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A study of selected endocrine disrupting chemicals and their binding to host molecules with molecular modelling

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ECOLE DOCTORALE

Titre: Une étude de certains perturbateurs endocriniens et de leur liaison aux molécules hôtes avec modélisation moléculaire

Mots clés: estradiol, cyclodextrine, champ de force AMOEBA, paramétrisation du champ de force, DFT, perturbateurs endocriniens

Résumé: Les perturbateurs endocriniens (Endocrine Disrupting Chemicals, EDC) sont des substances qui présentent des effets néfastes en raison d'un mode d'action endocrinien. Cela inclut souvent une interaction avec les récepteurs de la même manière que les ligands naturels des récepteurs. Parmi les EDC, il existe des ingrédients pharmaceutiques actifs (API) tels que les stéroïdes hormonaux. Les cyclodextrines (CD) sont des oligosaccharides cycliques utilisés comme systèmes d'administration de médicaments pour les API à faible solubilité dans l'eau et comme agents d'élimination des toxines. Le but de cette étude était de développer différentes techniques de modélisation moléculaire pour analyser les interactions entre les EDC choisis et les récepteurs d'œstrogènes ou CD. Les méthodes suivantes ont été appliquées : paramétrisation des EDC choisis (estradiol, progestérone, biephénol A) et CD dans le champ de force polarisable AMOEBA et simulation dynamique moléculaire réussie du système récepteur d'œstrogène + EDC ; tests de référence de diverses approches de calcul basées sur la mécanique quantique (DFT, MP2, semi-empirique) et la mécanique moléculaire (MD/MMGBSA) et les paramètres applicables, sur l'exemple du système estradiol+βCD.

Title: A study of selected endocrine disrupting chemicals and their binding to host molecules with molecular modelling

Keywords: estradiol, cyclodextrin, AMOEBA force field, force field parametrization, DFT, endocrine disrupting chemicals

Abstract : Endocrine Chemical Disruptors (EDCs) are substances that exhibit adverse effects as a consequence of an endocrine mode of action. It often includes interaction with receptors in the same way as receptor's natural ligands. Among EDCs there are Active Pharmaceutical Ingredients (APIs) such as steroid hormones. Cyclodextrins (CDs) are cyclic oligosaccharides used as drug delivery systems for APIs of a low solubility in water, and as toxin removing agents. The goal of this study was to develop different molecular modelling techniques to analyze interactions between chosen EDCs and Estrogen Receptor or CDs. Following methods have been applied: parametrization of chosen EDCs (estradiol, progesterone, biephenol A) and CD in AMOEBA polarizable force field and succeeding Molecular Dynamics simulation of the Estrogen Receptor + EDC system; benchmark tests of various Quantum Mechanics (DFT, semi-empirical) and Molecular Mechanics (MD/MMGBSA) based computation approaches and applicable parameters, on the example of estradiol+βCD system.

Tytuł: A study of selected endocrine disrupting chemicals and their binding to host molecules with molecular modelling

Słowa kluczowe: estradiol, cyclodextrin, AMOEBA force field, force field parametrization, DFT, endocrine disrupting chemicals

Streszczeenie : Substancje zaburzające funkcjonowanie układu hormonalnego (tzw. Endocrine Disrupting Chemicals, EDC) to substancje, które wykazują niekorzystny wpływ na funkcjonowanie układu hormonalnego. Często spowodowane jest to interakcją EDC z receptorami w taki sam sposób, w jaki wiążą się z nim naturalne ligandy receptora. Wśród EDC znajdują się aktywne substancje farmaceutyczne (Active Pharmaceutical Ingredients, API), takie jak hormony steroidowe.
Cyklodekstryny (CD) to cykliczne oligosacharydy stosowane jako nośniki dla API o niskiej rozpuszczalności w wodzie oraz jako substancje usuwające toksyny. Celem tego badania było opracowanie różnych technik modelowania molekularnego w celu analizy interakcji pomiędzy wybranymi EDC a receptorem estrogenowym lub CD.
Zastosowano następujące metody badawcze: parametryzację wybranych EDC (estradiol, progesteron, bisfenol A) i CD w polaryzowalnym polu siłowym AMOEBA, a następnie symulację dynamiki molekularnej układu Receptor Estrogenu + EDC; testy porównawcze różnych podejść obliczeniowych opartych na mechanice kwantowej (DFT, podejścia półempiryczne) i mechanice molekularnej (MD/MMGBSA) jak i testowanie wybranych parametrów obliczeń, na przykładzie układu estradiol + βCD.

This international PhD project, realized in cooperation between École Polytechnique and Medical University of Warsaw, was possible thanks to the French Government Scholarship (Bourse du gouvernement français, BGF) issued by Campus France.

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I would like to thank my both supervisors: **Prof. Łukasz Szeleszczuk** and **Prof. Thomas Simonson** for their constant support and, to paraphrase J.W. Goethe, for being this good teacher who treated me as if I was what I needed to be, to bring me where I needed to get.

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‘’Science is a very human form of knowledge.

We are always at the brink of the Known,

We always feel forward for what is hoped.

Every judgement in science stands on the edge of error, and is personal.

Science is a tribute to what we can know although we are fallible.’’

Jan Bronowski, Polish-British mathematician and philosopher

Contents

Introduction

According to the European Commission Regulation from 2018 [1], Endocrine Disrupting Chemicals (EDCs) are substances that exhibit adverse effects as a consequence of an endocrine mode of action. EDCs bind to receptors due to the similarity of their chemical structure shared with natural hormones. Examples and sources of EDCs are presented in Fig. 1.

Those are among others: dichlorodiphenyltrichloroethane (DDT) present in pesticides, parabens from cosmetics, phthalates which are products originating from plasticizers' depolymerization, bisphenol A which is a depolymerization product of polycarbonates and epoxyd resins present e.g. in water bottles, dioxins from paper industry, pharmaceuticals, plant/mushroom derivatives etc. [2].

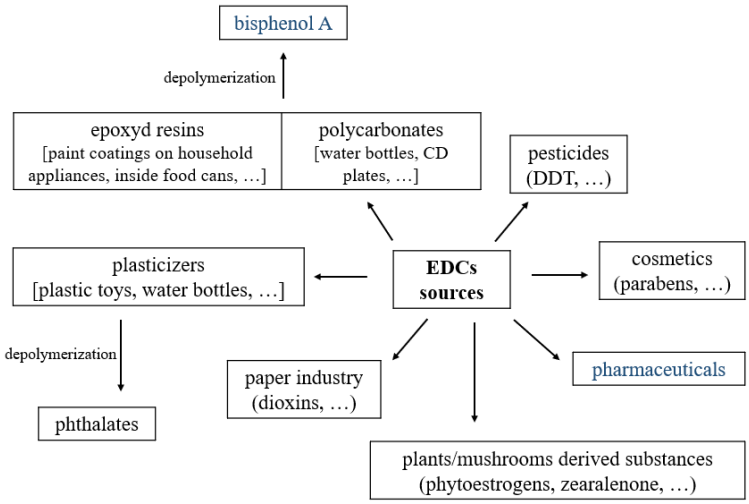

Fig. 1 Examples of EDCs sources.

A separate important source of EDCs are pharmaceuticals. When delivered as medication, hormones are described as EDCs as they alter the natural hormonal homeostasis in a human body. An important group of hormonal drugs are estrogens. In case of such external application of the naturally occurring estrogens, they are sometimes described as 'xenoestrogens' [3]. The same name is applied to other substances mimicking estrogens, like plant or mushroom derived substances (phytoestrogens, zearalenone etc.) [4,5].

This particular type of EDCs, xenoestrogens, poses a specific problem especially in the densely inhabited regions. Xenoestrogens can be found in wastewater and drinking water. Although the issue of removing xenoestrogens from water is not new, it is nevertheless a recurrent one and the subject of conflicting views. In 2009 in a broadly cited publication Daniel J. Caldwell *et al.* reported that the level of estrogens present in drinking waters in the United States does not exceed the margins of safety [6]. However, from today's perspective, two aspects must be taken into account. Firstly, already 15 years has passed since this study was performed. Secondly, even if this study is concerning a huge country, these results cannot be extrapolated for other regions in the world, for instance less-developed countries where the wastewater purification methods are less technically developed. A much more recent publication from 2020 about the occurrence of EDCs in Malaysian drinking water serves as a good example [7]. According to this report, the reproductive system connected hormones like testosterone, progesterone, estrone, 17β-estradiol and 17α-ethynylestradiol were observed to reach mean concentrations from 0.03 to 0.83 ng/L and 0.20 to 1.59 ng/L in river and tap

water, respectively. However, it has also been demonstrated that certain substances, such as 17α-ethynylestradiol can exert a triggering effect towards the endocrinal disfunction already at concentrations below 1ng/L [8]. Higher EDCs levels in the tap water then in river water are explained in the study by the water supply chain and purification methods malfunction.

As it was mentioned above, the scientific results are not unambiguous and coincident depending on the region of the measurements. The one thing is the level of xenoestrogens in waste and tap water, the other is their influence on human health if delivered in such quantities as detected. In 2020 a comprehensive review has been published on the influence of the present in water EDCs on the reproductive system [9]. With regards to estrogens, the authors state that ‘’estrogens that contaminate surface waters worldwide can negatively influence the fertility and reproductive capacity of humans’’ but at the same time they claim that ‘’data are limited on the levels and types of estrogens in the environment’’. This explains why the water contamination with EDCs, and especially with estrogens, is still a current scientific topic.

