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Review Report on the doctoral thesis of Ying Zhou entitled:

Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study

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Project background

Among the most recent challenges facing modern pharmacy is the design of new cancer drugs, where compounds that bind strongly to deoxyribonucleic acid (DNA) are sought. Such compounds include anthracyclines (namely: doxorubicin, daunorubicin, epirubicin, and idarubicin hydrochlorides), which are considered the most effective drugs in cancer therapy because they induce apoptosis of cancer cells by binding to their DNA. In order to quantify how strongly anthracyclines can bind to DNA, it becomes necessary to determine the equilibrium constants of anthracycline-DNA interaction (association and dissociation constants). However, there are currently several problems with appropriate determination of the equilibrium constants for the mentioned compounds because: 1) they are characterized by low values; 2) the limits of detection of the studied compounds depend on their concentration (the phenomenon of aggregation at high concentrations of anthracyclines), 3) the mechanisms of action of anthracyclines are not fully understood, 4) the crowding effect of macromolecules on anthracycline-DNA interactions. These unresolved problems gave rise to the research topic of Ying Zhou, who addressed the issues by studying the changes in fluorescence properties of anthracyclines during their interactions. It can be unequivocally concluded that the research topic presented in the

reviewed doctoral dissertation falls into the most current and important topics from a scientific point of view.

Evaluation of formal aspects of the thesis

The reviewed dissertation is written in English and comprises 102 pages, without adding the title page, table of contents, abstract, and the list of abbreviations and appendices included at the end of the dissertation. It is illustrated with 28 figures and includes six tables. The dissertation has a classical layout and is divided into an introduction combined with a literature section (34 pages), an experimental section in which three subsections can be distinguished (total of 32 pages) a summary and conclusions (4 pages). I think paper's layout is appropriate, the content of the chapters is consistent with the titles given, and the chapters are a logical development of the dissertation's main topic.

The dissertation does not clearly specify the primary objective of the work, where the PhD Candidate should demonstrate the ability to clearly define and formulate the scientific problem.

The dissertation ends with the literature list, which includes 137 items. The references list is well done, particularly the latest scientific reports are included.

General description of the thesis

In the introduction to her dissertation (Chapter 1), Ying Zhou provided background information on anthracyclines and mechanisms of anthracycline-DNA interactions (1.1), equilibrium constants of anthracycline-DNA interactions (1.2), equilibrium constants in the crowded environment (1.3) and the techniques and methodologies used to determine the equilibrium constants in this work (1.4). Discussion of these issues became essential to the research tasks.

The thesis main part (experimental part) consists of three chapters (Chapters 2-4), each of which deals with a specific scientific problem. All of them have the same structure and are divided into sections typical for scientific papers: introduction, experimental, discussion, conclusion and references. The advantage of this structure is that the chapters, consisting of parts typical for the scientific papers, can be read and analyzed separately.

However, there is a disadvantage in that some portions of the information contained in the introduction, materials, experimental, and discussion chapters are repeated.

The first experimental chapter (Chapter 2) deals with the study of the anthracycline self-aggregation process, which, according to the Author, can compete with anthracycline-DNA interactions and affect *K* values in anthracycline-DNA interactions. For this purpose, the analysis of the fluorescence absorption and emission spectra for all anthracyclines at different concentrations was performed. The results obtained allowed The Author to confirm that anthracyclines do not have the ability to self-aggregate at the micromolar level. Ms. Zhou proved that the *K* values of anthracycline-DNA interactions are not altered by the anthracycline self-aggregation process.

The third chapter describes the determination of the *K*-value for the formation of anthracycline-DNA complexes. The imperative of this part of the study was to exclude the influence of macromolecules on the kinetic studies. The Candidate performed the preliminary studies in the aqueous medium and after that - under physiological conditions. She determined equilibrium constants at the nanomolar level using fluorescence correlation spectroscopy and molecule brightness. The results obtained in these studies clearly confirmed the course of two types of reactions in doxorubicin-DNA interactions.

