EXPERIMENTAL AND COMPUTATIONAL INVESTIGATION OF CLUSTERING BEHAVIOR OF CYCLODEXTRIN-PERFLUOROCARBON INCLUSION COMPLEXES THROUGH MOLECULAR DYNAMICS SIMULATIONS

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Betül Kaymaz

June, 2021

EXPERIMENTAL AND COMPUTATIONAL INVESTIGATION OF CLUSTERING BEHAVIOR OF CYCLODEXTRIN-PERFLUOROCARBON INCLUSION COMPLEXES THROUGH MOLECULAR DYNAMICS SIMULATIONS

By Betül Kaymaz

24 June 2021

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a dissertation for the degree of Master of Science.

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CONTENTS

Page

ACKNOWLEDGEMENT ..... **iv**
CONTENTS ..... **v**
LIST OF FIGURES ..... **vi**
LIST OF TABLES ..... **vii**
LIST OF SYMBOLS ..... **viii**
ABBREVIATIONS ..... **ix**
ÖZET ..... **x**
ABSTRACT ..... **xii**
1. INTRODUCTION **1**
2. THEORETICAL PART **4**
2.1. Histotripsy 4
2.1.1. Usage areas of histotripsy 6
2.1.2. Limitations 7
2.1.2.1. Nanoparticle mediated histotripsy 8
2.2. Cyclodextrins 10
2.2.1. Cyclodextrin aggregations 12
2.2.2. Computational investigation of cyclodextrins and its aggregates 13
2.3. Perfluorocarbons 14
2.4. Nanocones 15
3. EXPERIMENTAL PART **17**
3.1. Materials 17
3.2. Characterization 17
3.3. Preparation of Nanocone Cluster (NCC) 18
3.4. Recovery of NCC from Solution 18
3.5. Molecular Dynamics Simulations 18
3.6. Histotripsy Study 20
4. RESULTS AND DISCUSSION **21**
4.1. Preparation of Nanocone Clusters 21
4.2. Molecular Dynamics Simulations 26
4.3. Recovery of NC Clusters from Solution 37
4.4. Histotripsy Study 40
5. CONCLUSIONS AND FUTURE WORK **41**
BIBLIOGRAPHY **43**
CURRICULUM VITAE **49**

LIST OF FIGURES

Figure 2.1: a) Ultrasound-guided histotripsy system and **b)** experimental histotripsy set up. 5
Figure 2.2: Image of targeted tissue ablation performed by histotripsy. 6
Figure 2.3: Response of the tissue phantoms at different acoustic pressures by using nanodroplet-mediated histotripsy. 9
Figure 2.4: Schematic of a glucopyranose unit (left) and natural cyclodextrins with their size properties. 10
Figure 2. 5: Schematic of CD inclusion complex and its aggregation. 12
Figure 2.6: Initial **1** and final **2** structures of **a)** βCD, **b)** γCD, **c)** HPβCD, and **d)** HMβCD. 14
Figure 2.7: Chemical presentations of different PFCs. 14
Figure 2.8: Nanocones mediated histotripsy application. 15
Figure 4.1: All cyclodextrin derivatives that are experimentally tested. 22
Figure 4.2: Appearance of CD derivatives with and without PFH after overnight stirring at RT. 23
Figure 4.3: Size of different cyclodextrin solution itself and in the presence of PFH of 1/1:CD:PFH ratio. 24
Figure 4.4: Z-Average size comparison of CD derivatives with 1 to 5 molar ratio PFH addition. 25
Figure 4.5: Distances between the center of mass of CDs and PFCs. 28
Figure 4.6: Energy values for 1:1 inclusion complexation investigation by **a)** Electrostatic and **b)** van der Waals interactions. 29
Figure 4.7: βCD (cyan)-PFH (green) and αCD (orange)-PFH (green) complexes comparison. There is 1 to 5 molar ratio between CD and PFH. 34
Figure 4.8: Energy values for clusters with 1:1 ratio are shown by **a, c, and e)** Electrostatic interactions and **b, d, and f)** van der Waals interactions. Energy values between CD and PFC, CDs, and PFCs with each other are presented by the first, second, and third rows respectively. 36
Figure 4.9: Energy values for clusters with 1:5 ratio are shown by **a, c, and e)** Electrostatic interactions and **b, d, and f)** van der Waals interactions. Energy values between CD and PFC, CDs, and PFCs with each other are presented by the first, second, and third rows respectively. 37
Figure 4.10: Histotripsy experiment results for βCD-PFC NCCs 40

LIST OF TABLES

Table 2.1: Cyclodextrin Derivatives with Their Basic Properties. 11
Table 4.1: Matching of computational and experimental results for 1:1 and 1:5 ratio clusters. 30
Table 4.2: Atomic Content Percentage of βCD-PFH, αCD-PFH, and HPβCD according to EDX. 39

LIST OF SYMBOLS

α : Alpha
β : Beta
γ : Gamma
Å : Angstrom
q : Charge
μ : Micro

ABBREVIATIONS

CD : Cyclodextrin
βCD : Beta Cyclodextrin
αCD : Alpha Cyclodextrin
γCD : Gamma Cyclodextrin
LMβCD : Low Degree Methylated Beta Cyclodextrin
HMβCD : High Degree Methylated Beta Cyclodextrin
HPβCD : Hydroxypropyl Beta Cyclodextrin
HMγCD : High Degree Methylate Gamma Cyclodextrin
HPγCD : Hydroxypropyl Gamma Cyclodextrin
PFC : Perfluorocarbon
PFP : Perfluoropentane
PFH : Perfluorohexane
DLS : Dynamic Light Scattering
EDX : Energy-Dispersive X-ray Spectroscopy
GC : Gas Chromatography
NC : Nanocone
NCC : Nanocone Cluster
NMH : Nanoparticle-Mediated Histotripsy
HIFU : High-Intensity Focused Ultrasound
FPGA : Field-Programmable Gate Array
ND : Nanodroplet
EPR : Enhanced Permeation and Retention
ADV : Acoustic Droplet Vaporization
TFA : Trifluoroacetic Acid
DMF : Dimethylformamide
BP : Boiling Point
PDB : Protein Data Bank
ID : Identifier
CID : Compound Identifier
^{19}F NMR : Fluorine Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR : Hydrogen Nuclear Magnetic Resonance Spectroscopy

SİKLODEKSTRİN-PERFLOROKARBON KONUK-KONAK KOMPLEKSLERİNİN KÜMELENME DAVRANIŞININ DENEYSEL VE MOLEKÜLER DİNAMİK SİMÜLASYONLARI İLE İNCELENMESİ

ÖZET

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Yeni bir teknik olan histotripsi, ultrason dalgalarını kullanarak dokularda doğal olarak bulunun gaz baloncuklarının kavitasyonuyla mekanik olarak dokuların parçalanmasına olanak sağlar. Diğer ablasyon yöntemlerinin aksine histotripsi ablasyon sırasında ısı çıkışına neden olmaz ve parçalanan doku sıvılaşarak vücuttan noninvazif olarak atılır. Odaklanılan histotripsy kullanım alanlarından biri olan kanser tedavisinin üçüncü klinik fazda olan insan testleri başarılı bir şekilde devam etmektedir. Sonuçlara göre histotripsi kanser tedavisi için kullanıma uygun, gelecek vadeden bir tekniktir. Tüm olumlu sonuçların yanı sıra histotripsi umut verici bir yöntem olmasına rağmen sınırlayıcı faktörlere sahiptir. Dokuların parçalanması için yüksek eşik basınç değeri gerekliliği, seçici olamayan ablasyon ve çoklu tümor tedavisi için geniş alanları tarama imkanı sağlayamaması bu sınırlayıcı faktörlerdendir. Yüksek basınca maruz kalan dokular sağlıklı olup olmaması fark etmeksizin parçalanırlar. Bu sebeple kanser tedavisi için doku ablasyonu sırasında odaklanmış ultrason ile uygulanan yüksek kavitasyon basıncı tümor dokunun çevresinde bulunan sağlıklı dokuya da zarar verebilir. Bu problemi çözmek için nanoparçacıkların kavitasyon çekirdeği olarak kullanılarak kavitasyon eşik değerini düşürdüğü, nanoparçacık aracılığıyla histotripsi (NMH) olarak adlandırılan metot geliştirilmiştir. NMH, FDA (Food and Drug Administration) onaylı perflorokarbon (PFK) içeren, ultrason uygulandığında vücut içinde gaza dönüşebilen nanoparçacıklar kullanarak düşük basınçta doku parçalanmasını sağlar. Ayrıca bu parçacıklar tümörün sebep olduğu bozuk damar yapısını kullanıp tümor dokuda birikerek nanoparçacıkların lokal yoğunluğunu arttırır.

