (**Figure S6**).

In our last step, we decided to analyse the effect of DAM against the same cell lines with identical combinations of PtNPs as those applied for MMC (**Figure 13**). In contrast to MMC, DAM was a less potent compound, however, it still showed a notable cytotoxicity up to ~78% for MCF-7 and ~68% for SK-BR-3.

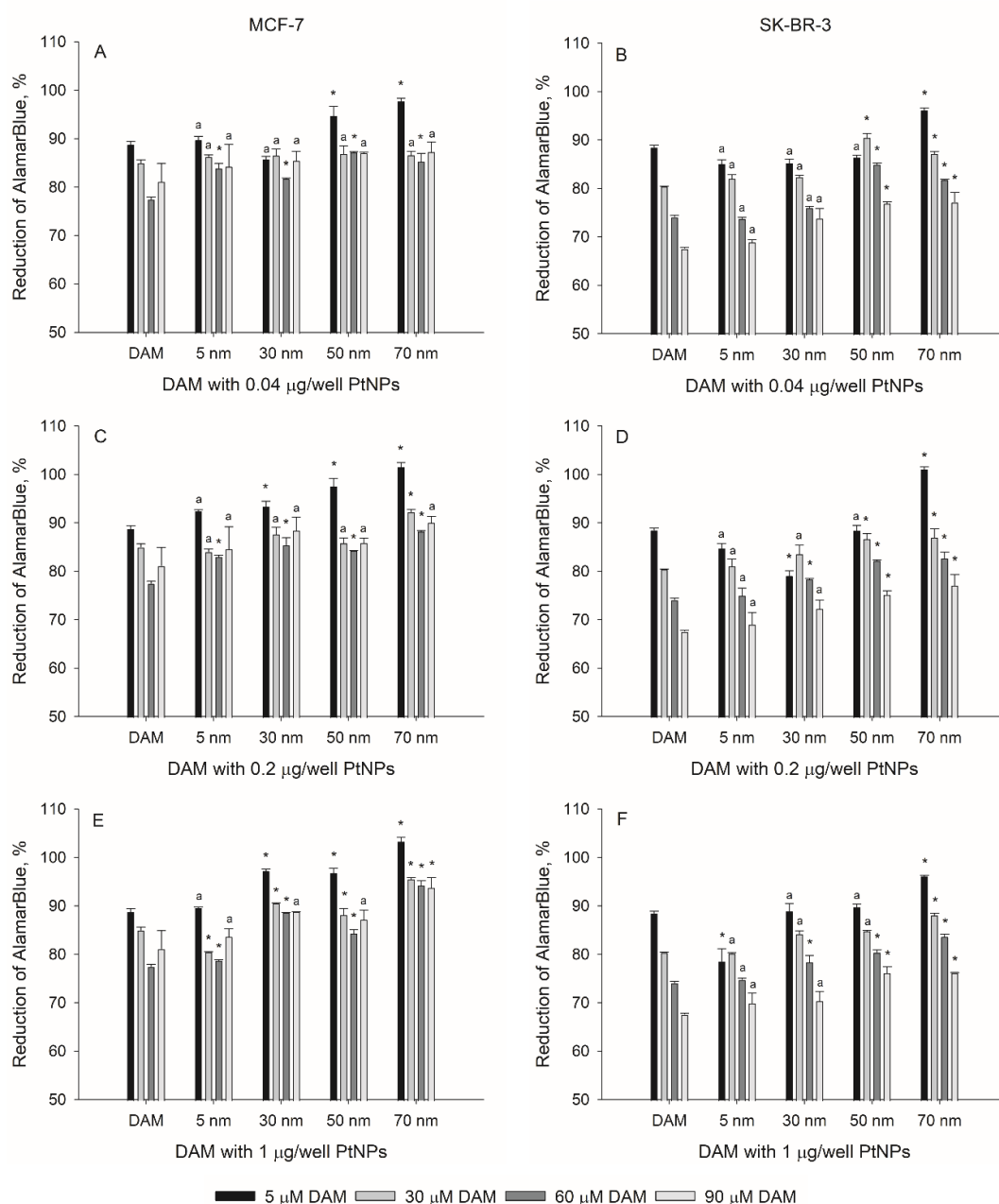


Figure 13. Influence of platinum nanoparticles (PtNPs) on the cytotoxicity of mitosenes (DAM) on MCF-7 and SK-BR-3 cell lines. Diverse MMC concentrations combined with 0.04, 0.2, and 1 μg/well PtNPs against MCF-7 and SK-BR-3 cell lines (panels A, C, E, and panels B, D, F, respectively). Results are reported as the mean reduction of alamarBlue in regard to the cells treated with the same concentration of mitomycin alone ± standard deviation (SD). a – no significant difference, * – significant difference ($p < \alpha$, $\alpha = 0.05$).

Unlike MMC, the addition of PtNPs generally resulted in increased cell viability and attenuation of DAM's cytotoxicity. This effect was most pronounced for larger nanoparticles, particularly 50 and 70 nm PtNPs, suggesting that larger PtNPs interact more efficiently with DAM molecules. A possible explanation for this phenomenon may be related to the aggregation behaviour previously observed in the DLS and AFM experiments, where clear interaction patterns were detected for PtNPs-DAM complexes as opposed to the PtNPs-MMC conjugates. This interpretation is additionally supported by the ITC results, which demonstrated significantly higher interaction enthalpy values for PtNPs-DAM complexes compared to PtNPs-MMC conjugates, indicating stronger intermolecular interactions between DAM and PtNPs.

Overall, the observed differences between the two cell lines are likely due to their distinct biological characteristics. The HER2-positive SK-BR-3 cell line appears to respond more dynamically to PtNPs-MMC conjugates than the MCF-7 cell line. Similar differences in susceptibility between these cell lines were observed in other systems containing gold [33] and silver nanoparticles [34]. In literature, the differences between MCF-7 and SK-BR-3 cell lines' responses to chemotherapeutics and other cytotoxic agents have been described. Although the susceptibility can vary, SK-BR-3 cells are known for their aggressiveness and invasiveness due to a higher proliferation rate, stronger metabolic activity, and greater membrane endocytosis [35–37].