As the toxicological studies have defined the predicted no-effect concentration for estradiol to be ranging from 1 to 5 ng L/1 [10,11] Caldwell et al. 2012; Laurenson et al. 2014) and for ethinylestradiol (contraception) from 0.035 to 0.35 ng L/1 [10,11], in 2011 the European Commission proposed environmental quality standards for estradiol and ethinylestradiol as 0.4 and 0.035 ng L/1, respectively [12,13]. A drinking water quality standard of 1 ng L/1 was proposed for estradiol [14], as advised by the World Health Organization. This means that right now there are well-defined levels which are acceptable at least in the EU. In the recent years, numerous water purification systems targeted at steroidal hormones have been developed as reviewed in 2023 [15]. It seems that, thanks to quite a few adjustments and technological progress over the last few years, we have arrived at the systems which are able to eliminate hormones like estrone, 17β-estradiol and 17α-ethinylestradiol to almost non-detectable levels [16,17]. As previously mentioned, there are still areas where those techniques are not used, and research is still being done to find better, more affordable, and more efficient technology.

After pharmaceuticals, another substantial EDCs group are pesticides, defined by the European Union (EU) as Endocrine Disrupting Pesticides (EDPs). After almost 15-year-long procedure, the first EDP was banned in EU only in 2023 [18]. Even though EDPs are similarly well-described and regulated, the removal of EDPs from water is a much more complex topic because, unlike estrogens, EDPs frequently exhibit significant structural differences from one another, making it more difficult to develop one method applicable to all molecules.

One of the toxin removing agents are cyclodextrins (CDs). Those are non-toxic cyclic oligosaccharides which can form inclusion complexes [19]. Moreover, complexation between a CD and a molecule characterized by a low solubility in water, enhances the bioavailability of the molecule

[20]. This fact is widely known and used in the pharmaceutical industry. It will be explained in details in the further part of the thesis.

When it comes to the objects, the main concern in this work has been put on 17-β-estradiol, also known as estradiol. The goal of the project was to obtain and analyze the structure of the estradiol+β-cyclodextrin complex. If successful, this would be the first time when a steroidal hormone encapsulated in a cyclodextrin has been described. This could be also a beginning for further analysis of steroidal hormones and cyclodextrin complexes for potential both pharmaceutical and toxicological uses. This, as it will be explained later, requires examination both in the water solution and in the solid state. The same concept could be applied to other, non-pharmaceutical EDCs.

17-β-estradiol (EST) is the most potent form of naturally occurring estrogens [21]. Therefore, it has found wide application in hormonal contraception, hormone replacement therapy (HRT), and treatment of menopausal and postmenopausal symptoms [22]. Oral administration of EST in a solid dosage form is the most favourable form of HRT [23]. While in the European Pharmacopoeia only the hemihydrate form of EST is described, recently its anhydrous form was successfully obtained [24]. Moreover, numerous cocrystals of EST have been designed [25,26] to solve one of the major problems associated with the application of EST: its poor oral bioavailability caused by very low water solubility (0.2–5 μg mL) [27]. This issue could be potentially solved by EST complexation with β-cyclodextrin (βCD).

The case of EST being an example of EDC is a well-known, described and explained fact, also at the molecular level because EST is a natural ligand binding to the Estrogen Receptor. However, there are numerous EDCs whose mode of action is not known or which have not even been defined as EDCs yet. And this all in the situation when more and more potential EDCs are being put on the market yearly. For so numerous cases, the molecular modelling approach is probably the best choice: it will help to understand the interactions between the given chemical substance and the impacted receptor. Moreover, computational approach, if properly constructed, could be used before the experimental examination as a first screening method for detection of possible EDCs. In order to create such computational verification model, firstly the best theoretical approaches [Fig. 2] and technics for such analysis must be chosen and developed.

In such studies there are always two general areas of interest: the structure (geometry and intermolecular interactions) and the energy of the system. Interaction absolute energy between host and guest is described by the following basic equation (eq. 1 [28]), where a host can be e.g. a protein, DNA, CD and a guest is a ligand, e.g. drug or toxin molecule:

$$E_{system} = E_{complex} - \left(E_{host} + E_{guest}\right) \quad (1)$$

The thermodynamic properties like enthalpy (ΔH) and entropy (ΔS) can be derived from the computation and they sum up to the Gibbs free energy of binding (ΔG), eq. 2 [28]:

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

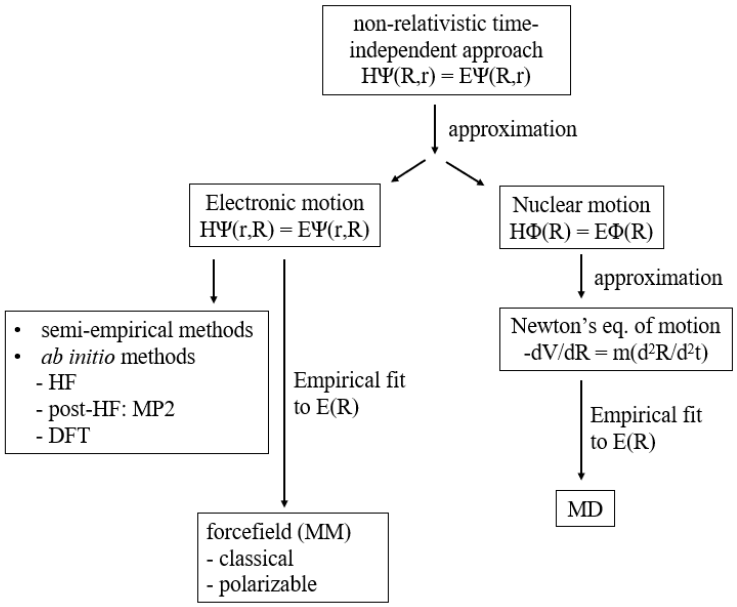

Fig. 2 A general scheme of non-relativistic time-dependent molecular modelling approaches.
Ψ and Φ - wave function in, respectively: position and momentum representations, H - Hamiltonian, E - potential energy surface, R - coordinates of the nuclei, r - coordinates of the electrons, V - velocity.

The first and already signalized topic is interaction between an EDC and a CD. In silico methods are widely applied to different aspects regarding CDs, xenoestrogens and toxins in general. Good example of the variety of objects and wide spectrum of methods used for this purpose are the following recent works: β-CD complexation with methyldrostanolone [29] which is both a toxin and estradiol derivative (conformational analysis, 2022), encapsulation of sarin by heptakis(2,3,6-tri-O-methyl)-β-CD (MD simulation and QM structural analysis, 2021) [30]. For years, CD complexes have been analyzed using the Molecular Mechanics (MM) approach as the computational power available at the time was not sufficient to apply the Quantum Mechanical (QM) methods. This has begun to change in the course of the last few years. CD complexes are started to be examined using QM-based methods, however there is no consistency in the techniques and parameters applied. Therefore, there was a need to perform benchmarking tests on the chosen example of CD-including complex. One of the aims of this work was to analyze the structure and thermodynamic properties of EST-βCD complex in water solution and in solid state using different computational approaches (semi-empirical, Moller-Plesset, DFT) and testing various computation parameters. The results were compared to the experimental data which was obtained in the first step of this work.

Nevertheless, to make this study complete, a previously standard approach used for the analysis of CD complexes, MD-MMGBSA calculations, has been applied, as well.

The second already mentioned aspect is interaction between EDC and a receptor. For the analysis of such systems MM-based approach must be introduced. Here, the forcefield (FF) term is used. FF is a set of mathematical potentials and parameters extracted from ab initio and/or experimental data and is used to calculate the energy of the inter- and intramolecular interactions between atoms [31]. There are two main types of FFs: classical or additive and polarizable.

Additive FFs describe electrostatic interactions using fixed point atomic charges and treat van der Waals interactions via Lennard-Jones potentials or other simple functions [32]. This means that the influence of polarization is averaged, hence the transferability of such fixed-charge is low. Moreover, lack of higher order atomic multipoles prevents an accurate description of the anisotropic electrostatic potential around molecules [33]. What is more, as additive force fields do not include explicit representation of induction, they may poorly represent the electrostatics of molecules which often play a crucial role in the intermolecular interactions [34].

On the contrary, polarizable force fields, that is those which treat electronic polarization explicitly, allow the electronic structure of a molecule to change with regards to alterations of the local electric field. In other words, in such models multi-body contributions are included in the electrostatic interactions. As J. A. Lemkuhl has described [35]: ‘’if a molecule is removed from the system, the dipoles of the other species will be aligned differently and will have different magnitudes, leading to different interaction energies among the remaining molecules’’. This is an answer for the non-transferability characteristic for the additive force fields. Superiority of polarizable force fields over the classical ones has been depicted on a great variety of objects [36-67].

In polarizable force fields the many-body interaction energy is explicitly treated through the introduction of electronic polarization. This can be implemented through application of [68]:

- fluctuating charge models: fluctuating charge represents the response of the system to the electrostatic potential [69]
- Drude oscillator models: Drude particles on polarizable sites describe the response of the system to the surrounding [70]
- atomic induced dipole models: induced dipoles respond to the surrounding electrostatic field [40,47].

A force field which uses the third approach is AMOEBA FF. It is being developed since 1990s and currently there is available a full set of parameters for proteins, nucleobases, organic molecules [71-73]. The parametrization process has been automatized and for this purpose the Tinker software is frequently used [74]. However, still there have been published only few studies applying this approach to big systems like a receptor-ligand complex. More research is needed in this direction.
Therefore, one of the purposes of this work was to, in the first place, parametrize selected EDCs and secondly, perform a receptor-EDC simulation using AMOEBA FF. The chosen molecules are: estradiol, progesterone and bisphenol A. Both estradiol and bisphenol A are model representatives of EDCs, with estradiol being a natural hormone whose receptor binding is mimicked by EDCs. Progesterone has been chosen for the two reasons. Firstly, it is another example of a potent pharmaceutical EDC. But even though, we know significantly less about progesterone’s binding to the progesterone receptor than about the estradiol + estrogen receptor interaction. This makes the

‘progesterone+progesterone receptor’ an interesting system to analyze. Secondly, so far there were no parameters available for the steroid fused rings core which is a basis for multiples molecules including hormones. Therefore, the first challenging element of this part of the work was parametrization of the three molecules. In the next step, the assumption was to use at least one of those molecules (preferably estradiol as a model molecule) to perform Molecular Dynamics simulation with receptor, in this case estrogen receptor, using AMOEBA FF.