En son geliştirilmiş mekanik doku ablasyon ajanlarından biri olan nanokaplar (NK), siklodekstrin (CD) ve PFK konuk-konak kompleksleri kolay üretim, kararlı yapı ve hesaplanabilir PFK miktarı olanakları sunarak NMH için gelecek vadeden sonuçlar göstermiştir. İlk NK örnekleri βCD (beta siklodekstrin) ve düşük derece metilli βCD (LMβCD) kullanılarak oluşturulmuştur. PFK molekülleri gibi FDA onaylı olan siklodekstrinler barındırdıkları halkasal glikoz birimi sayılarına göre gruplara ayrılırlar, bu çeşitlilik farklı PFK kullanımlarında farklı kompleksleşme davranışları gözlemlenmesine sebep olabilir. Buna ek olarak da siklodekstrin türevlerinin sahip oldukları fonksiyonel gruplar kümelenme ve konuk-konak kompleksi oluşumlarına katkısını aydınlatmak için daha fazla araştırmaya gerek olduğu bilinmektedir. Siklodekstirinler ile perfloropentan (PFP) ve perflorohekzan (PFH) kullanılarak oluşturulan kütüphane, kompleks ve kümelenme oluşturma davranışlarının tespiti ve CD-

PFK kümeleri hakkında detaylı bilgi elde edebilmek için ilk olarak deneysel olarak incelemeler yapılmıştır. Sınırlı optik özellikler ve yapılardaki PFK tespitindeki zorlukluklar gibi deneysel sınırlayacı faktörlerden ötürü, NMH ajanı olabilecek en uygun yapıyı bulmak için hesaplamalı inceleme yapılmıştır. Deneysel olarak; gaz kromatografisi kullanılarak verim yüzdesi hesaplanmış, enerji dağılımlı x-ışını floresans spektrometresi kullanarak flor varlığı kontrol edilmiş, ve dinamik ışık saçılım spektrometresi kullanılarak boyut çalışması yapılmış, hesaplamalı olarak; görsel desteğin yanında, kütle merkezleri arasındaki uzaklıklar ölçülerek nanokap oluşturma potansiyelleri kıyaslanmış, elektrostatik ve van der Waals etkileşimlerinin meydana getirdiği enerji değerleri hesaplanmıştır. Sonuçlar yalnızca βCD ve γCD (gamma siklodekstrin) türevlerinin PFK ile konuk-konak kompleksi oluşturduğunu ve deneysel olarak sadece LMβCD, βCD, and γCD çökelti oluşturarak kullanılabilir örnekler elde edilmesine olanak sağlamıştır. Bu üç siklodekstrin türevi arasında βCD en çok kullanılan türev olması ve kullanım öncesi modifikasyona ihtiyacı olamaması sebebiyle NK kümelenmeleri oluşturmak için en iyi aday olarak gözükmektedir. Elde edilen sonuçlarla βCD ve PFK moleküllerinden oluşan nanokapların kararlılık, üretim ve saflaştırma kolaylığı açısından en etkin kombinasyon olduğu ortaya çıkmıştır. PFH, PFP ile kıyasla daha yüksek bir kaynama noktasına sahip olduğu için üretim ve saklama aşamalarında daha kararlı NK kümeleri meydana getirmektedir. Simülasyon sonuçlarında hidroksipropillenmiş βCD (HPβCD) nanokapları gibi daha yüksek enerjiyle daha kararlı yapılar oluşturan durumlar olduğu ispatlanmış olsa da, konak moleküllerinin yüksek çözünürlüğü sebebiyle deneysel olarak çökme gözlemlenmeyen örneklerin genel laboratuvar pratikleri kullanılarak içerdikleri suyun uzaklaştırılması mümkün olmamış, PFK içeriği kaybedilmiştir. Son olarak elde edilen NMH kullanımı için en iyi NK kümesi adayı olan βCD-PFK kombinasyonlarının histotripsi etkinlikleri de test edilmiş, kavitasyon eşik değerini başarıyla düşürdükleri ispatlanmıştır.

Anahtar sözcükler: Histotripsi, Nanoparçacık Aracılığıyla Histotripsi, Nanokap, Siklodekstrin, Perflorokarbon.

EXPERIMENTAL AND COMPUTATIONAL INVESTIGATION OF CLUSTERING BEHAVIOR OF CYCLODEXTRIN-PERFLUOROCARBON INCLUSION COMPLEXES THROUGH MOLECULAR DYNAMICS SIMULATIONS

ABSTRACT

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MSc in Biomedical Engineering and Bioinformatics

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June, 2021

Histotripsy is a new approach which can ablate the tissue mechanically by using ultrasound waves which cavitate the pre-existing gas pocket in the tissue. Oppose to other ablation method, histotripsy is considered non-invasive since the ablation process does not cause heat generation and ablated tissue gets liquefied and eliminated by the body. Cancer applications one of the focus areas of histotripsy and human trial on third clinical stage has been successfully going on and results support that histotripsy is a promising method for cancer treatment. Even though histotripsy is a promising method to ablate tissue in the body, it has also limitations such as high cavitation threshold pressure, non-selective ablation, and scanning the large area includes multifocal tumors. Regardless of cancer or healthy tissue, all the tissue can be ablated if high cavitation pressure is applied with focus ultrasound which might cause damage on surrounding healthy cells during cancer tissue ablation. This problem was addressed via nanoparticle mediated (NMH) which uses nanoparticles as cavitation nuclei to lower the cavitation threshold. NMH uses perfluorocarbons (PFCs) filled nanoparticles that can become gas in the body under the ultrasound is applied. Moreover, these nanoparticles have the ability to accumulate tumors through leaky vasculature structure and increase the localized density of nanoparticles that can act like cavitation nuclei to lower the cavitation threshold pressure.

Recently developed nanoparticles called nanocones (NCs) which are inclusion complexes of cyclodextrin (CD) and PFCs showed promising results for NMH applications due to easy production, stable formation, and determinable PFC amount. A primary example was shown using βCD and low degree methylated βCD, however, cyclodextrins are FDA approved molecules likewise PFCs, and have different cavity size according to the number of glucopyranose units that can accommodate different PFCs molecules. Furthermore, it is known that functionality of CD derivatives contributes on aggregation and complexation behavior which need to be a further investigation for guest molecule of PFCs. The complexation and aggregation behavior of a library of CDs with perfluoropentane (PFP) and perfluorohexane (PFH) was first experimentally examined in order to get deeper information about the aggregation behavior of CD-PFC. Because of limited optical properties and the difficulty of determining PFC in the structure experimentally, a computational study is further investigated to determine the most suitable structure as an NMH agent.

The encapsulation efficiencies were experimentally determined by gas chromatography (GC), the presence of F (fluorine) atom in the aggregate was confirmed by energy dispersive x-ray analysis (EDX), and the size of clusters was determined by using dynamic light scattering spectroscopy (DLS). The distances between the center of masses were computationally compared to investigate the tendency of molecules to form an inclusion complex cluster, called nanocone cluster (NCC). Since there are only non-bonded interactions, the energies due to electrostatic and van der Waals interactions were calculated separately. Results showed that only βCD and GCD derivatives form an inclusion complex with PFC and only LDβCD, βCD, and GCD form NCC that can be precipitate and collect as samples for further use. Since βCD is most commonly used and does not require any further modification, it seems like a better candidate for NCC formation. Collectively data also supported that βCD and PFC showed the best formation due to stability, easy production, and recovery opportunities indicating higher boiling point PFH as better PFCs due to the better stability during production and storage. Even though, simulation data indicate there are more stable formations such as hydroxypropyl beta cyclodextrin (HPβCD), the high water solubility of host molecules prevents precipitation of cluster and makes the recovery process impossible using general laboratory practices to remove water without losing PFC content in the NCC. Finally, it was shown that cavitation threshold pressure was successfully decreased using the best NCC candidate for NMH.