Altogether, these results show a fundamentally different interaction pattern and influence of PtNPs towards MMC and DAM. In the case of MMC, combining it with nanoparticles tended to increase the cytotoxicity, possibly by improving the drug delivery or facilitating cellular uptake. In contrast, PtNPs seemed to reduce the biological activity of DAM, most likely through adsorption, aggregation, or sequestration of DAM molecules, resulting in a lowering of their biological activity combined. These findings may indicate that PtNPs preferentially support the activity of MMC, yet they can act as a neutralising agent for reactive MMC degradation products such as diaminomitosenes. Since DAM retains its biological activity, including both cytotoxic and potentially nonspecific effects, the sequestration of these molecules may reduce off-target interactions. On the other hand, this process may also partially diminish the overall cytotoxic contribution of MMC metabolites. Although the present results clearly demonstrate strong interactions between PtNPs and DAM, the overall biological consequence of this phenomenon remains uncertain and requires further, more mechanistic-focused investigation. Nevertheless, the

cytotoxicity results suggest that PtNPs can modulate the activity of both MMC and DAM, resulting in enhancing or diminishing their activity, respectively. The effect was dependent on nanoparticle size and concentration, with 5 nm tending to have the greatest influence overall, and the highest concentration leading to the least effectiveness of both types of API.

4. Conclusions

To conclude, we investigated the interactions between mitomycin C (MMC), its degradation metabolite 2,7-diaminomitosene (DAM), and platinum nanoparticles (PtNPs) of unique sizes 5, 30, 50 and 70 nm. While the aggregation patterns observed for MMC in combination with PtNPs were inconclusive, DAM consistently exhibited pronounced aggregation with nanoparticles in all tested sizes. The spectroscopic analyses revealed the formation of weak non-covalent interactions between PtNPs and MMC, which was further supported by thermal analyses, which demonstrated the endothermic nature of interactions in both inactive and active forms of API, though more noticeable in the latter. Moreover, all tested PtNPs sizes influenced the mutagenic potential of MMC and DAM in a dose-dependent manner, with more pronounced linearity in the case of the latter. Cytotoxicity analyses performed on MCF-7 and SK-BR-3 breast cancer cell lines demonstrated that PtNPs enhanced the toxicity of MMC, whereas interactions with DAM, likely associated with a stronger aggregation phenomenon, reduced its anticancer potential in a dose-dependent manner. Among all the sizes, 5 nm PtNPs were assessed to provide the most favourable enhancement of MMC activity. Overall, the obtained results suggest that platinum nanoparticles interact relatively weakly with MMC, while exhibiting strong aggregation tendencies toward its metabolite DAM. These findings support the concept of a potential nanoparticle-assisted drug delivery system, in which inactive MMC may exert synergistic anticancer effects intracellularly, whereas sequestration of the non-specific DAM metabolite by PtNPs could potentially reduce off-target activity. Nevertheless, further, more mechanistic-focused studies are required to fully elucidate the biological relevance and therapeutic applicability of the described interactions.

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6. Data availability

The data is available upon request.

7. Declaration of competing interests

None.

8. Author contribution statement

M.Z.: Project administration, Investigation, Methodology, Formal analysis, Visualisation, Writing – Original Draft, Review & Editing; **I.K.:** Investigation; **P.B.:** Writing – Review & Editing; **K.G.:** Writing – Review & Editing; **K.B.:** Investigation, Visualisation; **M.J.:** Investigation, Writing – Review & Editing; **D.W.:** Investigation, Writing – Review & Editing; **G.G.:** Writing – Review & Editing; **J.P.:** Conceptualisation, Resources, Supervision, Writing – Review & Editing.

All the authors have approved of the final version of the manuscript.

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Supplementary materials

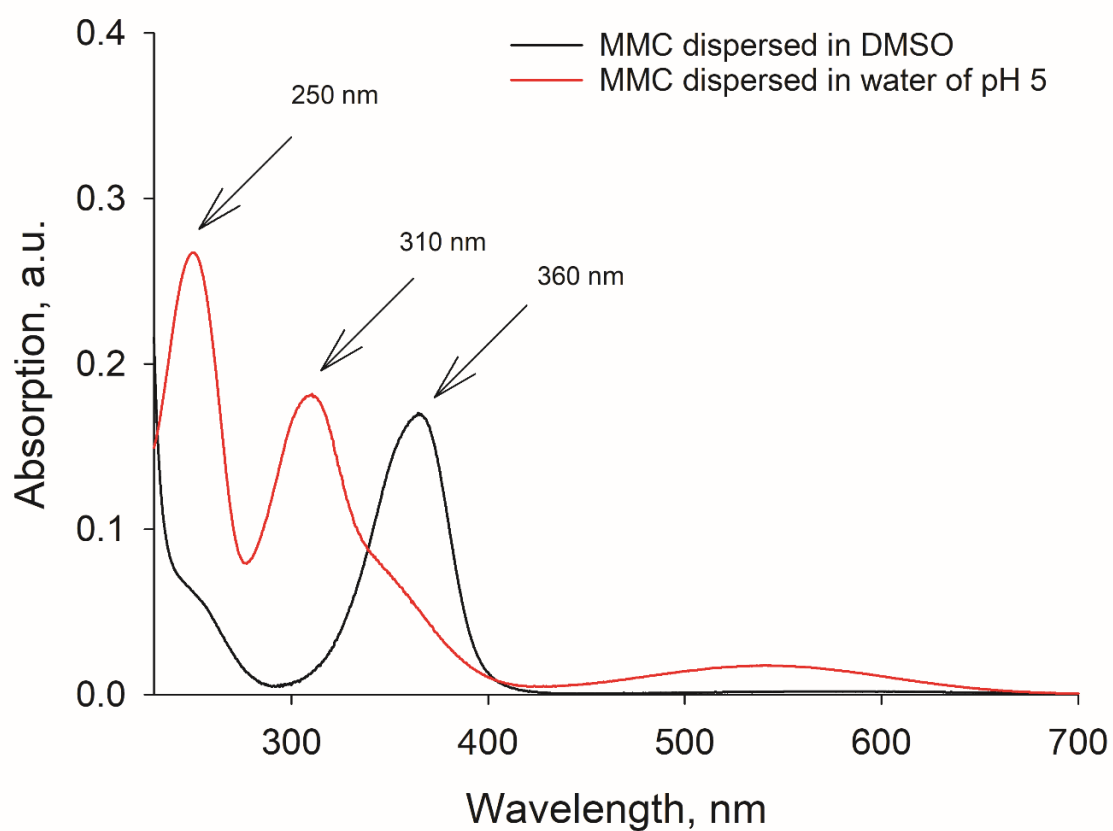


Figure 1. UV-Vis characterisation of mitomycin C (MMC) dispersed in DMSO and under acidic conditions. Arrows indicate peaks characteristic of both intact mitomycin (360 nm) and its degradation products, predominantly 2,7-diaminomitosesene (250 and 310 nm).