As it is explained in the further part of this work, when Molecular Dynamics calculations including cyclodextrins are performed, a carbohydrates-targeted additive GLYCAM force field is used [75]. The limitations of the classical approach are well-known and already when the latest version of GLYCAM was published, the authors mentioned works on the polarizable version of this force field. However, having already a well-functioning polarizable AMOEBA FF, it has been decided to include a cyclodextrin molecule in the parametrization process. This would allow to perform MD simulations on the cyclodextrin-EDC complexes using the polarizable FF approach. This would be also complementary to other previously mentioned computational approaches used in the benchmark analysis of CD-including systems.

Endocrine Disrupting Chemicals – mechanisms of action

A highly cited article from 2020 [76] points out that one of the issues regarding EDCs is lack of well-defined characteristics of such hazardous substances. This is especially crucial as the regulatory agencies use various approaches to evaluate the hazard coming from potentially endocrine disrupting chemicals. Michele A. La Merrill et. al. propose 10 EDCs key characteristics based on the end points of their acting. According to this research, as an EDC can be defined a substance which:

- interacts with or activates hormone receptors
- antagonizes hormone receptors
- alters hormone receptor expression
- alters signal transduction in hormone-responsive cells
- induces epigenetic modifications in hormone-producing or hormone-responsive cells
- alters hormone synthesis
- alters hormone transport across cell membranes
- alters hormone distribution or circulating hormone levels
- alters hormone metabolism or clearance
- alters fate of hormone-producing or hormone-responsive cells

It is worth mentioning that two of the most well-known and described EDCs: bisphenol A and already withdrawn diethylstilbesterol, fulfil 9 out of 10 above mentioned key characteristics.

A thorough discussion of the EDCs and the disease endpoints, including reproductive, metabolic, neurologic and cardiovascular disorders, can be found in a recent review on the topic [77].
EDCs can be absorbed by a human body via digestive system, skin and inhalation or even via placenta to the foetus. An example for the latter, is a perinatal exposure to bisphenol A which causes physiological and functional underdevelopment of genitalia, tracts and glands that may result in reduced fertility, aspermia, immature reproductive systems and the growth of several cancers such as breast, ovary and prostate cancer [78].

In a human body EDCs target primarily 6 receptors: estrogen, androgen, progesterone, thyroid hormone, glucocorticoid, peroxisome proliferator-activated receptors gamma and aryl hydrocarbon receptors [79,80]. There are two main mechanisms of interaction between natural activators and receptors: direct (known also as 'genomic') and indirect (or 'non-genomic') [78,79]. The same mechanisms are used by EDCs. In the direct mechanism, the ligand binds directly to a receptor and therefore affects the transcription of target genes in the nucleus. In the indirect mechanism, the ligand interacts with the components of the hormone signalling pathways, for instance with G protein-coupled receptor (GPR30) located in the cytoplasmic membrane. Activation of GPR30 by a ligand leads to downstream cellular signalling like protein kinase activation and phosphorylation what in turn may affect the transcription of target genes. In fact, what is observed, is the pleiotropic effect induced by a ligand via different pathways (nuclear and extracellular) and by interactions with different receptors, like ERα, ERβ, GPR30, depending on the location within the cell and the body [81]. The same differentiation in used mechanisms is observed for EDCs [81,82]. Regardless of the mechanism, EDCs alter the endogenous synthesis of hormones. This leads to toxic effects like hormonal imbalance, decrease of fertility, alterations in sperm quality and fertility, abnormalities in sex organs, endometriosis, early puberty, altered nervous system function and immunity, sex organ cancers etc. [83].

R.K. Gupta et al. underlines that the reproductive hormones, such as progestins, androgens, and estrogens are the primary targets of EDCs such as: pesticides (e.g. dichlorodiphenyltrichloroethane (DDT)), methoxychlor, vinclozolin, atrazine), detergents and surfactants (e.g. octyphenol, nonylphenol, bisphenol A (BPA)), plasticizers (e.g. phthalates), industrial compounds (e.g. polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)), natural plant derivatives (e.g. genistein, coumesterol) [84].

Estrogen Receptor

There are two subtypes of the estrogen receptor: ERα and ERβ, each of them characterized by a tissue-specific expression [85]. Despite being encoded by different genes, both estrogen receptors show high homology, and in both of them the E domain contains the ligand-binding domain (LBD)

and C domain, which is the DNA binding domain (DBD) [86]. In ER structures, two transactivation functions are present, called AF-1 (located in N-terminal domain) and AF-2 (located in LBD) [87]. They contain the nuclear location signals. After proper exposure of their surface, happening as the result of the ligand binding, they are responsible for incorporation of the co-activators, which is a necessary step to induce activation of the intercellular signalling pathways [Fig. 3].

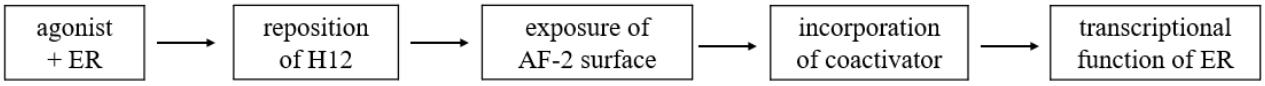

Fig. 3 A general scheme of ER activation.

Binding of an agonist to the ER requires creating a hydrogen bond with His524 (in ERα) [88] or His475 (in ERβ) [89]. This leads to a unique agonist-bound conformation of the receptor's LBD, characterized by a specific repositioning of the H12 helix, which is the most C-terminal helix of the LBD (molecular switch) [90]. On the contrary, selective ER modulators (SERMs) such as raloxifene or tamoxifen induce relocation of H12 into the co-activator binding cleft, which blocks AF-2 activity. Finally, pure antagonists completely destabilize H12 [91].

There are four natural steroid hormones which act as activators towards ER. Those are: estrone (E1), estradiol (E2, EST), estriol (E3), and estretrol (E4) [92]. The last one is produced only during pregnancy by the foetus liver [93]. Among these four compounds, E2 plays the most important role in the human organism and, therefore, is of high importance in breast or ovarian cancer progression. With regard to their relative binding affinity (RBA) to ER, with the exclusion of estretrol, estrogens are ranked in the following order: estradiol > estrone > estriol. In comparison to estradiol, the activity and potency of estrone and estriol are, respectively, 10 and 100 times lower [94].

E1 functions mainly as estradiol's metabolite and serves as its precursor (the estrone-to-estradiol transformation is reversible) [95] [Fig. 4]. E3 is a metabolite of E1 [Fig. 4], however, in non-pregnant women, estriol levels in the blood are hardly detectable. During pregnancy its amount distinctively grows because it it is produced by the placenta as well [96]. All estrogens are used as medication in menopausal hormone therapy, although estradiol is the most applied [97,98].

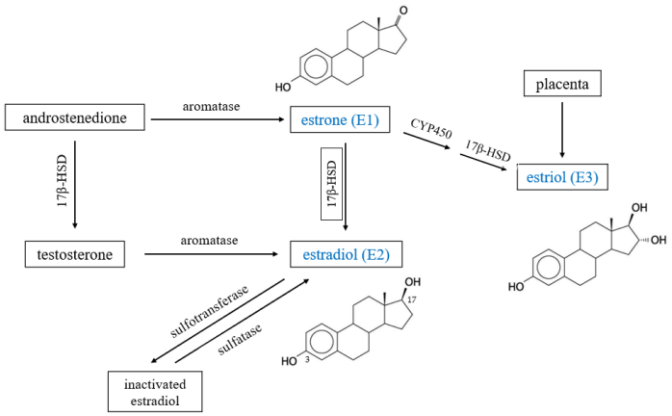

Fig. 4 A general scheme of estradiol's metabolism.

When compared to ERβ, ERα plays a more prominent role in the mammary gland and uterus as well as the regulation of metabolism. ERα LBD is composed of 12 α-helices (H1-H12) [99-103].

Estradiol (EST) binds as an agonist in the pocked formed by 22 residues. EST hydroxyl groups play a decisive role in the hormone positioning within the pocket. The hydroxyl group of the A ring [Fig. 5] creates a hydrogen bond with Glu353 from H3, Arg394 from H5 and water molecule, whereas hydroxyl group of the ring D creates a hydrogen bond with His 524 from H11 [99-103]. Creation of the hydrogen bond with H11 allows repositioning of the H12 what in turn generates a ligand-dependent activation function 2 (AF-2). It is necessary for the interaction with co-activators and later initiation of the intercellular signalling pathway [104,105].

Except for the already mentioned hydrogen bond interactions with Glu353, Arg394 and His524, EST molecule position is stabilized also by the π- π stacking with Phe404 [99-103].