In the last experimental chapter, Ms. Zhou developed a method based on the bleaching of immobilized fluorescent molecules to determine the equilibrium constant in the nucleus of a single living cell. The obtained results enabled to propose three mechanisms such as histone hindrance, side reactions and ionic strength. In addition, the PhD student found that the affinity of doxorubicin varies by 50% from cell to cell, which may be due to the intracellular heterogeneity of cancer cells.

The results of this research are two publications in highly rated journals: The Analyst, IF=3.978 (*Single molecule brightness analysis for determination of anticancer drugs interactions with DNA*) and Physical Chemistry Chemical Physics, IF=3.43 (*Fluorescence correlation spectroscopy for multiple-site equilibrium binding: a case of doxorubicin-DNA interaction*). In addition to these articles, the Candidate has three more articles in her academic record outside the topic of her dissertation.

The general conclusions of the thesis are summarized in the concluding section. The thesis is prepared to a good editorial standard. All figures are carefully prepared and clearly presented. The language is comprehensive and consistent, and errors and inaccuracies are relatively rare.

To summarize this review part, I conclude that the obtained results are positive and scientifically relevant. The most important achievements of the discussed dissertation, which mainly used three different methods based on fluorescence properties of anthracyclines to determine equilibrium constants in the anthracycline-DNA interactions, include:

1) demonstrating that anthracyclines do not self-aggregate at the micromolar level, so the *K* values of anthracycline-DNA interactions are not altered by the anthracycline self-aggregation process;
2) suggesting two mechanisms of doxorubicin action - intercalation and electrostatic binding confirmed by single-MB method;
3) development of a method based on bleaching of immobilized fluorescent molecules to determine the equilibrium constant in the nucleus of a single living cell;
4) confirmation that doxorubicin is a non-affinity specific anticancer drug to cells.

In general, the presented results seem to be reliable and well documented. However, it should be noted that always in the case of recent studies, the interpretation and discussion could be broader and more in-depth. Undoubtedly, this is a common feature of all experimental work related to the search for new anticancer drugs, with particular emphasis on studies of binding of the drug with DNA. I would like to emphasize that these papers cover a wide area of research, and their main value lies in the detailed description of the obtained results. This makes it easy to plan further work in this area, which the Ms. Zhou has undoubtedly made very clear in Chapter 6 dealing with future work to be done to achieve more accurate *K* determination in cells. In my opinion, the greatest achievement of the PhD student should be the work related to the description of anthracycline-DNA interactions at the single-molecule level, which gives a very valuable contribution to the search for new drugs and cancer treatment. It is in this direction that the latest trends in research on new drugs are heading.

Final evaluation statement

Ms. Ying Zhou's dissertation is an original research paper. The obtained research results enrich the knowledge in the field of physical chemistry, which can be used in biochemistry, pharmacy and medicine. The reviewed dissertation contains elements of scientific novelty, which makes it possible to positively evaluate it.

The assessed dissertation is the result of a huge amount of work and the results obtained are well presented. The interpretation of the data is at a very good scientific level. I highly appreciate the experience of Ms. Ying Zhou in physical chemistry (determination of equilibrium constants) necessary to understand the drug-DNA interaction. This research was performed at a very high scientific level.

In my opinion, the reviewed paper entitled: *Equilibrium constants determination for anthracycline-DNA interactions: from aqueous solution to single cell study* fulfills the requirements for a doctoral dissertation (Article 13 of the Act of 14 March 2003 on Academic Degrees and Academic Title and Degrees and Title in Art, Journal of Laws No. 65, item 595, as amended). In view of the above, I recommend the Council of the Discipline of Chemical Sciences, Institute of Chemical Physics, Polish Academy of Sciences in Warsaw, to admit Ms. Ying Zhou, M.Sc. to further stages of the PhD degree conferment procedure.

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