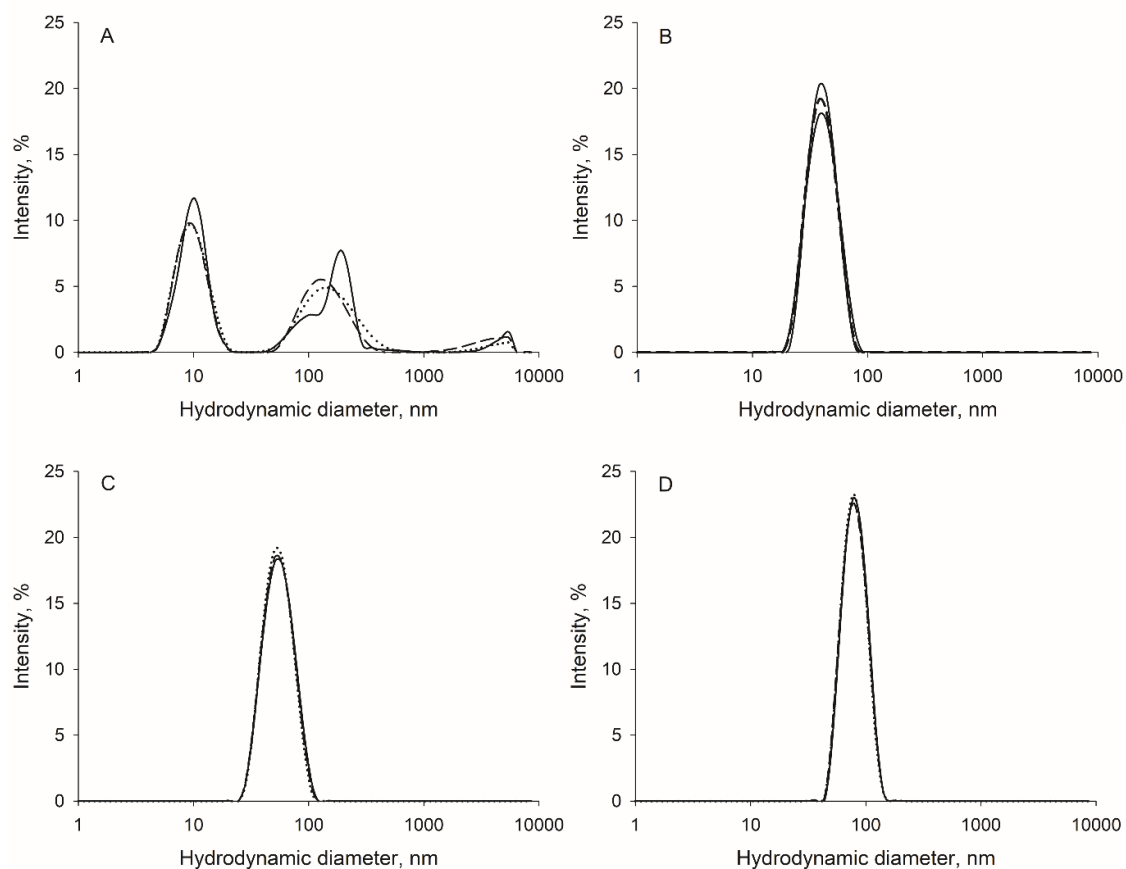


Figure S2. Hydrodynamic diameter of platinum nanoparticles (PtNPs) upon addition of DMSO. (A) – 5 nm PtNPs, (B) – 30 nm PtNPs, (C) – 50 nm PtNPs, (D) – 70 nm PtNPs. Solid line – PtNPs alone, dotted line – PtNPs with identical volume as 6 μ M of MMC, dashed line – PtNPs with identical volume as of 30 μ M MMC.

Table S1. Hydrodynamic diameter of platinum nanoparticles (PtNPs) upon addition of mitomycin C (MMC) dispersed in DMSO. Pdl – polydispersity index, SD – standard deviation.

Sample	Peaks, nm $\pm$ SD	Area, % $\pm$ SD	Pdl $\pm$ SD
5 nm	10.13 $\pm$ 0.34	53.27 $\pm$ 7.38	0.52 $\pm$ 0.12
	164.2 $\pm$ 28.42	42.53 $\pm$ 11.07	
	4574.5 $\pm$ 391.03	4.2 $\pm$ 4.25	
5 nm + 6 μM MMC	9.95 $\pm$ 0.45	57.57 $\pm$ 2.31	0.52 $\pm$ 0.06
	136.47 $\pm$ 18.52	35.7 $\pm$ 3.73	
	4150.33 $\pm$ 750.53	6.73 $\pm$ 5.38	
5 nm + 30 μM MMC	10.18 $\pm$ 0.03	53.47 $\pm$ 1.14	0.61 $\pm$ 0.02
	199.67 $\pm$ 20.32	43.7 $\pm$ 0.95	
	3952.33 $\pm$ 139.75	2.83 $\pm$ 0.4	
30 nm	43.38 $\pm$ 0.7	100	0.08 $\pm$ 0.01
30 nm + 6 μM MMC	41.37 $\pm$ 0.72	100	0.08 $\pm$ 0.01
30 nm + 30 μM MMC	42.44 $\pm$ 0.52	100	0.09 $\pm$ 0.01
50 nm	58.48 $\pm$ 0.75	100	0.09 $\pm$ 0.01
50 nm + 6 μM MMC	55.52 $\pm$ 0.43	100	0.06 $\pm$ 0.01
50 nm + 30 μM MMC	56.54 $\pm$ 0.57	100	0.08 $\pm$ 0.01
70 nm	83.91 $\pm$ 0.15	100	0.07 $\pm$ 0.03
70 nm + 6 μM MMC	80.73 $\pm$ 1.3	100	0.03 $\pm$ 0.01
70 nm + 30 μM MMC	83.22 $\pm$ 0.56	100	0.05 $\pm$ 0.03

Table S2. Hydrodynamic diameter of platinum nanoparticles (PtNPs) upon addition of identical volumes of DMSO as of MMC. Pdl – polydispersity index, SD – standard deviation.