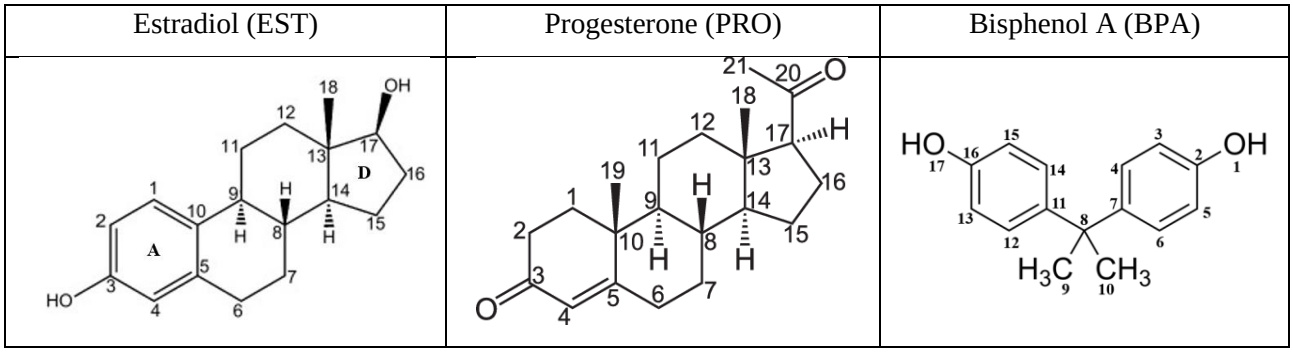

Fig. 5 Structures of selected EDCs.

Due to the structural similarity with EST, bisphenol A (BPA) [Fig. 5] binds to both types of ER. It displays 1000- to 2000-fold less affinity to ER then EST does [81]. BPA is ERα activator via the same mechanism as EST. Towards ERβ, it acts as an antagonist because it prevents LBD from obtaining the activated type of conformation [106]. BPA shows also a high binding affinity towards GPR30 [81]. This shows that the disruptive influence of BPA on the hormonal homeostasis happens via multiple mechanisms. It has been also proven that BPA interacts with other hormonal receptors like androgen, pregnane X, and peroxisome proliferator-activated receptors [81]. This example highlights to which extent a single EDC can disrupt the functioning of a human hormonal system.

On the contrary to BPA, progesteron (PRO) does not bind to ER. This is due to the absence of hydroxyl groups at carbons 3 and 17 of PRO molecule. PRO binds to progesterone receptor (PR). The molecule is anchored in the PR binding pocket via net of hydrogen bonds created around carboxyl oxygen attached to PRO's carbon 3 [107,108]. PRO binds to PR and causes activation of its transcriptional function in a mechanism similar to the one described for ER: AF-2 activity is mediated by a hormone-dependent interaction with steroid receptor coactivators (Src) [109].

Both ER and PR undergo a dimerization which happens after binding of the agonist [110,111]. With regards to this process, an important element of the ERα LBD structure is Tyr537. Its phosphorylation has been proven to influence the hormone binding, ER dimerization and transcriptional activity [112]. Src family tyrosine kinases were shown to specifically phosphorylate

ER's Tyr537 [113]. This estradiol-dependent ER phosphorylation at Tyr537 plays a crucial role in the nuclear export of ERα.

Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of glucose (α-D-glucopyranoside) subunits joined by α-1,4 glycosidic bonds [Fig. 7]. The so-called native CDs are not substituted and are composed of 6 (α-CD), 7 (β-CD) or 8 (γ-CD) units. CDs are non-toxic and therefore can be used as drug delivery agents [114]. CDs are used in pharmaceutical formulations due to their ability to form inclusion complexes. Due to the presence of hydroxyl groups, the external fragments of CDs are polar. When a non-polar substance enters the molecular hole of CD, the formed host–guest complex is polar and more soluble than a separate guest molecule [115]. Therefore, CDs are commonly used to increase the solubility of API (Active Pharmaceutical Ingredient) or protect it from external factors like light, humidity and heat. Worldwide, more than 100 original drugs have been ever manufactured with CDs as excipients [116–118]. One of the APIs groups characterized by poor solubility in water are hormonal steroids like estradiol or progesterone. Encapsulation in CDs may enhance their solubility in water and as a result also their bioavailability. Based on the same principle of the encapsulation CDs found a second application which is their usage as toxin (e.g. EDCs) removing agents.

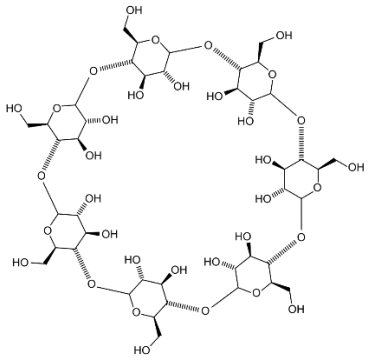

Fig. 7 Structure of β-CD.

According to the Web of Science, within the last decade each year more than 1000 articles concerning the CD-including drug delivery systems have been published and since 2018 this number is visibly rising. The topics encompass such inventions like liposomes+CD+ligand, nanotubes+CD+ligand or gold layer+CD+ligand. Already even a couple of review articles has been written on this subject [119-121]. According to the EMA (European Medicine Agency) [122], there are some CD-complexed drugs at the European market, formed with SBE-β-CD (sulfo-butyl-ether-CD) or 2-hydroxypropylo-β-CD (2-HP-β-CD). Currently, on the European market there is one CD-hormone medication. It is RM-β-CD nasal spray for hormone replacement therapy by 17-β-estradiol [123]. Nevertheless, still new attempts are made in this topic and CDs are generally considered as good non-toxic agents enhancing solubility of the low water soluble chemical compounds.

In terms of extraction, they are often used in organic solvents being attached to the chromatographic columns [124,125]. Both in the experimental and computational studies apart from the 'natural' CDs (α, β, γ) also the ones with attached different side chains are used, for instance the already mentioned SBE-β-CD [126,127] and 2-HP-β-CD [128-131] or 2,6-dimethylo-β-CD [132,133], methyl-β-CD [134,135]. Among all CDs, the most often used ones are 2-HP-β-CD and β-

CD. This is due to the fact that most of chemical compounds (potential drugs and toxins) are too big to enter the cavity of the α-CD. In turn, the γ-CD is in most of the cases too wide and therefore the binding affinity between the CD and the guest is weaker. The 2-HP-β-CD is typically chosen among the CD derivatives because, from a synthetic perspective, a structural alteration from the β-CD is relatively easy and still, in many cases, the 2-HP-β-CD's solubility enhancing abilities are sufficient enough. In experimental works methylation or 2-hydroxypropylation happens randomly. In the *in silico* research such attempt is not that common as it would require specifying places at which a side chain is added so it would not be 'random' anymore. It is more popular to use fully substituted CDs (per-methylated, per-2-HP-hydroxypropylated etc.) [136-138]. In this project β-CD was used.

AMOEBA forcefield

AMOEBA FF uses the concept of ***atomic multipoles***. Atomic multipole term defines that each atomic centre consists of partial charge, dipole vector and quadrupole tensor. For the dipole and quadrupole description ***local coordinate frames*** are constructed at each site. They are constructed according to the z-then-x convention [47,73], as described in Fig. 6. The multipole moments are derived directly from ab initio quantum mechanical electron densities for small molecules and molecular fragments. For this purpose the ***Distributed Multipole Analysis*** (DMA) of wavefunctions expressed in terms of Gaussian atomic orbitals is used. It is carried out in the Gaussian software (GDMA) [139].

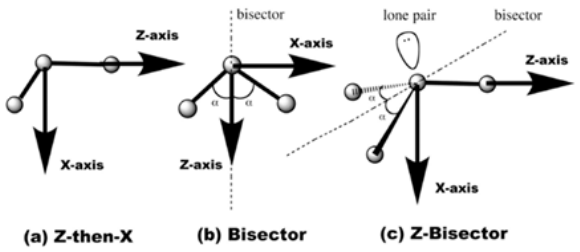

Fig. 6 Local coordinate frame definitions for atomic multipole sites.

Adapted from [32] under the CC BY 4.0. licence.

a) The Z-then-X frame is used for general sites, and with addition of a third orthogonal y-axis can treat chiral centers. The majority of AMOEBA multipole sites are defined using this local frame. (b) The Bisector frame is useful for molecules with 2-fold local symmetry or pseudo-symmetry, such as water and aliphatic methylene carbon atoms. (c) The Z-Bisector frame is used for sites such as the sulfur atom of dimethylsulfoxide, which have a distinct primary ("Z") axis and symmetry or pseudo-symmetry along a secondary direction.

Induced dipoles (μind i,α) are described by atomic polarizability (αi) and influence of the electric field on the atom i (Ei,α):

$$\mu ind\ i,\alpha = \alpha i\ Ei,\alpha. \quad (3)$$

Polarization term is defined as a sum of atomic multipoles' response terms for the electric field created by non-connected atoms and interaction terms between inducted atomic dipoles. Polarization is explicitly treated by mutual induction of dipoles at polarizable sites (located at atomic

centers) [47]. A point dipole moment is induced at each polarizable site with regards to the electric field experienced by that site, according to the eq. 4:

$$\mu_i^{ind} = \alpha_i\left(E_i^{dir} + E_i^{mut}\right) \quad (4)$$

where α_i is the atomic polarizability on site i; E_i^{dir} is the "direct" electric field generated by permanent multipoles of other sites; E_i^{mut} is the "mutual" field generated by induced dipoles of other sites [68].

In other words, induced dipoles produced at the atomic centers mutually polarize all other sites. Based on Thole's model, [140] polarization at a very short range is damped, what delivers energies in a better agreement with ab initio results and allows to avoid the so-called polarization catastrophe [71]. Atomic polarizabilities are assigned based on the element type of each atom [73]. When short-range polarization between bonded atoms is ignored, use of intramolecular polarization delivers only marginal improvement when compared with the nonpolarizable potentials. To overcome this problem, a group-based intramolecular polarization scheme has been introduced. Those ***polarization groups*** are usually functional groups with limited conformational degrees of freedom [32,71]. They are partitioned between rotatable bonds [73]. This concept prevents permanent multipoles from polarizing other atoms within their group but induced-induced polarization occurs between all atoms.