Keywords: Histotripsy, Nanoparticle Mediated Histotripsy, Nanocones, Cyclodextrin, Perfluorocarbon.

CHAPTER 1

1. INTRODUCTION

Cyclodextrins (CDs) and their derivatives are supramolecules known for their ability to form stable inclusion complexes and self-assembled aggregates [1], [2] which has enabled them to become an essential part of research and pharmaceutical applications over recent decades [3]. Aggregation in CDs and their inclusion complexes, as evidenced in Dynamic Light Scattering (DLS) studies, Transmission Electron Microscopy (TEM), Nuclear Magnetic Resonance (NMR), and dialysis membrane permeation methods in multiple researches [4]–[8] has been explained to occur as a result of a balance between hydrophilic and hydrophobic moieties of involved molecules driving towards the growth of self-assembled and ordered structures [9]. These researches further indicate that the resulting aggregates are much complex in nature as they can be of various sizes, differ in their stability and solubility depending on multiple factors such as the nature of the CD molecule or the modification on it e.g. hydroxypropyl or methyl groups, solvent system (aqueous or organic), concentration of CD and/or guest molecule, pH of solution, temperature at which a CD is exposed and binding equilibrium of guest molecule with the hydrophobic cavity of the CD. Moreover, introduction of other factors such as mixing of soluble polymers and adding functionalities may also affect this aggregation and complexation leading to completely different aggregate and drug solubility profiles [5], [10]. The methods of forming and collecting these aggregates are critical as well for the bulk synthesis varying in time scales from hours to days depending upon the nature of the guest molecule and type of complexes (A or B) formed thereof [11]. The advantage of having a natural CD being able to form an inclusion complex as well as a nanoparticle has been capitalized to form nano sized acoustically active agents in a modern therapeutic technique such as histotripsy [12]–[16], an ultrasound-based technique that employs ultrasound waves with repeating high amplitude short-duration pulses to strain and ablate tissues at a focal point inside the body without disrupting neighboring tissues for

advanced medical treatments [17]. Nanodroplets (NDs) have been developed as a histotripsy agent and their success for lowering required cavitation threshold has been published [15], [16], [18]. Due to complex production steps requirement and determination of exact PFC amount uncertainty, further studies have been done and another nanoparticle has been developed. This study revealed the potential of natural beta cyclodextrin (βCD)'s derivative, low degree methylated βCD (LMβCD), as a host molecule which can engulf in its core the ultrasound agents such as perfluorocarbon (PFC) namely perfluorohexane (PFH) as a guest molecule to form an inclusion complex referred as "nanocone (NC)" resulting in nanosized particles (<50 nm) as mediating histotripsy agents and that the overall cavitation threshold was significantly reduced from 25-30 MPa to 10-15 MPa. The size of individual cyclodextrin derivatives was clearly different than their inclusion complexes with PFC (~40-100 nm) which was a clear indication of a variation in the mode of aggregation or cluster formation due to complexation [1]. Although the potential of combining CD and PFH as host-guest for histotripsy application was established in a previous study, the structure and nature of PFH makes the complexation reaction hard to monitor both at molecular and nano scales due to lack of characteristic optical absorbance and limited characterization ways and therefore, strands us with a lack of understanding of the mode and type of aggregate or cluster formed thereof and its relative stability. This limitation becomes more crucial as experimental evidence discussed formerly suggests that CD aggregates are susceptible to varying physio-chemical conditions and therefore, may affect the histotripsy agent's aggregate stability, a fundamental parameter affecting its potential future use. Moreover, it is yet to be determined whether only LMβCD is the only or best CD derivative suitable for histotripsy or do other members of the CD family hold any prospect. The current study addresses these fundamental issues in order to move further the discussion and research to realize the potential of these ultrasound active nanoparticles by investigating inclusion complex formation and potential aggregation/clustering of these inclusion complexes. In addition to be all being commercially available except LMβCD, cyclodextrin derivatives were selected based on their cavity size and functionality that affect not only solubility feature in water but also it has contribution to complexation behavior. For instance, αCD might not be the best candidate for PFH but might have a perfect fit for PFP, or γCD with a wider cavity could accommodate more than one PFC molecule in its cavity. Moreover, it has been also stated in the literature that different functionality of cyclodextrin affects the complexation behavior by changing cavity size and solubility [19].

In this study, the formation potential of nanocone cluster (NCC) between 8 different CD derivatives and 2 PFCs (perfluoropentane (PFP) and perfluorohexane (PFH)) is experimentally and computationally investigated. The experimental conditions will mainly invoke green chemistry criterion and the formation of any collectible nanoaggregates will be considered practical for this study and further characterized. Inclusion complexation and aggregate/cluster formation will be tested computationally using molecular dynamics simulation to prove whether there forms a stable NC and its aggregate or just a physical assembly. This difference also can affect the histotripsy mechanism. To enlighten this complexation mechanism as well as the clustering mechanism a detailed study, both experimental and computational, was done by starting 1 to 1 interactions of CD and PFC derivatives to investigate the inclusion complex formation potential of individual CD derivatives with different PFCs through molecular dynamics simulations. After that to see clustering behavior and also the effect of complexation capacity on the cluster formation two different molar ratios were tested to see the effect of free PFC presence. Visual results were supported by energy calculations through Lennar-Jones and Coulomb interaction models. In the case of 1 to 1 inclusion complex tests, additionally, the center of mass values were calculated. Hence, the predisposition to stay together or close could be shown. Finally, the potential of lowering cavitation threshold of histotripsy was tested for BCD:PFC ratios of 1:1 and 1:5 for the best composition to see whether cluster structure with and without free PFC has an effect on cavitation threshold or working mechanism of NC as nanoparticles for NMH.

CHAPTER 2

2. THEORETICAL PART

2.1. Histotripsy

Histotripsy is a method that can destruct a tissue for several applications. This method provides the ablation of desired soft tissue mechanically in the body by using ultrasound waves. The name also describes the method, in Greek, *histo* means soft tissue, and *tripsy* means break down [20]. Before this technology, to ablate a tissue another method called high-intensity focused ultrasound (HIFU) was found and is used clinically. In the case of HIFU thermal ablation of tissue is carried out by raising the temperature above 55°C and retain for 1 second [21]. Decreasing the temperature and maintenance of it to complete the ablation treatment for the whole target area causes a huge problem; thermal injuries due to non-targeted application [22]. For this reason, further studies were needed. Histotripsy was created to ablate tissue in non-thermal and non-invasive ways. In this technique, ultrasound waves are used to ablate tissues mechanically.

For the histotripsy, a special ultrasound transducer was designed. Histotripsy transducers differ from the regular transducer by their concave shape which is consists of smaller ultrasound transducers. Basically, histotripsy uses an ultrasound transducer which is constituted by a sheer number of regular ultrasound transducers. This special architecture serves for the mechanical ablation of the targeted tissue. The highest pressure value is obtained in only targeted area. When the waves go through the surrounding tissue there is no ablation or any harm due to low pressure value. To ablate a tissue there is a pressure threshold ~25–30 MPa for the soft tissues [23]. The high pressure causes rapid expansion and eventually, explosion of free gas pockets in the focal area by evaporation.

Figure 2.1 shows both experimental setup and clinical appearance of histotripsy. In the experimental setup, 1-MHz focused ultrasound transducer produces required ultrasound pulses when a field-programmable gate array (FPGA) board generates input signals.

When the transducer is triggered, a marine hydrophone is used to acquire acoustic waveforms. The process is also verified by using a high-speed camera with a light source. In a water tank, there is a tissue sample or agarose tissue phantom which mimics tissue was placed as a target. The tank is filled with water since water can mimic the acoustic behavior of the soft tissue and allows passing of the pulses [17], [24].

Since ultrasound also an imaging tool, as it presents in **Figure 2.1a**, by adding an imaging probe to the transducer process turns to an ultrasound-guided histotripsy treatment with this addition second image guide is supplied.