Sample	Peaks, nm $\pm$ SD	Area, % $\pm$ SD	Pdl $\pm$ SD
5 nm	10.13 $\pm$ 0.34	53.27 $\pm$ 7.38	0.52 $\pm$ 0.12
	164.2 $\pm$ 28.42	42.53 $\pm$ 11.07	
	4574.5 $\pm$ 391.03	4.2 $\pm$ 4.25	
5 nm + identical as 6 μM DMSO	10 $\pm$ 0.23	53.07 $\pm$ 1.65	0.62 $\pm$ 0.02
	168.53 $\pm$ 12.13	43.77 $\pm$ 0.78	
	4205.67 $\pm$ 327.48	3.16 $\pm$ 1.05	
5 nm + 30 identical as μM DMSO	9.8 $\pm$ 0.17	51.1 $\pm$ 1.65	0.65 $\pm$ 0.02
	145.37 $\pm$ 3.27	41.8 $\pm$ 3.3	
	3788 $\pm$ 487.12	7.1 $\pm$ 2.72	
30 nm	41.39 $\pm$ 0.24	100	0.06 $\pm$ 0.01
30 nm + identical as 6 μM DMSO	40.98 $\pm$ 0.55	100	0.07 $\pm$ 0.01
30 nm + identical as 30 μM DMSO	41.89 $\pm$ 0.13	100	0.09 $\pm$ 0.01
50 nm	56.87 $\pm$ 0.41	100	0.08 $\pm$ 0.01
50 nm + identical as 6 μM DMSO	55.54 $\pm$ 1.12	100	0.06 $\pm$ 0.01
50 nm + identical as 30 μM DMSO	56.01 $\pm$ 0.5	100	0.07 $\pm$ 0.01
70 nm	82.26 $\pm$ 0.72	100	0.03 $\pm$ 0.01
70 nm + identical as 6 μM DMSO	80.96 $\pm$ 1.38	100	0.03 $\pm$ 0.03
70 nm + identical as 30 μM DMSO	81.16 $\pm$ 0.35	100	0.04 $\pm$ 0.02

Table S3. Hydrodynamic diameter of platinum nanoparticles (PtNPs) upon addition of mitosenes (DAM). Pdl – polydispersity index, SD – standard deviation.

Sample	Peaks, nm $\pm$ SD	Area, % $\pm$ SD	Pdl $\pm$ SD
5 nm	9.74 $\pm$ 0.45	55.3 $\pm$ 2.08	0.59 $\pm$ 0.02
	134.97 $\pm$ 23.14	41.63 $\pm$ 3.19	
	4785.67 $\pm$ 290.9	3.07 $\pm$ 1.82	
5 nm + 6 μ M DAM	133.8 $\pm$ 9.43	90.6 $\pm$ 5.95	0.47 $\pm$ 0.1
	13.58 $\pm$ 6.08	8.97 $\pm$ 6.07	
	4402.67 $\pm$ 403.6	0.43 $\pm$ 0.75	
5 nm + 30 μ M DAM	279.83 $\pm$ 46.5	100	0.21 $\pm$ 0.02
30 nm	41.81 $\pm$ 0.64	100	0.08 $\pm$ 0.01
30 nm + 6 μ M DAM	91.62 $\pm$ 12.13	100	0.14 $\pm$ 0.02
30 nm + 30 μ M DAM	132.07 $\pm$ 1.46	100	0.17 $\pm$ 0.01
50 nm	56.44 $\pm$ 0.99	100	0.07 $\pm$ 0.01
50 nm + 6 μ M DAM	78.63 $\pm$ 13.4	100	0.1 $\pm$ 0.01
50 nm + 30 μ M DAM	120.03 $\pm$ 9.77	100	0.16 $\pm$ 0.04
70 nm	81.77 $\pm$ 0.44	100	0.02 $\pm$ 0.01
70 nm + 6 μ M DAM	105.97 $\pm$ 4.5	100	0.08 $\pm$ 0.01
70 nm + 30 μ M DAM	142.47 $\pm$ 5.85	100	0.1 $\pm$ 0.01

Table S4. Data from atomic force microscopy analysis for PtNPs alone and in combination with either mitomycin C (MMC) or mitosenes (DAM). SD – standard deviation.

	5 nm PtNPs			30 nm PtNPs			50 nm PtNPs			70 nm PtNPs		
	Alone	With MMC	With DAM	Alone	With MMC	With DAM	Alone	With MMC	With DAM	Alone	With MMC	With DAM
Mean	11.7	162.2	285.4	38	565.6	1105.2	55	1200.2	1502.9	78	1001.4	1829.7
SD	2.6	104.6	171.2	6.8	372.4	602.7	4.1	1019.3	1143.1	6.6	594.6	1784.8