The ***polarization energy*** between induced dipoles and permanent multipole moments is computed fully between atoms separated by three (1-4) or more bonds, and completely neglected for any closer separation [71].

In the AMOEBA FF atomic interactions are defined as ***bonded and non-bonded interactions***, according by the following equations:

$$U = U_{bond} + U_{angle} + U_{b\theta} + U_{torsion} + U_{oop} + U_{vdw} + U_{ele\ perm} + U_{ele\ ind} \quad (5)$$

where the first five terms describe the short-range valence interactions (bond stretching, angle bending, bond-angle cross term, torsional rotation, out-of-plane bending, please see full equations: eq. 2-7) and the next three terms describe: nonbonded vdW and electrostatic contributions.

$$U_{bond} = K_b(b - b_0)^2[1 - 2.55(b - b_0) + 3.793125(b - b_0)^2] \quad (6)$$

$$U_{angle} = K_\theta(\theta - \theta_0)^2[1 - 0.014(\theta - \theta_0) + 5.6x10^{-5}(\theta - \theta_0)^2 - 7.0x10^{-7}(\theta - \theta_0)^3 + 2.2x10^{-8}(\theta - \theta_0)^4] \quad (7)$$

$$U_{b\theta} = K_{b\theta}[(b - b_0) + (b' - b_o')](\theta - \theta_0) \quad (8)$$

$$U_{torsion} = \sum_n K_{n\varphi}\left[1 + \cos(n\varphi \pm \delta)\right] \quad (9)$$

$$U_{oop} = K_\chi \chi^2 \quad (10)$$

$$U_{vdw(ij)} = \varepsilon_{ij}\left(\frac{1.07}{\rho_{ij}+0.07}\right)^7 + \left(\frac{1.12}{\rho_{ij}^7+0.12} - 2\right) \quad (11)$$

Equations 6-11 describe: bond stretching, angle bending, bond-angle cross term, out-of-plane bending, torsional rotation energy and vdW terms in AMOEBA FF, where K_b is bond force constant, b-b0 is distance

from equilibrium after atom movement, K_θ is angle force constant, $\theta - \theta_0$ is angle from equilibrium between 3 bonded atoms, $K_{n\varphi}$ is dihedral force constant, n is multiplicity of the function, φ is dihedral angle, δ is phase shift, K_χ is out-of-plane bending constant, χ is an angle created between 4 atoms; R_{ij} is separation distance between atoms i and j ($\rho_{ij}=R_{ij}/R_{0ij}$ where R_{0ij} is minimum energy distance and is combined for heterogeneous atom pairs); εij is potential minimum combined for heterogeneous atom pairs

Additive forcefields

- **CHARMM forcefield**

In comparison to the polarizable FF, a potential energy function of an additive FF is composed as presented in equation 12, on the example of CHARMM FF (Chemistry at HARvard Macromolecular Mechanics). The main difference lies in the absence of the electrostatic contribution description which is a core element of a polarizable FF [141]. In this work, CHARMM FF has been used to evaluate the parametrization process of AMOEBA FF. [ttp://dx.doi.org/10.1016/j.bbagen.2014.08.004].

$$V = \sum_{bonds} k_b(b - b_0)^2 + \sum_{angles} k_\theta(\theta - \theta_0)^2 + \sum_{dihedrals} k_\Phi[1 + \cos(n\Phi \pm \delta)] + \sum_{impropres} k_\omega(\omega - \omega_0)^2 + \sum_{Urey-Bradley} k_u(u - u_0)^2 + \sum_{nonbonded} \varepsilon \left[\left(\frac{R_{\min ij}}{r_{ij}}\right)^{12} - \left(\frac{R_{\min ij}}{r_{ij}}\right)^{6}\right] + \frac{q_i q_j}{\varepsilon r_{ij}} \quad (12)$$

where in bond stretches term: k_b is bond force constant, $b\text{-}b_0$ is distance from equilibrium after atom movement; in bond angles term: k_θ is angle force constant, $\theta - \theta_0$ is angle from equilibrium between 3 bonded atoms; in dihedrals (torsion angles) term: k_Φ is dihedral force constant, n is multiplicity of the function, Φ is dihedral angle, δ is phase shift; in impropers (out of plane bending) term: k_ω is force constant, $\omega\text{-}\omega_0$ is out of plane angle; Urey-Bradley term is cross-term accounting for angle bending using 1,3 nonbonded interactions: k_U is respective force constant, U is distance between 1,3 atoms in harmonic potential; last two terms account for nonbonded interactions between pairs of atoms i and j.

- **AMBER forcefield**

Another additive force field applied in this work is AMBER FF (Assisted Model Building with Energy Refinement) [142]. The potential energy function is calculated according to the eq. 13 which, similarly as in other additive force fields, consist of terms for bonds, angles, dihedrals, van der Waals interactions and electrostatics.

$$V = \sum_{bonds} k_b(b - b_0)^2 + \sum_{angles} k_\theta(\theta - \theta_0)^2 + \sum_{dihedrals} \frac{V_n}{2}[1 + \cos(n\Phi - \delta)] + \sum_{ij} \frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}} + \frac{q_i q_j}{r_{ij}} \quad (13)$$

These terms are derived with use of the Antechember software which uses general AMBER FF for organic molecules (GAFF) [143]. The terms are assigned based on the atoms connectivity [144].

In this work AMBER FF has been used due to its particularity: an adjustment called GLYCAM which is AMBER FF adapted for the carbohydrates [75]. In the newest version, GLYCAM06j, bond and valence angle deformation force constants, dihedral angle rotational barriers, electrostatic properties were obtained with QM calculations, as those parameters are hardly obtainable experimentally.

Partial atomic charges are derived by fitting to the QM molecular electrostatic potentials (ESP-fitting). However, in contrast to older GLYCAM versions, partial charges are not fitted to aliphatic hydrogen atoms. In the GLYCAM06j version, for the ono-bonded interactions the 1-4 scaling has been removed.

In this work GLYCAM force field was applied to perform MD/MMGBSA analysis of the cyclodextrin-estradiol complex. The underlying theory for the MD/MMGBSA calculations is described in the further part of this thesis.

Molecular Dynamics / MD-MMGBSA approach

- **Molecular Dynamics – receptors in solution and crystal structures**

For the Molecular Dynamics (MD) calculations the underlying physics is defined by the Newton equation of motion [145]. The analyzed system might be either objects placed into a solvent box which is replicated into infinity (for solutions, for instance: receptor simulation) or a whole crystal structure recreated thanks to an infinite replication of the crystal unit cell (for solid state). Calculations are performed in one of the ensembles: NVE (microcanonical), NVT (canonical), NPT (isothermal-isobaric), where N states for number of particles, V for volume, T for temperature, P for pressure. Each time, the given parameters (N, V, E, P) are restrained to the imposed values. First stage after solvent box / crystal unit cell preparation is system's heating up to the desired temperature and later two-stage equilibration, till firstly temperature and later pressure oscillates around the imposed values [145]. The following step is the production run. Positions and velocities from the MD trajectories which define movements of atoms, are used to compute the structural and thermodynamic properties. The above described method is referred to as a classical MD. Several variations have been already constructed, among them ab initio MD, which is said to be probably the most precise approach, as it starts from the QM-optimized structures, is however, restricted to small systems [**Publication 5: A Review on Combination of ab Initio Molecular Dynamics and NMR Parameters Calculations**]. Therefore, this method was not used in this work.

In all cases a proper representation of the entropy term is a crucial aspect. Its measurement is dependent on the space sampling. The extended space sampling methods are among others SMD (Steered Molecular Dynamics) and FEP (Free Energy Perturbation calculations). The idea of the former is based on application of the biased coordinates and the free energy of binding (ΔG) is calculated from the non-equilibrium work [146,147]. The principle of the latter is application of the biased paths and ΔG is calculated based on the alchemical transformation [148,149]. In the current work neither SMD nor FEP approach is used. However, this work is a preparation for future application of those methods to analyze both the EDC-ER and EDC-CD complexes. More detailed information on the topic can be found in **Publication 1: Application of Various Molecular**

Modelling Methods in the Study of Estrogens and Xenoestrogens and in the 'Conclusions and perspectives' part at the end of this work.

- **MD-MMGBSA -cyclodextrin complexes**

So far, in many cases in order to prepare a CD structure for further simulations, the geometry optimization has been performed using Molecular Mechanics (MM) methods. Often a special Glycam06 forcefield (adjusted AMBER forcefield) dedicated for carbohydrates has been used [75]. In the works published even a couple years ago it has been often referred to as a 'standard procedure'. The review about the computational methods used for CD-complexes simulations [**Publication 4: Application of Molecular Dynamics Simulations in the Analysis of Cyclodextrin Complexes**] cites at least 25 articles from the recent years where Glycam06 has been applied. However, now, the energy minimization of a CD structure can be handled by the DFT calculations which are much more accurate and therefore have been used in this project.