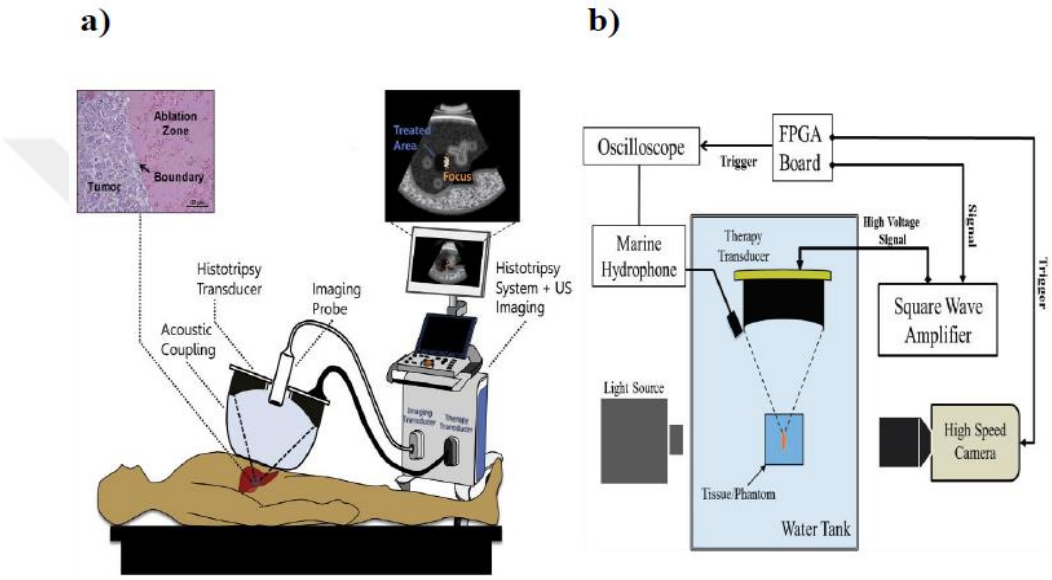


Figure 2.1: a) Ultrasound-guided histotripsy system and **b)** experimental histotripsy set up [17], [24].

As a result, the process ends up with the ablation of desired area in tissue. **Figure 2.2** provides images of targeted tissue ablation by histotripsy without harming surrounding tissue. Targeting is done by clinician manually with the help of an imaging probe.

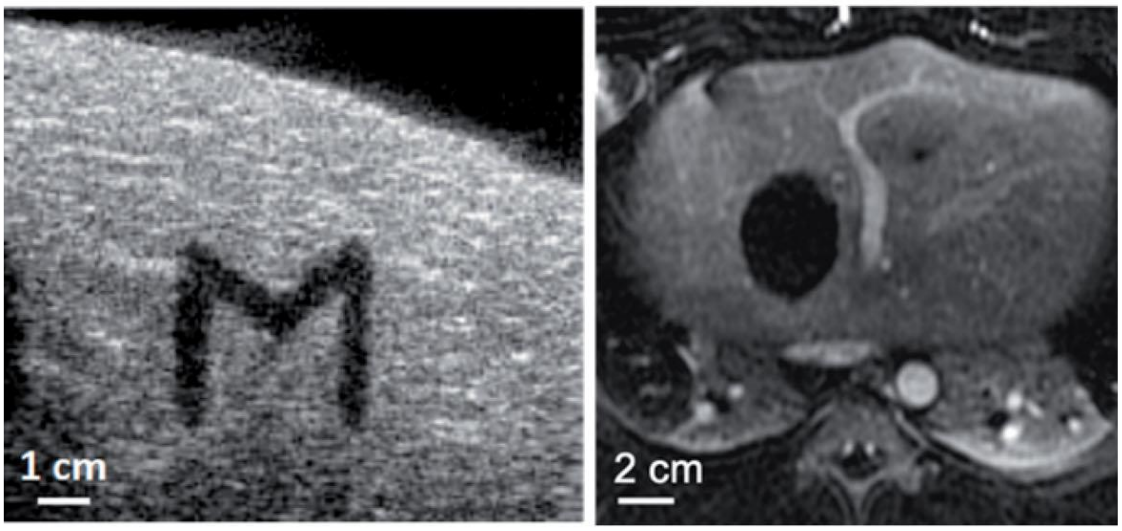


Figure 2.2: Image of targeted tissue ablation performed by histotripsy [25].

Non-invasive and non-thermal behavior of the histotripsy opens a wide scope for the applications.

2.1.1. Usage areas of histotripsy

In a body, there might be several unwanted tissues in the veins or organs. In some cases to treat a disease alteration can be done by removing tissues medically. Surgeries one of the most common ways to do this kind of intervention. In modern days, with the help of technology, a surgery does not comprise very high risks the way it used to be. Still it has risks and if it is possible to avoid them again with the help of technology the risk can be reduced to the minimum. The problems with a surgery are infection risks, congelation problems and for the surgery, the patient must be under anesthesia. Besides these risks, it is an expensive process and mostly after a surgery patient will need time to full recovery. To overcome these limitations histotripsy can be the answer for some cases. Various studies have assessed the efficacy of histotripsy for diverse tissue types which can be a good candidate for this new approach. By using histotripsy even doing medical intervention to fetuses without taking surgery risks is possible. Kim et al. tried to test histotripsy's fetal activity by using a sheep model. The liver, kidney, and heart were chosen as target organs. The study showed that ablation and removal of the tissue are possible by histotripsy and this is the only method for tissue removal non-invasively from fetal organs [26], [27]. Renal applications another area for histotripsy usage. Several studies have investigated histotripsy for tissue ablation in the renal area. Hall et al. performed renal tissue ablation in vivo by using a rabbit model [28]. Winterroth et al.

tested histotripsy by using ex vivo porcine kidney tissue [29]. Both of these studies and some other studies came to the following conclusion, histotripsy is an effective way to ablate renal tissues and facilitates the treatment procedure comparing to other methods i.e. invasive surgery. Prostatic and cardiac tissue ablations are also popular topics for this approach. Lake et al. and Wheat et al. reported their findings as histotripsy is a promising method and requires future study. Wheat et al. also reported that treatment of the prostate causes minimal bleeding and to test limits of the bleeding, aggressive treatment was performed by targeting large volumes and anticoagulation was done to the canine. As a result, they ended up with only minimal bleeding [30]. In the literature, it is possible to find examples of other tissue types such as liver [31] and heart [32]. This method is not only usable for soft tissues but also opens up a new treatment method for urinary calculi [33]. Much of the current literature on histotripsy pays particular attention to cancer treatment in several organs. Histotripsy also opens up a new treatment way for cancer treatment. Several conditions were tried not only ex vitro but also in vivo and the results are promising. Different type of cancer treatment is possible with this approach. In a study which set out to determine in vivo activity of histotripsy, implantation of a VX-2 tumor which is a rabbit papilloma cell line for malignant cancer model into the kidney in a rabbit model and they showed the potential of a new cancer treatment successfully [34]. Data from several studies also support the success of cancer treatment by histotripsy. It is possible to find example for renal [35], prostate [36], liver [37], breast [38], pancreatic, musculoskeletal cancers treatment. These researches examine the emerging role of histotripsy in the context of cancer treatment.

2.1.2. Limitations

These findings suggest histotripsy as a promising cancer treatment method. However, this approach also has limitations. The biggest issue is, targeting the focal area is done by a technician. Since there is no other targeting mechanism, the process highly opens to human error. Due to high pressure and the absence of a specific targeting mechanism, destruction of tissues in the focal area happens. In this case, healthy tissue can be damaged. Irregular shaped tumors make the targeting hard and make precise ablation almost impossible. The efficacy would decrease even though all the attention. Another problem, ultrasound is not the best way for imaging certain tumors. Even though in the case of cancers which can visualize by ultrasound some metastatic tumor tissues cannot be detected due to their small size.

2.1.2.1. Nanoparticle mediated histotripsy

The main focus of this study will be reducing the high energy requirement for ablation in the focal area. If a way would be found to reduce only the targeted tissue pressure threshold, healthy surrounding tissues cannot be affected even though they are scanned too. But here is the problem, how we will reduce this threshold just in the target area. ~25–30 MPa [23] ultrasound pressure is required to destroy tissue with the help of microbubbles. Perfluorocarbons (PFCs) are well-known molecules which are used as ultrasound contrast agents. They are mainly used for diagnosis. PFCs can evaporate easily with the pressure decrease without reaching high pressure values. This property of PFCs makes them applicable for cavitation applications and it is possible to see examples in the literature. More information will be supplied in a future chapter called 'perfluorocarbons'.