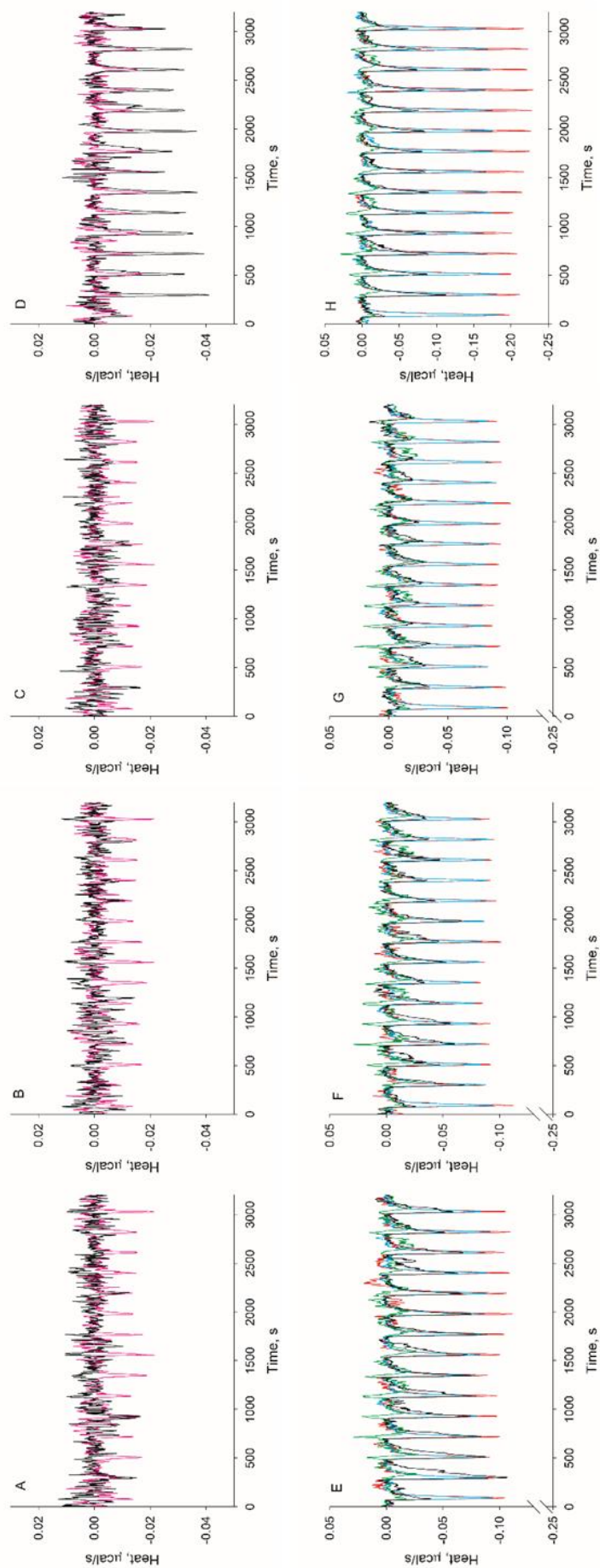


Figure S3. Thermograms for the ITC analyses on platinum nanoparticles (PtNPs) with mitomycin C (MMC) dispersed in sodium citrate (A – D) and DAM (E – H). (A, E) – 5 nm PtNPs, (B, F) – 30 nm PtNPs, (C, G) – 50 nm PtNPs, (D, H) – 70 nm PtNPs. PtNPs with MMC – black, PtNPs with appropriate sodium citrate – pink, water with PtNPs – red, water with appropriate sodium citrate – blue, MMC/DAM with appropriate sodium citrate – green.

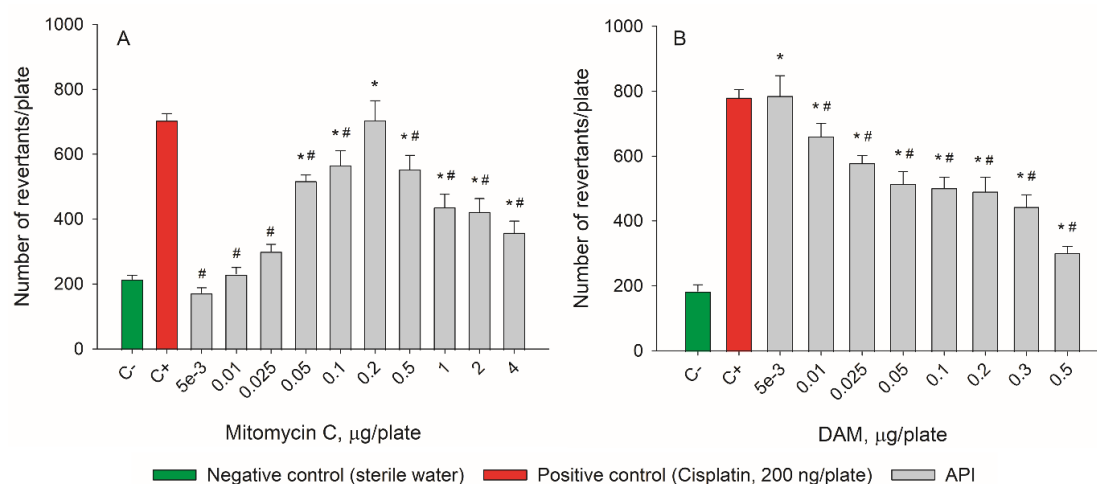


Figure S4. Mutagenicity of mitomycin C (MMC) and mitosenes (DAM) on *Salmonella enterica* serovar Typhimurium TA102. C- negative control, C+ positive control, API – active pharmaceutical ingredient (either mitomycin C or mitosenes). * - significant difference from the negative control, # – significant difference from the positive control; $p < 0.05$, $\alpha = 0.05$.