Alongside with MD the often used method is MMGBSA (MM Generalized Born Surface Area). This approach allows to obtain the free energy of binding (ΔG). Firstly, MD using an explicit solvent model is performed. Secondly, from the last snapshots of MD the solvent molecules are extracted. On these snapshots, MMGBSA calculation in the implicit solvent is conducted. In MMGBSA the entropy term (ΔH) is calculated as a sum of MM-based electrostatics energy term (bonded and non-bonded energy terms) and two solvation related energy terms (calculated in the implicit model) [eq. 14] [150]. In the eq. 14 ΔG_{pol} corresponds to the Generalized Born (GB) approximation of the Poisson-Bolzmann equation, which in turn describes the electrostatic environment of the solute in a solvent containing ions [150]. ΔG_{nonpol} relates to the Solvent Accessible Surface Area (SASA) which is an implicit approach describing the relationship between ΔG and surface area of a solute molecule.

$$\Delta H = \Delta E_{MM} + \Delta G_{pol} + \Delta G_{nonpol} \quad (14)$$

The change between explicit and implicit solvent model which happens before MMGBSA is performed, requires energies' reweighting and several approximations. What is more, for each simulated system several parameters must be arbitrary decided on. All these factors and the fact that the implicit model is less accurate then the explicit one, results in MMGBSA methods being very differently assessed: for some systems they reflect the experimental ΔG very accurately, for others not at all. A number of adjustments has been tried on the MMGBSA model, among others application of the polarizable FF QM/GBSA approach. MMGBSA is still a widely chosen method, especially to calculate ΔΔG in the protein-ligand systems, where the MMGBSA score is used to rank the ligands' binding affinity to the receptor. However, this approach has a better equivalent in form of the FEP calculations. This fact has been known for years but the FEP method is computationally demanding.

When it comes to the MD-MMGBSA calculations, there is not much information on the simulations concerning specifically CD complexes [151-154]. There is one relatively recent (publication year: 2018) example where the computation object is a CD complex with genistein, a natural EDC characterized by a structural similarity to EST. In this work a high level of theory, M06-2X/6-31+G(d,p), is applied to perform calculations on the snapshots extracted from MD [155]. However, as it will be presented later, for the purpose of this work the standard MMGBSA method was applied, without the QM approach after MD run.

Quantum Mechanical approaches

In this work for different purposes, two types of QM computational approaches have been used: semi-empirical methods and Density Functional Theory calculations (DFT).

Semi-empirical methods accuracy is generally considered to be lower than the accuracy of DFT. However, with regards to CD complexes no real comparison between different QM-based approaches has ever been made. Moreover, CD complexes are not small systems and still semi-empirical calculations are often a preferred approach. This has been shown through a thorough literature review **Publication 6: Current Status of Quantum Chemical Studies of Cyclodextrin Host-Guest Complexes**.

DFT approaches provide a high calculational accuracy. However, they are computationally costly, when compared to semi-empirical methods. For years DFT-based methods have been not-correct enough due to the neglection of the dispersion (London) effects [156]. For example, in the condensed matter studies, this was not a major problem in the case of systems characterized by strong electrostatic interactions such as ionic solids, while it was a serious limitation for molecular crystals, where dispersion forces such as van der Waals interactions greatly contribute to the overall binding energy. The most popular method to overcome this problem is the application of "dispersion corrections" (DFT-D), i.e. in the form $C6R^{-6}$ in the DFT formalism [157]. These semiempirical approaches provide the best compromise between the cost of first principles evaluation of the dispersion terms and the need to improve non-bonding interactions in the standard DFT description [158]. Implementation of the dispersion corrections (e.g. D3, TS, MBD) [159] made DFT approach one of the most desirable option for the analysis of small systems. However, it must be stated that application of the empirical dispersion corrections does not increase the accuracy of the results in 100% of cases. Therefore, their application should be tested for each new system.

DFT approach describes the total energy of the system (Et) by the Hohenberg-Kohn-Sham equation [145] [eq. 15]:

$$Et[\rho] = T[\rho] + U[\rho] + Exc[\rho] \quad (15)$$

where T stands for kinetic energy of non-interacting particles, U for classical electrostatic energy due to the Coulombic interactions, Exc for the exchange-correlation energy, ρ is charge density.

A crucial element, on which the accuracy of the DFT methods depends, is the exchange-correlation energy presented as the exchange-correlation functional [145] which can be approximated in several ways: as Local-Density Approximation (LDA), Generalized Gradient Approximation (GGA) e.g. PBE-TS, PBE-SOL or hybrid functionals, e.g. B3LYP, M06-2X. In other words, this energy term is represented as a functional of the electron density ρ.

In the DFT approach, the electronic structure is evaluated on the basis of a potential acting on the electrons in the system. The DFT potential is constructed as the sum of external potentials, determined solely by the structure of the system and an effective potential resulting from interelectronic interactions. All-electron DFT methods treat core and valence electrons in the same way. However, the DFT calculations can be very much simplified and accelerated if electrons are divided in two groups: valence electrons and inner core electrons. In most cases, the electrons of the inner shells (core electrons) are tightly bound and are not involved in the chemical binding. In most organic molecules, binding is solely due to the valence electrons [160]. This separation means that in a large number of cases the atom can be reduced to an ionic core that interacts with the valence electrons. In the pseudopotential approach, widely used for the solid-state DFT calculations, ion cores are considered to be frozen, meaning that the properties of solids are calculated on the assumption that the ion cores are not involved in chemical bonding and therefore they do not change as a result of structural modifications or presence of other atoms. A pseudopotential represents an effective interaction that approximates the potential felt by the valence electrons [161].

Another aspect which must be decided on and which has a huge influence on the calculation results, is choice of a basis set. A basis set is set of basis functions which represent the electronic wave function in form of the algebraic equations what makes them readable for a computer [145]. In the non-periodic DFT calculations the localized basis sets are used.

- **DFT calculations in solid state**

Solid state substances have either amorphous or crystalline character. In order to properly represent the crystalline ones during the calculations their periodicity must be taken into account. This happens when the periodic DFT approach is used. In such case, plane-wave basis sets are usually applied. They are commonly used in calculations involving three-dimensional periodic boundary conditions. The main advantage of a plane-wave basis sets is that it is guaranteed to converge in a smooth, monotonic manner to the target wavefunction [162]. Additional benefit resulting from the application of plane-wave basis set is the introduction of periodic conditions to the studied system. For accurate and computationally feasible approximation of a large system such as macroscopic

crystals, periodic boundary conditions are often applied using crystal unit cells as simulation boxes. During the computations only the properties of the original unit cell need to be calculated and then propagated in the chosen dimension. Additionally, the main advantage of imposing periodic boundary conditions relates to Bloch's theorem, which states that in a periodic system each electronic wavefunction can be written as a product of a cell-periodic part and a wavelike part. The cell periodic part can then be expanded using a basis set consisting of a discrete set of plane waves whose wave vectors are reciprocal lattice vectors of the crystal. Therefore, each electronic function can be written as a sum of plane waves [163]. Periodic DFT calculations are used among others in the procedure of the Crystal Structure Prediction, to explain the crystallization and solvation processes, analyze polymorphs, verify the experimentally obtained structures etc. A detailed description and numerous examples on the topic can be found in **Publication 3: Periodic DFT Calculations-Review of Applications in the Pharmaceutical Sciences** and **Publication 10: Pharmaceutical Hydrates Analysis—Overview of Methods and Recent Advances**.

A particular application of periodic DFT approach is calculation of NMR properties. NMR data is of high importance for the description of CD complexes. Only small number of these complexes has crystalline form and only for few of them it is possible to obtain a crystal of a size suitable for single-crystal X-ray measurements. Therefore, the ssNMR (solid state Nuclear Magnetic Resonance) technique is often the best choice to analyze the inner structure of the complex. Moreover, ssNMR technique delivers information unobtainable by any other experimental technique. In particular, ssNMR can provide the information on orientation of the guest molecule inside the cavity and the complex stability in the solid state. It also enables the quantitative analysis of the phases, especially the complexed and non-complexed guest molecules. In addition, this technique allows for the study of the local molecular dynamics of a guest molecules and the nature of intermolecular interactions between the host and the guest. The thorough description of the topic including numerous examples can be found in **Publication 7: A Review of Applications of Solid-State Nuclear Magnetic Resonance (ssNMR) for the Analysis of Cyclodextrin-Including Systems**.

Already for over a decade the computation of NMR shielding tensors is performed using the Gauge Including Projector Augmented Wave Density Functional Theory (GIPAW) method of Pickard et al. [164] and not by previously used Gauge Invariant Atomic Orbitals (GIAO) [165]. To compare the theoretical and experimental data, the calculated chemical shielding constants (σiso) are converted to chemical shifts (δiso) using the following equation:

$$\delta iso = (\sigma Gly + \delta Gly) — \sigma iso \quad (16)$$

where σGly and δGly stand for the shielding constant and the experimental chemical shift, respectively, of the glycine carbonyl carbon atom (176.50 ppm), if glycine is used as external standard.

The accuracy of combined ssNMR and DFT NMR (GIPAW calculation) is already confirmed so well that such an approach is used as a verifying tool for other techniques, like X-ray photoelectron spectroscopy (XPS) [166].

Combined ssNMR and DFT NMR approach can be especially helpful in the structural analysis in case of a huge disorder within the crystal structure. The GIPAW NMR calculations facilitate peak assignment in the ^{13}C CP MAS NMR spectra. Such approach has been used in this work and has been described in details in **Publication 8: 17-β-Estradiol—β-Cyclodextrin Complex as Solid: Synthesis, Structural and Physicochemical Characterization**.

- **DFT calculations in solution**

In the QM molecular modelling approaches, solvent is presented as a continuum using implicit models. There are two main types of implicit solvent models: Polarizable Continuum Model (PCM) and SMD (Solvation Model Density). In both cases, the structureless polarizable medium is characterized mainly by its dielectric constant ε. PCM is the most often used solvent model in the computational analysis of CD complexes and one of the most used continuum models in general. It defines the molecular free energy as a sum of electrostatic (es) and the dispersion-repulsion (dr) contributions to the free energy, and the cavitation energy (cav) [167] [eq. 17]:

$$Gsol = Ges + Gdr + Gcav \quad (17)$$

In order to calculate the Gcav, the surface of the van der Waals sphere is used. Van der Waals sphere is defined as a function of atom type, connectivity, overall charge of the molecule, and the number of attached hydrogen atoms. To obtain Gdr, the solvent accessible surface is used. Ges is obtained thanks to use of an approximate version of the solvent excluding surface constructed through scaling all radii by a constant factor (e.g. 1.2 for water) and then adding some more spheres not centered on atoms in order to arrive at a somewhat smoother surface [Fig. 8] [167].