In the literature, there are examples of usage of some nanoparticles which can accumulate in tumor tissue and reducing required pressure threshold as a result of ultrasound active agent presence. Detailed study was done about a nanoparticle called nanodroplets (NDs) by Yüksel Durmaz et al. It is proved that reducing the threshold locally is possible with these particles. Nanodroplets are polymeric micelle which can carry ultrasound active agent in its core. Cancer environment differs from healthy tissues with its particular characteristic. The acidic cellular environment can be an example for these features, but we will focus on another feature for our content. Due to uncontrolled propagation, cancer tissues form leaky vasculature. This leaky vasculature property of cancer is an opportunity for targeting studies. By using this, without adding a targeting agent a molecule can enter selectively to the tumor environment if the size of the molecule is convenient [39]. This event is called the enhanced permeation and retention (EPR) effect. Since it allows targeting by altering the size only, it is classified as passive targeting. The molecule size must be maximum 600nm for to use the EPR effect to target. Thus, it can penetrate in the tumor environment [39]. There is a large volume of published studies about using the EPR effect for cancer targeting including nanodroplets. Different polymers were used to design micelle and the maximum size for this formulations is 187.3 ± 10.8 nm at 37°C which makes the particle a good candidate for passive targeting [16].

PFC core changes the phase from liquid the gas by the presence of ultrasound pulses which can create required energy. A mechanism called acoustic droplet vaporization (ADV) leads to this phase change and does not require high pressure unlike using free gas pockets in the tissue [16], [40].

Histotripsy uses the free gasses in the body. With the high pressure, they are getting expended and eventually explode and destroy the surrounding cells. In the NDs case, gas bubbles with a larger initial volume are used instead of pre-existence gas pockets in the tissue. So, they are not only can evaporate with lower pressure but also closer to exposure due to their initial size.

Thus far, NDs are a good candidate to solve high pressure threshold problem theoretically. When the histotripsy experiments were examined, these results also corroborate the theoretical aspect as shown in **Figure 2.3**. In the figure, the pressure increased gradually between **a** to **e** and it is proven that even though at 8.2 MPa cavitation is possible by using NDs. While increasing the pressure, the cavitation area also increases as expected. The control image shows an application of the highest pressure value for the highest cavitation area to the tissue phantom without NDs. Nonetheless, there is no cavitation observed. These data prove that NDs can be used for targeted histotripsy with significantly lower pressure values.

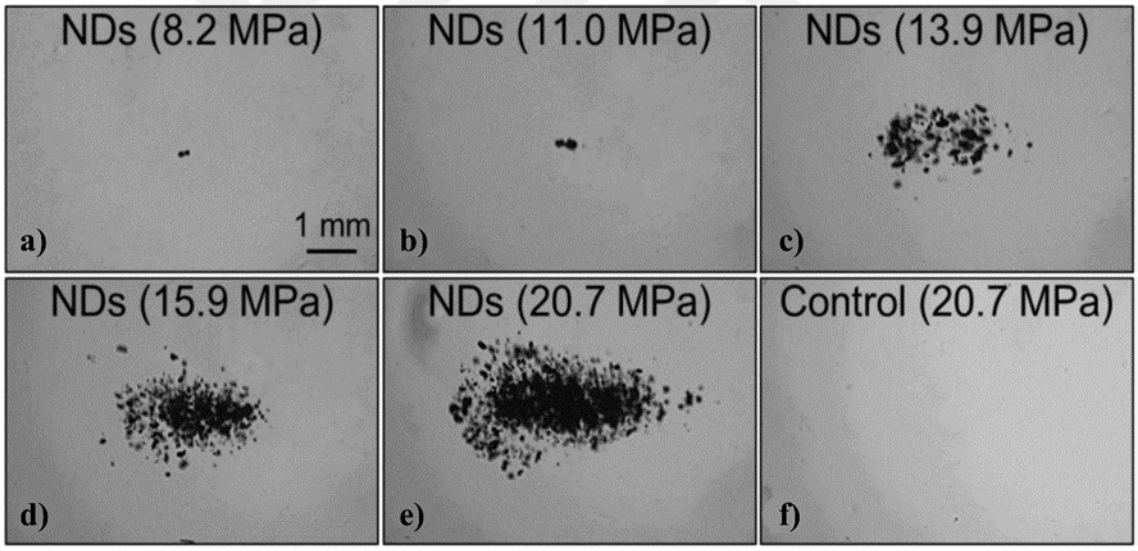


Figure 2.3: Response of the tissue phantoms at different acoustic pressures by using nanodroplet-mediated histotripsy [15].

The possibility of tissue ablation at low pressure values is proven by this far. As a further study, this method requires improvement. Despite of all advantages NDs also have limitations. The first problem is the complex preparation way of NDs which requires multiple steps of polymerization and the chemical reaction that also makes it unapproachable on big scales. NDs require cold storage conditions due to their low stability. Another problem is the amount of PFC inside the core cannot determine exactly however it is very important to arrange the dosage between different treatments. In

addition to these, even though the size of a ND is applicable for the EPR effect, to penetrate deep tumor tissue smaller size is required [12]. At this point, to overcome NDs' limitations another nanoparticle called nanocones (NCs) was created for histotripsy applications and successfully used as an NMH agent. They consist of cyclodextrins (CDs) which are cyclic sugar molecules and PFCs. Nanocones and cyclodextrins will be explained in detail in future sections.

2.2. Cyclodextrins

Cyclodextrins (CDs) cyclic molecules which consist of glucopyranose units. They are found in nature and come into existence by the enzymatic degradation of starch. Diversification is possible by functionalizing the natural cyclodextrins but mainly there three types of CDs and they are called alpha (α), beta (β), and gamma (γ) CDs. The only difference between αCD, βCD, and γCD is the number of glucopyranose units, they have 6, 7, and 8 units respectively [11]. Since the only difference the number of glucopyranose units they differ from each other only by means of size and solubility. These cyclic molecules have a hydrophobic cavity and hydrophilic external faces due to hydroxyl groups' configuration. The inner cavity diameter of αCD is 0.53 nm and since it has the smallest cavity. βCD and γCD have 0.65 and 0.83 nm inner cavity diameters respectively [5]. Hydrophilic cavity gives an opportunity for inclusion complexation. There is a large number of published studies that show CD usage to encapsulate hydrophilic molecules. There is no covalent bonding for this complex, due to hydrophobicity rapid equilibrium between CD and hydrophobic molecule occurs. The potential of forming stable inclusion complexes gives an opportunity to use CDs in pharmaceutical applications [41].

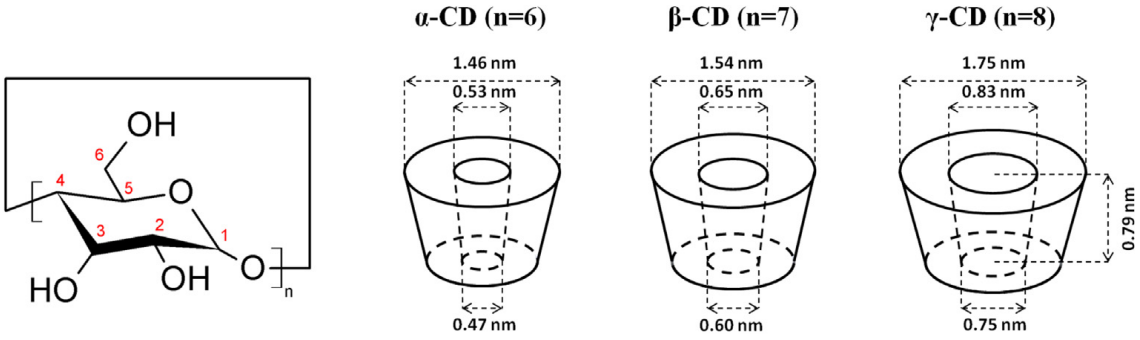


Figure 2.4: Schematic of a glucopyranose unit (left) and natural cyclodextrins with their size properties [5].

The solubility of CDs differs from each other and βCD has the poorest solubility which is 18.5 mg/mL [41]. This poor solubility is a problem for the complexation efficiency but on the other hand, provides more stable complexes than highly soluble CDs. Despite the higher stability, solubility is an important factor for a complex, especially for drug delivery purposes. For these reasons, functional group additions can be done to increase solubility. **Table 2.1** compares some CD derivatives with natural CDs. Data from this table shows adding functional groups can increase the solubility successfully and may cause a size difference for the inner cavity. CDs are completely safe and FDA (Food and Drug Administration (FDA) approved molecules. Since they originate from starch, in the body, they show starch-like behaviors [11].