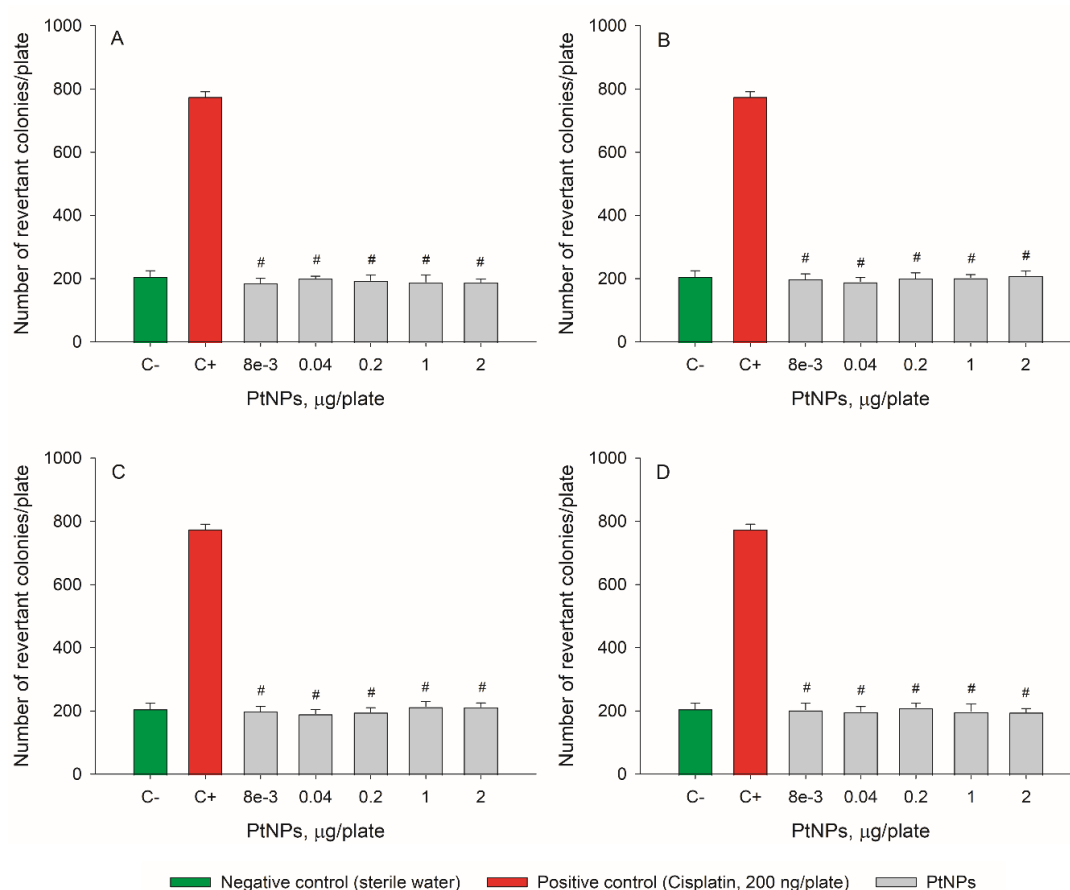


Figure S5. Effects of platinum nanoparticles (PtNPs) on *Salmonella enterica* serovar Typhimurium TA102. (A-D) – 5, 30, 50, and 70 nm, respectively. C- negative control, C+ positive control, PtNPs – platinum nanoparticles. # - significant difference from the positive control; $p < 0.05$, $\alpha = 0.05$.

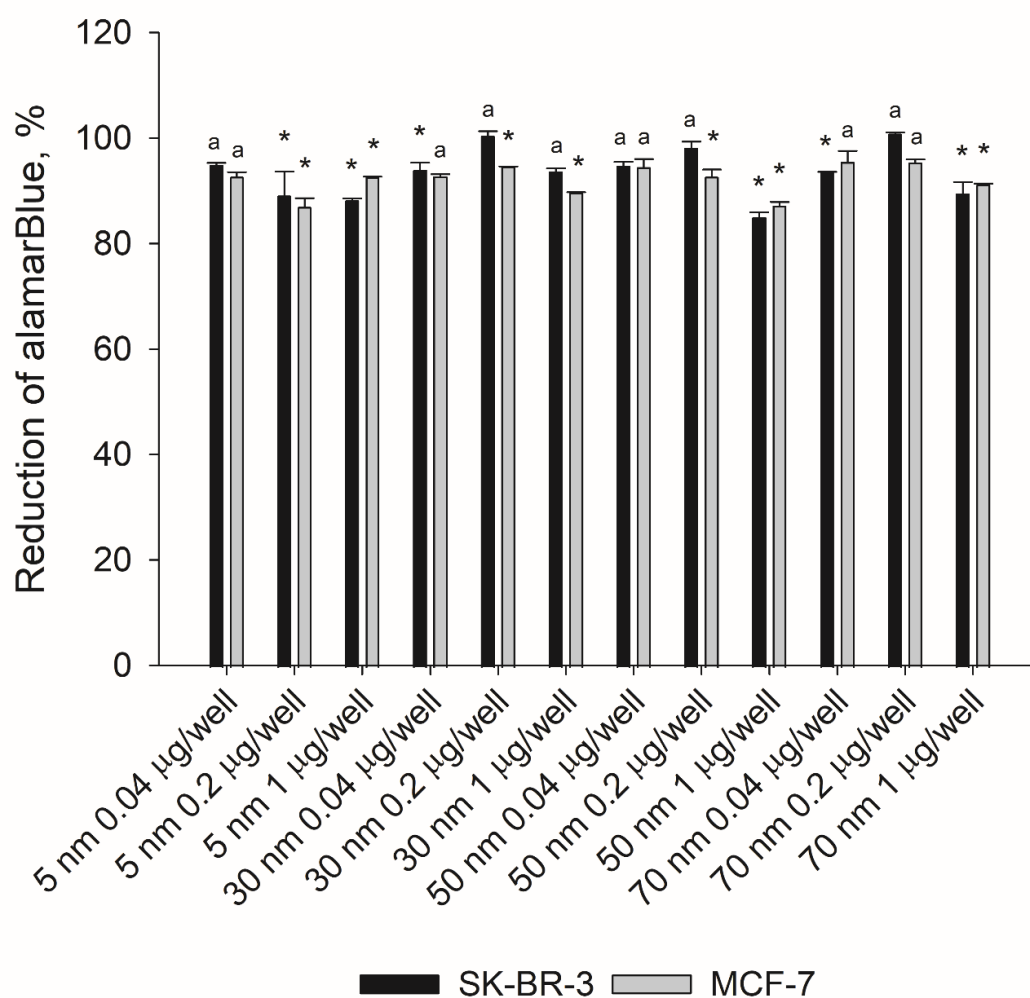


Figure S6. Cytotoxicity of platinum nanoparticles (PtNPs) on MCF-7 and SK-BR-3 cell lines. Results are reported as the mean reduction of alamarBlue in regard to untreated cells $\pm$ standard deviation (SD). a – no significant difference, * - significant difference ($p < \alpha$, $\alpha = 0.05$).

11. Author contribution statements

My contribution to the manuscripts that constitute the basis of this doctoral thesis are as follows:

1. The effects of platinum nanoparticles on physicochemical properties of daunorubicin

In this work I wrote the original version of the manuscript, and upon the review of the colleagues involved in this work, I applied the suggested corrects/comments. I contributed to each of these methods in the following way:

- DLS – planning, preparation of the samples, execution of analyses, data processing alongside it's interpretation, and preparation of graphs
- Fluorescence spectroscopy – planning, preparation of the samples, execution of analyses, data processing alongside it's interpretation, and preparation of graphs
- NTA – planning, preparation of the samples and data analysis, interpretation of the results
- FTIR, NIR and DSC – planning, preparation of the samples, co-interpretation of the results
- ITC – planning, preparation of the samples, data processing alongside it's interpretation, and preparation of graphs
- Release analysis – preparation of the samples, execution of analyses, data processing and it's interpretation, and graph preparation
- Ames mutagenicity assay – planning, preparation of samples, execution of analyses, data processing alongside it's interpretation, and graph preparation
- Cytotoxicity assays – planning, preparation of samples, data processing alongside it's interpretation, and graph preparation