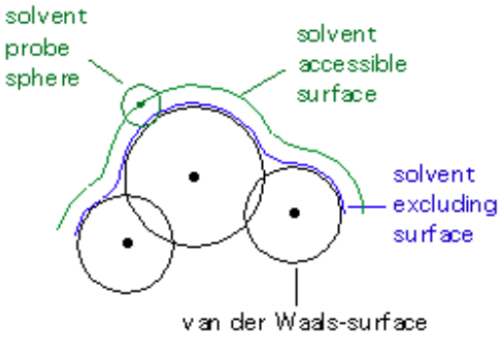

Fig. 8 Graphical representation of the PCM solvent model, description in the main text. Adapted from [167] under the CC BY 4.0. licence.

A different approach is presented by SMD. This model defines the free energy of solvation via two components: the one is electrostatic contribution arising from the self-consistent reaction field, the other comes from the short-range interactions between the solute and solvent molecules [168].

Results

For the first time the chosen EDCs (estradiol, progesterone and bisphenol A) and cyclodextrin have been subjected to the AMOEBA FF parametrization. The procedure was performed using Tinker software. The molecular information about the chosen molecules obtained after the parametrization process was compared with the data from the QM approaches and also using the classical CHARMM FF (NAMD software). The compared data stayed in a good agreement. 10-ns Molecular Dynamics simulation of EST with ERα was performed using Tinker-HP. The simulation was stable. Detailed information about methods and the results are presented in **Publication 2: Polarizable models for selected Endocrine Disrupting Chemicals and their hosts.**

For the first time, the crystal structure of the estradiol and β-cyclodextrin complex has been determined. Different approaches have been tested in order to obtain both crystalline and amorphous system. The complex has been analyzed using SCXRD, PXRD (powder X-ray diffraction), ^{13}C CP MAS ssNMR, FT-IR (Fourier transform infrared spectroscopy), TGA (thermogravimetric analysis), DSC (differential scanning calorimetry), Cryo-SEM experimental techniques as well as molecular modelling approaches: periodic DFT calculations and NMR parameters calculation (CASTEP software by BIOVIA). Detailed information about methods and the results are presented in **Publication 8: 17-β-Estradiol—β-Cyclodextrin Complex as Solid: Synthesis, Structural and Physicochemical Characterization**.

The EST-βCD complex has been also analyzed in the aqueous solution. Application of HRMS (high-resolution mass spectrometry) experimental technique allowed for the first time to thoroughly examine the structure of the complex and define its molar ratio as 1:2 (EST:βCD). Usage of the phase solubility phase studies delivered value of the complex stability constant what in turn enabled to obtain the experimental ΔG Gibbs free energy of the EST-βCD complex.
Moreover, the analyzed system was subjected to DFT and semi-empirical computational approaches (Gaussian16 software). The benchmark method was used to describe the influence of different computational QM-based parameters (B3LYP vs M06-2X functional / PM6 vs PM7 semi-empirical approaches; PCM / SMD water models / in vacuo; presence / absence of D3 dispersion correction) on the results concerning energy and thermodynamic properties. The parameters have been chosen based on the literature review **Publication 6: Current Status of Quantum Chemical Studies of Cyclodextrin Host-Guest Complexes**. At the end, Molecular Dynamics simulation and MMGBSA calculations were performed (AMBER software) to analyze the molar ratio and stability of the complex. Detailed information about methods and the results are presented in **Publication 9: 17-β-Estradiol—β-Cyclodextrin Complex as an aqueous solution: Structural and Physicochemical Characterization supported by MM and QM calculations**.

Conclusions and perspectives

The tasks defined at the beginning of this work have been successfully completed. For the first time, estradiol + β-cyclodextrin complex has been determined using both experimental and computational methods. The complex has been also analyzed in the aqueous solution using both experimental methods and a good variety of computational approaches which were compared to each other. For the first time the molar ratio of the complex has been definitely determined.

Estradiol, progesterone, bisphenol A and cyclodextrin have been successfully parametrized in the polarizable AMOEBA FF. Using AMOEBA FF, the estradiol + estrogen receptor system has been analyzed and the obtained simulation was stable.

This work constitutes a prelude to a complex analysis of the EDC-receptors and EDC-cyclodextrins systems what would be followed by formation of general guidelines on molecular modelling regarding such systems.

In the future, in the first place, progesterone + progesterone receptor and bisphenol A + estrogen receptor simulations using AMOEBA FF will be performed. Next steroidal hormones and selected small EDCs, like phthalates and polychlorinated biphenyls will follow. There is a need for further parametrization of such molecules and their simulation with the respective receptors. The former element will be easier now due to the already obtained steroidal fused rings parameters. These studies will not only deliver information on the applicability of polarizable force fields but will also contribute to the pre-experimental detection of possible EDCs and description of their mode of action.

With regards to the complexation between the low water solubility hormones and cyclodextrins, the list of both guests and hosts should be extended.

Potential objects of such studies would be steroidal pharmaceuticals such as progesterone, hydrocortisone, prednisolone, dexamethasone, testosterone etc. [Fig. 9].

First of all, till now structures of none of those hormones encapsulated in cyclodextrins have yet been identified. Secondly, similarly as in case of estradiol+β-cyclodextrin case, the stoichiometry of such complexes with β-cyclodextrin is not decisively determined, as the literature shows non-coherent data. So far, as a result of my additional research, two complexes: between progesterone and βCD as well as

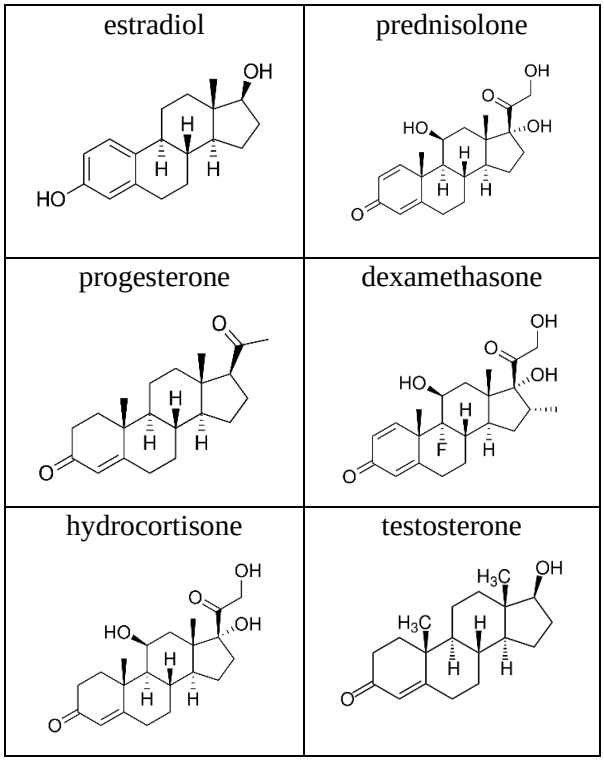

Fig. 9 Structures of selected EDCs, steroidal hormones of a pharmaceutical application.

between hydrocortisone and βCD have been already experimentally determined and analyzed using both experimental and computational methods. At the moment of writing this thesis, the results are not published yet.

The next step would be extension to other widely applied cyclodextrins like 2-HP-βCD. Additionally, the release of guests from the CD-complexes should be measured. Such a study has already been carried out for the estradiol+β-cyclodextrin complex as an addition to my PhD project. As it is described in the original publication attached to this thesis, two forms of this complex have been obtained: amorphous and crystalline. The first objective of the performed release study was to confirm that the solubility of the amorphous complex is higher, hence the release of the estradiol should be higher, as well. The second objective was to obtain the information how much the encapsulation in a cyclodextrin enhances the solubility of estradiol. Release study was performed in HCl solution of pH=2 according to the dissolution test for solid dosage forms as described in Ph. Eur. Monographs 2.9.3 [169] and 5.17.1. [170]. The paddle method was used.

The results confirmed that, as In the majority of cases, the solubility of the amorphous complex was higher than the crystalline one [Fig. 10]. However, the increase of the estradiol solubility after the encapsulation with cyclodextrin was very low [Fig. 10]. Those results have not been published yet, however the efforts to analyze different CD+steroidal pharmaceuticals may bring more welcomed results.

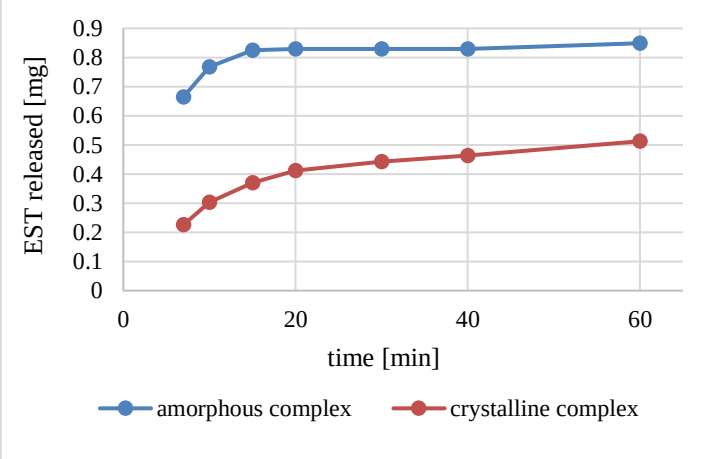

Fig. 10 EST release from amorphous and crystalline complex with β-cyclodextrin, unpublished results of PhD candidate.