Table 2.1: Cyclodextrin Derivatives with Their Basic Properties [5], [41]–[43].

Cyclodextrin	Abbreviation	# of glucopyranose units	Solubility in water at RT (mg/ml)	Inner Cavity Diameter (nm)	# of H Donors-Acceptors
Alpha Cyclodextrin	**αCD**	6	130	0.53	18-30
Beta Cyclodextrin	**βCD**	7	18.5	0.65	21-35
Low Degree Methylated Beta Cyclodextrin	**LMβCD**	7	-	-	-
High Degree Methylated Beta Cyclodextrin	**HMβCD**	7	> 600	-	9-35
Hydroxypropyl Beta Cyclodextrin	**HPβCD**	7	> 600	0.6	25-39
Gamma Cyclodextrin	**γCD**	8	249	0.83	24-40
High Degree Methylated Gamma Cyclodextrin	**HMγCD**	8	230	-	-
Hydroxypropyl Gamma Cyclodextrin	**HPγCD**	8	>500	0.8	24-45

2.2.1. Cyclodextrin aggregations

Besides the inclusion complex formation ability CDs also form self-assembled aggregates. At first, the thought was the reason of the aggregation must be an active drug in the system. It was found out later, without any other molecule CDs can built aggregates by intermolecular hydrogen bonds [44], [45]. Due to hydrophilic and hydrophobic moieties in a system which contains CDs and hydrophobic molecules, there are other interactions except inclusion complexation. Hydrophobic molecules and CDs interact within themselves. CDs form aggregates on their own even though there is no other molecule type in an aqueous environment and the existing literature on CDs is extensive and focuses particularly on self-assembled CD aggregates. It is proved that the tendency for building an aggregate of CD inclusion complexes is higher than the only CD involved aqueous solutions and also it is proportional to the concentration [5], [46]. The natural formation of the aggregation is an advantage for the production but this aggregate formation directly affects solubility and their complexation behavior. For some cases, to increase or enhance the solubility and stability adding functional groups or soluble polymers may be required [5], [10].

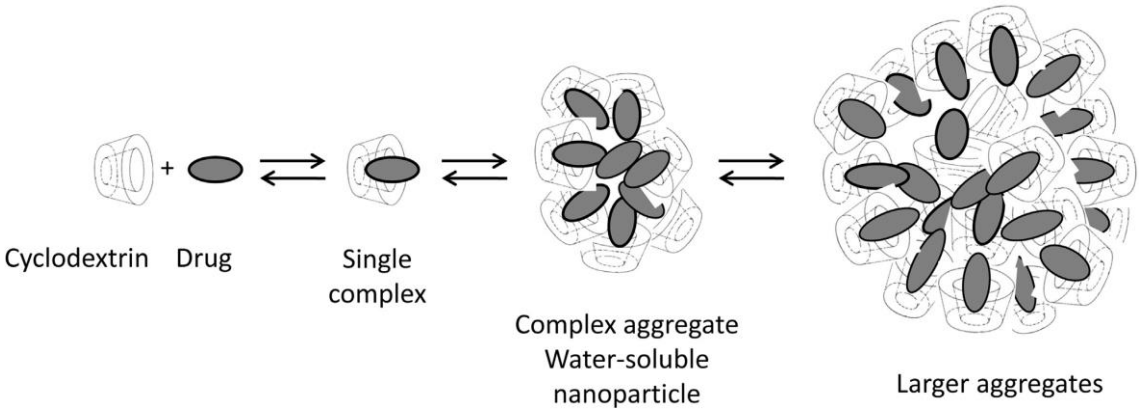


Figure 2. 5: Schematic of CD inclusion complex and its aggregation [5].

Investigations of pharmaceutical applications give promising results. To overcome solubility, toxicity, and stability problems for a drug, a delivery system can be used. Nanoparticles, micelles, dendrimers, hydrogels, and liposomes several well-liked examples for this purpose [47], [48]. CD aggregates thanks to their non-toxic and soluble features, have examples for different applications such as pharmaceuticals by inducing with an IgG [49] or a drug [50], or as an ultrasound contrast agent by inducing with perfluorocarbons [51] and encapsulate fish oil [52].

2.2.2. Computational investigation of cyclodextrins and their aggregates

Numerous studies have attempted to explain CD aggregations and their behaviors. Since the functionalities or other free molecules in the environment can affect the aggregation behavior, a completely different aggregate may be achieved. In the literature, it is possible to find indications about CD solutions behave differently from the theoretical expectation. Miyajima et al. found that the activity coefficient of some CD solutions decreases when the concentration increases [5], [53], [54]. Theoretically, the activity coefficient is directly proportional to the concentration. This contradiction was explained with the unknown mechanism of CD aggregations.

At the molecular level, the best way to see what exactly happens between CDs and other molecules molecular dynamics simulations may be a good choice for the investigation. Before examination, the aggregation behavior of CDs with a drug, investigation of 1:1 interaction between them is a rational step for a start. Thus, the aggregation effect to the complexation behavior can be enlightened. Fermeglia et al. studied the complexation tendency of βCD with several anticancer drugs. They did energy calculations of the complexes and found overlapping results with previously published experimental studies [55]. Mahalapbutr et al. did a similar study, the aim was to decrease the solubility of a cancer drug by forming CD inclusion complexes. Several βCD derivatives were tested separately and they found all derivatives form inclusion complexes with the drug and these results were combined and supported with experimental data [56]. A considerable amount of literature has been published similar to these examples. Computational examinations of CDs overlap with experimental results and in addition to that allows energy calculations to determine stability which is not possible to do experimentally. In the case of aggregation studies through molecular dynamics simulations, in addition to energy calculations, visualizing and analyzing the aggregation behavior is a big advantage since there is no other way to enlighten. Huang et al. made an investigation for aggregation mechanism of glipizide and four different CD derivatives and they showed that βCD and γCD had the strongest tendency for aggregation with the drug as seen in **Figure 2.6** [57].

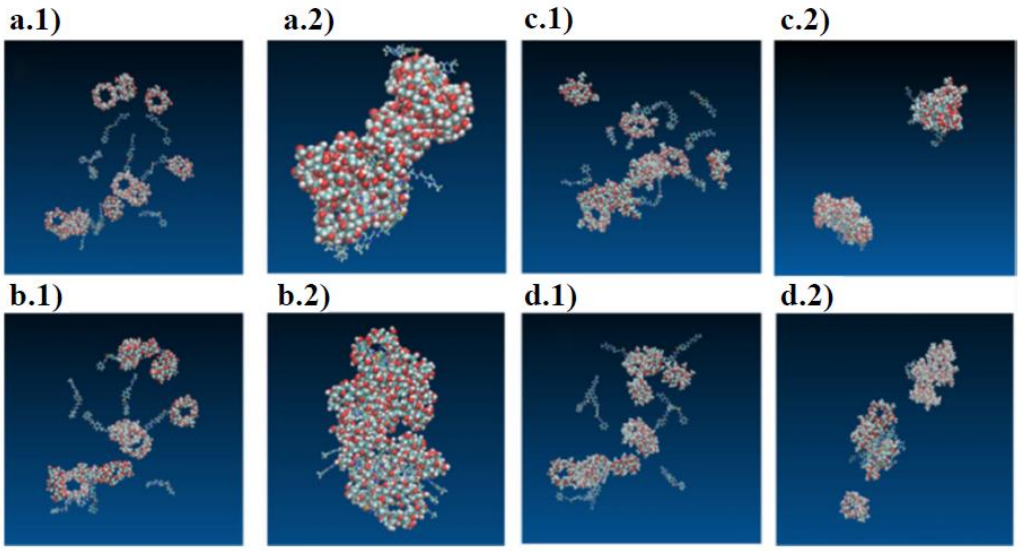


Figure 2.6: Initial **1** and final **2** structures of **a)** βCD, **b)** γCD, **c)** HPβCD, and **d)** HMβCD [57].

Zhu et al. performed molecular dynamics simulations to test βCD aggregates behavior in bulk water, bulk oil, and oil-water interface. In addition to this aim, they also investigated the aggregation effect to a toxic molecule called anthracene. As a result, they showed that 20% of the anthracene formed inclusion complexes and 10% was adsorbed on the hydrophilic outer surface of βCDs. Thus, CDs can be used for anthracene extraction [58].