2. Platinum nanoparticles as modulators of idarubicin activity: a physicochemical and *in vitro* biological study

In this work I wrote the original version of the manuscript, and upon the review of the colleagues involved in this work, I applied the suggested corrects/comments. I contributed to each of these methods in the following way:

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- AFM – planning, preparation of the samples, interpretation of the results
- FTIR, NIR and DSC – planning, preparation of the samples, co-interpretation of the results
- ITC – planning, preparation of the samples, data processing alongside it's interpretation, and preparation of graphs
- Ames mutagenicity assay – planning, preparation of samples, execution of analyses, data processing alongside it's interpretation, and graph preparation
- Cytotoxicity assays – planning, preparation of samples, execution of analyses, data processing alongside it's interpretation, and graph preparation

3. Comparative analysis of platinum nanoparticles interactions with mitomycin C and its activated metabolite

In this work I wrote the original version of the manuscript, and upon the review of the colleagues involved in this work, I applied the suggested corrects/comments. I contributed to each of these methods in the following way:

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- AFM – planning, preparation of the samples, interpretation of the results
- FTIR, NIR and DSC – planning, preparation of the samples, co-interpretation of the results, and preparation of graphs
- ITC – planning, preparation of the samples, data processing alongside it's interpretation, and preparation of graphs
- Ames mutagenicity assay – planning, preparation of samples, execution of analyses, data processing alongside it's interpretation, and graph preparation
- Cytotoxicity assays – planning, preparation of samples, execution of analyses, data processing alongside it's interpretation, and graph preparation

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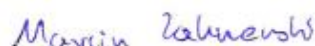
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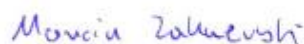
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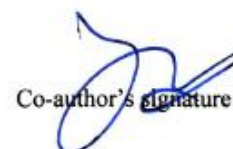
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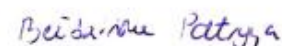
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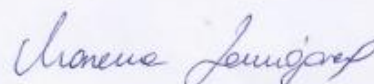
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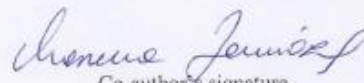
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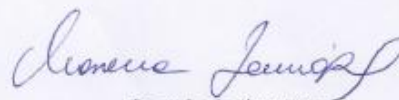
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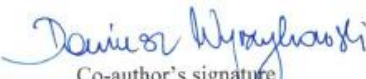
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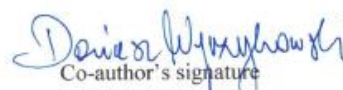
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Gdańsk, 12.06.2026

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AUTHOR CONTRIBUTION STATEMENT

I hereby declare my contribution to the following manuscripts:

1) Zakrzewski M., Beldzińska P., Gajdowska F., Gołński G., Gackowska K., Strankowska J., Jamróiewicz M., Wyrzykowski D., Grzyb K., Gutowska-Owsiak D., Synak A., Bojarski P., Piosik J. The effect of platinum nanoparticles on the physicochemical properties of daunorubicin. RSC Adv. 2025 Sep 25;15(42):35407-35420. DOI: 10.1039/d5ra02827b.

Investigation, Writing – review & editing

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AUTHOR CONTRIBUTION STATEMENT

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Resources

My contribution to this paper came down to the discussion of spectroscopic part of the results as well as some improvement of this part of the manuscript.



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12. Curriculum vitae

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Education:

2022 – 2026	Intercollegiate Biotechnology Doctoral School of University of Gdańsk and Medical University of Gdańsk, Poland	PhD candidate in Biotechnology
2021 – 2021	Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Belgium	Erasmus+ exchange programme
2020 – 2022	Intercollegiate Faculty of Biotechnology of University of Gdańsk and Medical University of Gdańsk, Poland	Master's degree in Biotechnology
2017 - 2020	Intercollegiate Faculty of Biotechnology of University of Gdańsk and Medical University of Gdańsk, Poland	Bachelor's degree in Biotechnology

Publications:

Manuscripts:

1. **Zakrzewski M.**, Beldzińska P., Gackowska K., Yurchak A., Jamróiewicz M., Wyrzykowski D., Bury K., Grzyb K., Gołuński G., Piosik J. Platinum nanoparticles as modulators of idarubicin activity: a physicochemical and *in vitro* study.
2. **Zakrzewski M.**, Kalisz I., Beldzińska P., Grzyb K., Bury K., Jamróiewicz M., Wyrzykowski D., Gołuński G., Piosik J. Comparative analysis of platinum nanoparticles interactions with mitomycin C and its activated metabolite.

Published

1. **Zakrzewski M.**, Beldzińska P., Gajdowska F., Gołuński G., Gackowska K., Strankowska J., Jamróiewicz M., Wyrzykowski D., Grzyb K., Gutowska-Owsiak D., Synak A., Bojarski P., Piosik J. The effect of platinum nanoparticles on the physicochemical properties of daunorubicin. RSC Adv. 2025 Sep 25;15(42):35407-35420. doi: 10.1039/d5ra02827b
2. Beldzińska P., **Zakrzewski M.**, Grzyb K., Hauer A., Jamróiewicz M., Wyrzykowski D., Bury K., Gołuński G., Piosik J. Platinum nanoparticles interact with epirubicin in size dependent manner and affect its biological activity. Eur J Pharm Biopharm. 2025 Nov;216:114866. doi: 10.1016/j.ejpb.2025.114866
3. Prylutsky Y., Beldzińska P., Derewońko N., Halenova T., Raksha N., **Zakrzewski M.**, Gołuński G., Prylutska S., Ritter U., Savchuk O., Piosik J. Biosafety and Blood Compatibility of Graphene Oxide Particles in In Vitro Experiments. Materials (Basel). 2025 May 6;18(9):2128. doi: 10.3390/ma18092128
4. Beldzińska P., **Zakrzewski M.**, Mruk I., Bogusławski M., Derewońko N., Bury K., Wyrzykowski D., Gołuński G., Rychłowski M., Piosik J. Size dependent impact of platinum nanoparticles on doxorubicin activity. Eur J Pharm Sci. 2025 Jun 1;209:107094. doi: 10.1016/j.ejps.2025.107094