Another group of EDCs which complexation with CDs might be useful are pesticides. The research in this topic has been already started. Thanks to the cooperation with the Agricultural University of Athens in Greece, a few complexes between CD and different derivatives of chlorophenoxyacetic acid have been already obtained during duration of my PhD project. The complexes have been analyzed with application of the molecular modelling approach. At the moment of writing this thesis, the results are not published yet.

All the above mentioned complexes should be analyzed using the in silico methods which were defined in this study as the most compatible with the experimental results. This will allow to create a good dataset of the results and confirm which methods are the most effective to predict structure, stoichiometry, stability and interactions within the CD-EDC complexes.

However, not only the already mentioned techniques should be applied. There are two MM-based extended space sampling methods which might be of an interest both for the CD-EDC and receptor-EDC complexes. Those methods are Steered Molecular Dynamics (SMD) and FEP (Free Energy Perturbation) calculations.

SMD applies an external steering force and in this way allows to move a ligand along a selected pathway (for example into and out of a host molecule). The moving is scheduled to stop at the given host-guest distances (called 'windows') in which in the equilibrated state, MD simulation is performed. The pulling velocity is applied to the selected ligand's atom [146,147]. SMD results have form of diagrams of the free binding energy vs host-guest distance, one for each window. WHAM (Weighted Histogram Analysis Method) [171] is used to connect these diagrams and arrive at one Potential of Mean Force profile (PMF) corresponding to the whole pulling process. Out of PMF, the overall ΔG is extracted [171].

SMD calculations deliver mechanistic information on the host-guest binding. In contrast to a binding site of a protein, each CD offers two entering modes: via its wider or narrower rim. This is well illustrated in the article from 2008 about β-CD and progesterone binding [172].
More than a decade ago, SMD has been checked for CD-complexes. One of the last articles using SMD for CD back then, in 2008 claimed that 'the energy analysis was in good agreement with the experimental results' (β-CD-progesterone complex) [172]. However, the CD input structure at the time could be optimized solely with the MM-based methods because the QM geometry optimization of such objects requires much more calculating power, the science lacked at the time. Though, the results obtained in 2008 may not be accurate. Now, it is possible to use the DFT-based methods for that purpose what means that the obtained results should be closer to the experimental data. In other words, SMD could be a good technique for the CD-complexes but it needs to be revisited with the new computer capabilities at hand.

Though, there are still just a few articles published on the subject. For the search 'cyclodextrin SMD' without any search constraints, the Web of Science database shows less than ten results, but interestingly, the used guests are similar to estradiol, for example pinostrobin in 2018 [173]. The newest article in the topic published in 2022 applied SMD for levodopa-CD complex [174]. All the cited studies omit description of a vital aspect which is geometry optimization. And it is already a well-known fact that the geometry of the initial structure has a huge impact on the SMD results. This is why the SMD method can be applicable for the CD-complexes only when the initial geometry is optimized with the newest QM-based approaches about which the benchmarking tests have been described in this thesis.

The next extended space sampling approach is FEP. It allows to calculate the difference of ΔG (ΔΔG) between two similar systems. This method is used for instance to calculate differences in

ligand binding to a wild and mutated protein or to obtain Potential of Mean Force profile for systems which differ in chemical structure, for example comparison of a couple of similar ligands binding to the same binding site [148,149].

In FEP, thermodynamic cycles of non-existing intermediate states are created [175] [Fig. 11]. At each state, after obtaining an equilibrium, the MD simulation is performed. Movement from one intermediate state to the other is regulated by the coupling parameter λ [176].

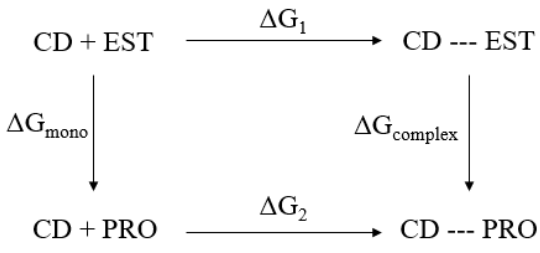

Fig. 11 $\Delta\Delta G = \Delta G_2 - \Delta G_1 = \Delta G_{complex} - \Delta G_{mono}$

Similarly to the SMD case, also FEP application for the CD systems is rare. Two articles refer to a double complexation of a ligand with a CD (imipramine [177], amphotericin B [178]). With regards to this thesis, the more interesting example is the FEP study for progesterone, testosterone and hydrocortisone [179] which delivers some concrete calculation parameters. However, this data has been published in 2009, so surely it needs to be revisited, taking into account even just the increase of the computational power which happened in the last decade. The last, and the most recent (2016), CD-including FEP study corresponds to S-β-CD complexed with either uranyl or uranyl ion [180]. This publication can be also a source of some basic calculational details but its objects are far different than EDCs.

The same methods, SMD and FEP, could be used also for the EDC-receptor systems. And both in CD-EDC and receptor-EDC cases, additive and polarizable force field (AMOEBA FF) should be used. The compilation of all above mentioned methods and objects, would deliver a complete view on the molecular modelling possibilities and challenges regarding the computational analysis of the interactions between EDCs and host molecules.

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PhD project publications

I Publications which are the core of the PhD project (basis for the defence)

a) Original research
   - Mazurek, A.H., Thirion V., Szeleszczuk Ł., Piquemal J.-P., Clavaguera C., Simonson T. Polarizable models for selected Endocrine Disrupting Chemicals and their hosts. J. of Comp. Chem. SUBMITTED (in this document named as Publication 2)
   - Mazurek A.H., Szeleszczuk Ł., Bethanis K., Christoforides E., Dudek M.K., Zielińska-Pisklak M., Pisklak D.M. 17-β-Estradiol—β-Cyclodextrin Complex as Solid: Synthesis, Structural and Physicochemical Characterization. Molecules. 2023, 28, 3747. Doi: 10.3390/molecules28093747 (in this document named as Publication 8)
   - Mazurek A.H., Szeleszczuk Ł., Bethanis K., Christoforides E., Dudek M.K., Wielgus E., Pisklak D.M 17-β-Estradiol—β-Cyclodextrin Complex as an aqueous solution: Structural and Physicochemical Characterization supported by MM and QM calculations. J. of Mol. Structure XXXX (in this document named as Publication 9)

b) Review articles
   - Mazurek A.H., Szeleszczuk Ł., Simonson T., Pisklak D.M. Application of Various Molecular Modelling Methods in the Study of Estrogens and Xenoestrogens. IJMS 2020, 21, 6411. DOI: 10.3390/ijms21176411 (in this document named as **Publication 1**)
   - Mazurek A.H., Szeleszczuk Ł. Current Status of Quantum Chemical Studies of Cyclodextrin Host–Guest Complexes. Molecules. 2022, 27, 3874. DOI: 10.3390/molecules27123874 (in this document named as **Publication 6**)

- For the above listed publications Polish Ministry of Science and Higher Education points is equal to 490 pkt
- For the above listed publications IF is equal to 18.6

II Additional publications

- Mazurek A.H., Szeleszczuk Ł., Pisklak D.M. Periodic DFT Calculations—Review of Applications in the Pharmaceutical Sciences. Pharmaceutics. 2020, 12, 415. DOI: 10.3390/pharmaceutics12050415 (in this document named as **Publication 3**)
- Mazurek A.H., Szeleszczuk Ł., Gubica T. Application of Molecular Dynamics Simulations in the Analysis of Cyclodextrin Complexes. IJMS. 2021, 22, 9422. DOI: 10.3390/ijms22179422 (in this document named as **Publication 4**)
- Mazurek A.H., Szeleszczuk Ł., Pisklak D.M. A Review on Combination of Ab Initio Molecular Dynamics and NMR Parameters Calculations. IJMS. 2021, 22, 4378. DOI: 10.3390/ijms22094378 (in this document named as **Publication 5**)
- Mazurek A.H., Szeleszczuk Ł. A Review of Applications of Solid-State Nuclear Magnetic Resonance (ssNMR) for the Analysis of Cyclodextrin-Including Systems. IJMS. 2023, 24, 3648. DOI: 10.3390/ijms24043648 (in this document named as **Publication 7**)
- Szeleszczuk Ł., Mazurek A.H., Milcarz K., Napiórkowska E., Pisklak D.M. Can We Predict the Isosymmetric Phase Transition? Application of DFT Calculations to Study the Pressure Induced Transformation of Chlorothiazide. IJMS. 2021, 22, 10100. DOI: 10.3390/ijms221810100
- Zielińska A., Mazurek A., Siudem P., Kowalska V., Paradowska K. Qualitative and quantitative analysis of energy drinks using 1H NMR and HPLC methods. J. Pharm. Biomed. Anal. 2022, 213, 114682. doi: 10.1016/j.jpba.2022.114682.
- Jurczak E., Mazurek A.H., Szeleszczuk Ł., Pisklak D.M., Zielińska-Pisklak M. Pharmaceutical Hydrates Analysis-Overview of Methods and Recent Advances. Pharmaceutics. 2020, 12, 959. doi: 10.3390/pharmaceutics12100959 (in this document named as **Publication 10**)

- Overall Polish Ministry of Science and Higher Education points is equal to 1428 pkt
- Overall IF is equal to 59.802

Appendix

The following appendix is composed of publications which are the basis for the defence. Those are:

1) Polarizable models for selected Endocrine Disrupting Chemicals and their hosts. (proof of submission)
2) 17-β-Estradiol—β-Cyclodextrin Complex as Solid: Synthesis, Structural and Physicochemical Characterization.
3) 17-β-Estradiol—β-Cyclodextrin Complex as an aqueous solution: Structural and Physicochemical Characterization supported by MM and QM calculations.
4) Application of Various Molecular Modelling Methods in the Study of Estrogens and Xenoestrogens.
5) Current Status of Quantum Chemical Studies of Cyclodextrin Host–Guest Complexes. Molecules.