2.3. Perfluorocarbons

Perfluorocarbons (PFCs) are chemically inert and stable molecules which have several pharmaceutical applications. They consist of carbon and fluorine atoms and different types of PFCs are available due to the number of atoms, in **Figure 2.7** there are some examples with their boiling points (BP).

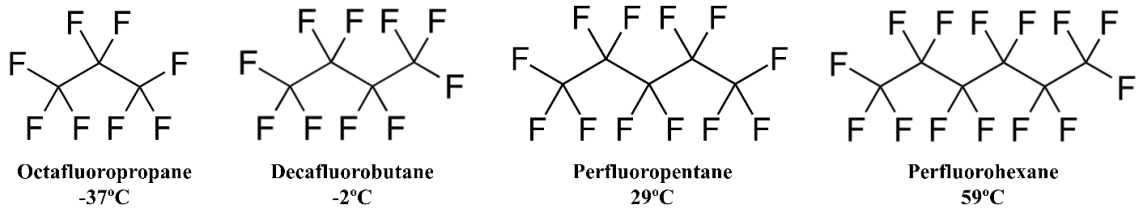


Figure 2.7: Chemical presentations of different PFCs.

They are synthetic molecules, substitution of hydrogen atoms by fluorine atoms provides extremely high stability since the C-F bond is extremely strong. Due to their inert ability, the body eliminates PFCs with expiration easily. They can be used as oxygen carriers and

bind 40-50 times more oxygen than hemoglobin [59], [60]. Also, PFCs are medically approved contrast agents for diagnostic ultrasound imaging [61], [62]. Due to their low boiling and melting points, PFCs can vaporize by a sufficient ultrasound pressure easily. This feature makes them acoustically active molecules and makes possible to diagnose and apply treatment for some cases [62].

2.4. Nanocones

The recently developed CD-PFC inclusion complexes are called nanocones (NC) have shown a promising potential on the histotripsy treatment [12], [13]. It is shown that PFCs can be used as ultrasound agents and can reduce the cavitation pressure threshold. In **Figure 2.8** there is a clear trend of threshold pressure decreasing with the presence of NCs and the concentration raise of NCs.

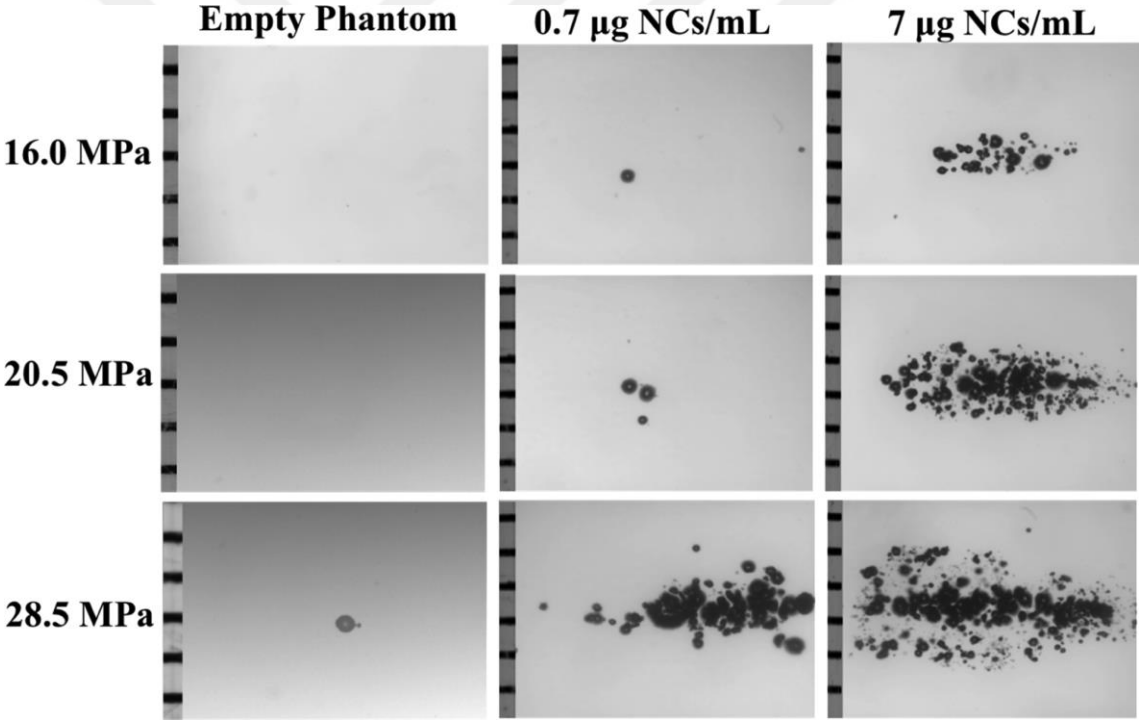


Figure 2.8: Nanocones mediated histotripsy application [12].

For this purpose, PFH and PFP were chosen since they showed the best result. NCs overcome the limitations of NDs and become the best candidate for histotripsy. The production process is quite simple and the PFC amount can calculate by using gas chromatography. Size of NCs also smaller than NDs which enhances tumor accumulation. Rehman et al. sized NCs as 37.75 ± 7 nm. Cell toxicity and hemolysis studies were also done and as expected NCs do not show hemolytic activities or cause

significant cell deaths [12]. LMβCD was used due to its high solubility for a very early example of NCs. As it is mentioned before βCDs are not only CDs, there are αCD and γCD as natural CD derivatives and their functional derivatives including methyl and hydroxypropyl group. At this point, we ended up with an extensive CD library to scan. Since NCs are the most promising NMH agent, a detailed study to elaborate the information about NCs is crucial at this point.

CHAPTER 3

3. EXPERIMENTAL PART

3.1. Materials

β-Cyclodextrin (βCD, Sigma-Aldrich, 97%), methyl-β-cyclodextrin (HMβCD, Sigma-Aldrich), α-cyclodextrin (αCD, Sigma-Aldrich, 98%), (2-hydroxypropyl)-β-cyclodextrin (HPβCD, Sigma-Aldrich), (2-hydroxypropyl)- γ-cyclodextrin (HPγCD, Sigma-Aldrich), γ-cyclodextrin (γCD, TCI Chemicals, 99%), tetradecafluorohexane (PFH, Sigma-Aldrich, 99%), perfluoro-n-pentane (PFP, Strem Chemical, 98%), agarose powder (Agarose Type VII, Sigma-Aldrich), saline solution (0.9% sodium chloride, Hospira), trifluoroacetic acid (TFA, AppliChem, 90%), N-N dimethylformamide (DMF, Sigma-Aldrich, 40%) were used as received.

3.2. Characterization

F atom content in the NCC was examined by using elemental mapping using energy-dispersive X-ray spectroscopy (EDX), Gemini 500, Zeiss. Gas chromatography (GC) method by Rehman et al. with some modifications was used to determine PFC amount in the complex solution [12]. Trifluoroacetic acid (TFA) was used instead of DMF to enhance the solubility of NCC. A PFC calibration curve was created by injecting 0.5-10 μL PFC in 400μL standard solution of TFA and anisole as internal standard. Injection of the samples was done while the temperature of the column was raised gradually from 40 °C to 290°C and the sample volume was 1 μL. For each sample, the process was repeated three times. The size of the clusters was investigated through dynamic light scattering (DLS) method by using Zetasizer Ultra, Malvern Panalytical. 1 to 1 and 1 to 5 (CD:PFH) molar ratios were used to seek cluster behavior of the complexes and the effect of the CD:PFC ratio to the cluster formation. The initial concentration of 16.6 mg/mL dissolved CD solutions were prepared likewise the nanocone preparation by adding the same mol

PFH with CD or 5 times more. Before and after overnight stirring measurements were accomplished at 90° by diluting the solutions to 1 mg/mL. As control, measurements were also done for overnight stirred CD solutions without PFH adding to see the difference of presence of PFH. The entire process was done at room temperature and triplicate measurements were done for each sample.