5. Beldzińska P., Galikowska-Bogut B., **Zakrzewski M.**, Bury K., Jamrógiewicz M., Wyrzykowski D., Gołński G., Sądej R., Piosik J. Platinum as both a drug and its modulator - Do platinum nanoparticles influence cisplatin activity? Chem Biol Interact. 2025 Feb 1;407:111365. doi: 10.1016/j.cbi.2024.111365
6. Gołński G., Konkel K., Galikowska-Bogut B., Beldzińska P., Bury K., **Zakrzewski M.**, Butowska K., Sądej R., Piosik J. Influence of silver nanoparticles' size on their direct interactions with doxorubicin and its biological effects. Sci Rep. 2024 Aug 9;14(1):18544. doi: 10.1038/s41598-024-69724-6. Erratum in: Sci Rep. 2024 Oct 14;14(1):24050. doi: 10.1038/s41598-024-75135-4
7. Jamrógiewicz M., Bray A., Gołński G., Beldzińska P., **Zakrzewski M.** Photodegradation of indomethacin and naproxen contained within commercial products for skin - RAP. J Pharm Biomed Anal. 2024 Aug 15;246:116201. doi: 10.1016/j.jpba.2024.116201

Conferences:

Main authorship:

1. Poster presentation entitled “Platinum nanoparticles interact with idarubicin and affect its biological activity” – **Marcin Zakrzewski**, Patrycja Beldzińska, Grzegorz Gołński, Dariusz Wyrzykowski, Katarzyna Bury, Jacek Piosik. 19th Congress of the Polish Biophysical Society PTBF2025, 23-26.06.2025, Warsaw, Poland.
2. Poster presentation entitled “Various size of platinum nanoparticles and their influence on daunorubicin” – **Marcin Zakrzewski**, Patrycja Beldzińska, Grzegorz Gołński, Jacek Piosik. 5th Congress of Polish Biosciences BIO2023, 13-16.09.2023, Szczecin, Poland.
3. Poster presentation entitled „Platinum nanoparticles influence on daunomycin” – **Marcin Zakrzewski**, Patrycja Beldzińska, Grzegorz Gołński, Jacek Piosik. IV Pomorskie Studenckie Sympozjum Chemiczne, 23-24.04.2022, on-line.
4. Oral presentation entitled “Platinum nanoparticles as potential modulators of the activity of selected anticancer drugs” – **Marcin Zakrzewski**, Patrycja Beldzińska. Seminar for the Polish Biophysical Society, 21.03.2023, Gdańsk, Poland.

Co-authorship:

1. Oral presentation entitled “Metallic nanoparticles as potential modulators of anticancer therapy” – Grzegorz Gołuński, Patrycja Bełdzińska, **Marcin Zakrzewski**, Barbara Galikowska-Bogut, Natalia Derewońko, Katarzyna Bury, Marzena Jamrógiewicz, Dariusz Wyrzykowski, Rafał Sądej, Jacek Piosik. 19th Congress of the Polish Biophysical Society PTBF2025, 23-26.06.2025, Warsaw, Poland.
2. Poster presentation entitled “How do platinum nanoparticles affect the biological activity of doxorubicin?” – Patrycja Bełdzińska, **Marcin Zakrzewski**, Inez Mruk, Natalia Derewońko, Katarzyna Bury, Grzegorz Gołuński, Michał Rychłowski, Jacek Piosik. 19th Congress of the Polish Biophysical Society PTBF2025, 23-26.06.2025, Warsaw, Poland.
3. Poster presentation entitled “Platinum nanoparticles as cisplatin modulators – direct interactions and biological effects” – Patrycja Bełdzińska, Kamila Butowska, Grzegorz Gołuński, **Marcin Zakrzewski**, Barbara Galikowska-Bogut, Rafał Sądej, Augustyn Moliński, Jacek Piosik. 5th Congress of Polish Biosciences BIO2023, 13-16.09.2023, Szczecin, Poland.
4. Oral presentation entitled “Silver nanoparticles as doxorubicin carriers – direct interactions and modulation of biological effects” – Kinga Konkel, Barbara Galikowska-Bogut, Patrycja Bełdzińska, Katarzyna Bury, **Marcin Zakrzewski**, Kamila Butowska, Rajmund Kaźmierkiewicz, Rafał Sądej, Jacek Piosik, Grzegorz Gołuński. 5th Congress of Polish Biosciences BIO2023, 13-16.09.2023, Szczecin, Poland.

Awards:

Third-degree Rector’s Award in 2025.

Cerificates and courses:

1. Master Pharma Regulatory Affairs: FDA/EMA & Global (Udemy) – 26.03.2026
2. Learn Excel, Canva, PowerBI or Photoshop (Santander Open Academy) – 09.05.2024
3. Intellectual Property & Innovation Week (University of Gdańsk) – 25.04.2024

4. English and IELTS with British Council 2023 (Santander Open Academy) – 02.06.2023 – 31.01.2024
5. INDEPTH Summer School 2023 "Deep-Sea Chat – 22-25.08.2023
6. Bioinformatics and Comparative Genomics – 13-17.03.2023
7. StatSoft Statistica course – 24.02.2023
8. Workshop on the use of Leica confocal microscope – 24.11.2022
9. Microscopy of Tomorrow – A Sea of Possibilities (KAWA.SKA) – 23.11.2022
10. Systems biology - computational biology – 8-18.11.2022
11. Good Laboratory Practices (LabISO) – 24.02.2022
12. Advanced Training in the Principles and Operation of the Malvern NanoSight NS300 Analyzer – 24.06.2021

Additional activities:

The PhD candidate is/was a member of the following groups:

- Doctoral Students Council of the University of Gdańsk (2023 – 2025)
- Disciplinary Committee for Doctoral Students of University of Gdańsk (2023 – 2025)
- Budget Committee for Doctoral Students of University of Gdańsk (2023 – 2025)
- Doctoral Students Council of Intercollegiate Biotechnology Doctoral School of University of Gdańsk and Medical University of Gdańsk (2023 – 2025)
- Polish Biophysical Society (2021 – now)
- Students Council at the Intercollegiate Faculty of Biotechnology (2019 – 2021)

Besides that, the candidate took part in the following events:

- Co-organizer of an on-line international conference “Natural Science Baltic Conference 2025”
- Educator at the Night of Biologists 2024