3.3. Preparation of Nanocone Cluster (NCC)

All cyclodextrin derivatives were commercially available except LMβCD which was synthesized as described before [12], [63], [64]. 100 mg/mL CD derivatives (in the case of βCD 100mg/6mL) were completely dissolved in double-distilled water by stirring at room temperature and PFC derivatives were added into these solutions with the ratio of 1:5 or 1:1 (CD:PFC). The stirring continued overnight, and the vial was rested in the fridge at 4°C for an hour. The solution was centrifuged at 5000 rpm for 10 minutes and precipitates were collected and dried under vacuum.

3.4. Recovery of NCC from Solution

To find a way to remove the water from NCC solution that does not precipitate, different practices used in the laboratory were applied and their effect on safety of PFC in the NCC was investigated. βCD (create a nearly opaque solution with PFH) and αCD (create a nearly clear solution with PFH) were chosen to test common practices in the laboratory such as centrifuge, rotary evaporation, low pressure vacuum evaporation, lyophilization, acetone precipitation, and air drying. Selected NCC (βCD and αCD) was prepared based on described NCC preparation method and the solution was divided into six different portions before water removal. Water in the system was removed using one of listed methods and the fluorine percentage of dried NCC powder was analyzed.

3.5. Molecular Dynamics Simulations

Native CD molecules, αCD, βCD, and γCD were obtained from RCSB Protein Data Bank (PDB) [65]–[68]. Their PDB IDs are 1CXF, 3CGT, and 1D3C respectively. PFP and PFH were obtained from PubChem and CIDs are 12675 and 9639. Low, high degree methylated, and hydroxypropylated versions of all of βCD and γCD (γCD only have high degree methylated version) were created using Biovia Discovery Studio [69]. In

particular, for hydroxypropylation of βCD 6 of H atoms, which are bound to OH group of 6' C atoms were replaced with C_3OH_7 whereas 5 of H atoms were replaced with the hydroxypropyl groups in γCD. Low degree methylation of βCD was done by adding methyl groups and 3 random H atoms, which are bound to OH group of 6' and 2' C atoms, respectively. For high degree methylation, all H atoms which bound OH of 6' and 2' C atoms, were replaced with methyl groups for both βCD and γCD. In total, 8 cyclodextrins were obtained to be tested. The resultant molecules were randomly placed in the simulation box by using VMD [70], three different box contents were tested. There were 1:1 interaction boxes which include only 1 CD with 1 PFC and for the clustering study, there were two different stoichiometric ratios. In the first one, CD and PFC were used as 1:1 ratio while in the second one the ratio was 1:5. Accordingly, simulation boxes were consisted of 20 CDs and 20PFCs or 20 CDs and 100 PFC. The simulation boxes were prepared by Charmm-GUI [71]. For 1:1 interaction study boxes edge distance is 12 Å and for clusters, 8Å. 0.15 M NaCl was placed by the Monte-Carlo method for preparation of the systems studied. As a force field, CHARMM36 was used [72] and the box was solvated by TIP3P water model [73]. The obtained boxes were simulated by GROningen MAchine for Chemical Simulations (GROMACS) package [74]. 298.15 K was used as a stable temperature through the simulation. The simulations were performed for 100ns for the systems that had 1:1 stochiometric ratio to investigate 1:1 interactions while the bigger systems were simulated for 200ns where the clustering was investigated. Each of the simulations were performed twice each of which started from a different velocity distribution. The second replicates of the simulations were performed until the same behavior was achieved as in the first ones.

The distance between center of masses of CDs and PFCs was calculated by using gmx distance command. Short-range non-bonded interactions, in particular, electrostatic and van der Waals interaction energies, which are modeled by Coulomb and Lennard-Jones potentials, respectively, were also calculated.

Electrostatic interactions were calculated by the following equation:

$$V_C(r_{ij}) = f\frac{q_i q_j}{\varepsilon_r r_{ij}} \qquad \textbf{(2.1)}$$

where r_{ij} presents $|r_i - r_j|$, the distance between particles' centers. f is a constant called the electric conversion factor which equals $1/4\pi\varepsilon_o$= 138.935458 kJ mol^{-1}nm e^{-2} and ε_r is another constant that equals 1. q presents charges of particle i (q_i) and j (q_j).

van der Waals interactions are calculated using the following equation:

$$V_{LJ}(r_{ij}) = \frac{C_{ij}^{(12)}}{r_{ij}^{12}} - \frac{C_{ij}^{(6)}}{r_{ij}^{6}} \quad \textbf{(2.2)}$$

where r_{ij} presents $|r_i - r_j|$, the distance between particles' centers. $C_{ij}^{(12)}$ and $C_{ij}^{(6)}$ are the parameters which depend on atom types and are taken from a matrix of Lennard-Jones parameters.

3.6. Histotripsy Study

The NCC formation which is chosen as the most efficient was tested to see histotripsy activity. Tissue phantoms were used which were made of agarose gel to mimic the tissue. Three different tissue phantoms were used including NCC with different CD-PFC ratios, 1:1 and 1:5 as samples, and empty phantom which is used as control. Agarose tissue phantoms were prepared by adding agarose powder into saline solutions while stirring and the temperature was held above 70ºC until it became transparent. Degassing was done to the solution under a 20.5 mmHg vacuum for 30 minutes when the temperature was above 37ºC. Testing groups were prepared by adding NCC into the agarose solutions while stirring. The concentrations of PFC amount in used NCC for 1:1 and 1:5 were arranged as same. After these steps prepared agarose mixtures were poured into rectangular polycarbonate holders and rested at 4ºC in the refrigerator. They became ready to use after they solidify. Before the experiments, agarose tissue phantoms were warmed to 37ºC for mimicking body temperature and maintained the temperature during the experiments. The pressure applied by single-cycle 500 kHz histotripsy pulses with 1Hz pulse repetition frequency at peak negative pressures of 9.96, 18.83, 18.4, 26.16, and 31.58 MPa.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Preparation of Nanocone Clusters

The first example of nanocone preparation was shown for βCD and LMβCD through a very simple method of mixing CD derivatives and PFC in water for a certain time yielded precipitate which can be easily filtrated and dried for further use [12], [13]. It was proven that this precipitate contains inclusion complex of CD and PFH using ^{19}F NMR and EDX analysis. The size of redispersed powder of nanocones was concentration dependent and it was possible to obtain as small as 38 nm. Moreover, the amount of PFH in the nanocones was able to calculate using gas chromatography. All these promising data clearly addressed the limitations of nanodroplets and it was worth of study deeper to understand the structural organization of inclusion complex of CDs and PFCs.

First, interactions between cyclodextrin derivatives and PFCs are experimentally investigated. Along with native CDs (αCD, βCD, and γCD), methylated and hydroxypropylated version of βCD and γCD were tested as host (**Figure 4.1**). It was hypothesized that αCD with the smallest cavity size either might not accommodate the PFC or might accommodate the short PFC chain. γCD with the biggest cavity size might host more than one PFC molecules which might result a product with more PFH that allows less material usage for the same treatment dosage. Since using LMβCD instead of βCD in a low volume of water resulted higher PFH encapsulation in a previously published study, it was thought that better water solubility of host molecules increases the chance of inclusion complex formation. However, it might also negatively affect the precipitation potential of the cluster. To answer all these hypotheses, selected cyclodextrin derivatives were mixed with either PFH or PFP at the same cyclodextrin concentration. After overnight stirring, all solutions of CD derivatives end up with turbidity in the presence of PFH indicating aggregation/clustering (**Figure 4.2).**

However, only turbidity of βCD-PFH, βCD-PFP, LMβCD-PFH, LMβCD-PFP, and γCD-PFH was dense enough to cause precipitate. To test the concentration effect, studies were done at the solubility limit of CD derivatives. In this case, each CD derivative was dissolved in different water volumes due to their solubility and the result were the same with an equal cyclodextrin concentration study. This data supports the hypothesis about the solubility of host molecules might affect the precipitation potential of inclusion complex aggregates. Among native CD; αCD and γCD have better solubility than βCD. While αCD did not give the precipitated, γCD resulted an unstable precipitate at room temperature that has the tendency to fall apart during centrifuge. Moreover, hydroxypropylated derivatives for βCD and γCD has the highest solubility followed with high degree methylated one which they all did not form the precipitate that can be recovered. Although this does not mean they do not form the inclusion complex or its aggregates, it means that formed structure not able to collect practically since the solvent is water and PFC is volatile. As it represented in **Figure 4.1**, βCD, LMβCD, and γCD form aggregates that can be easily filtrated from the solution which they are a potential candidate for the best NCC for desired application.

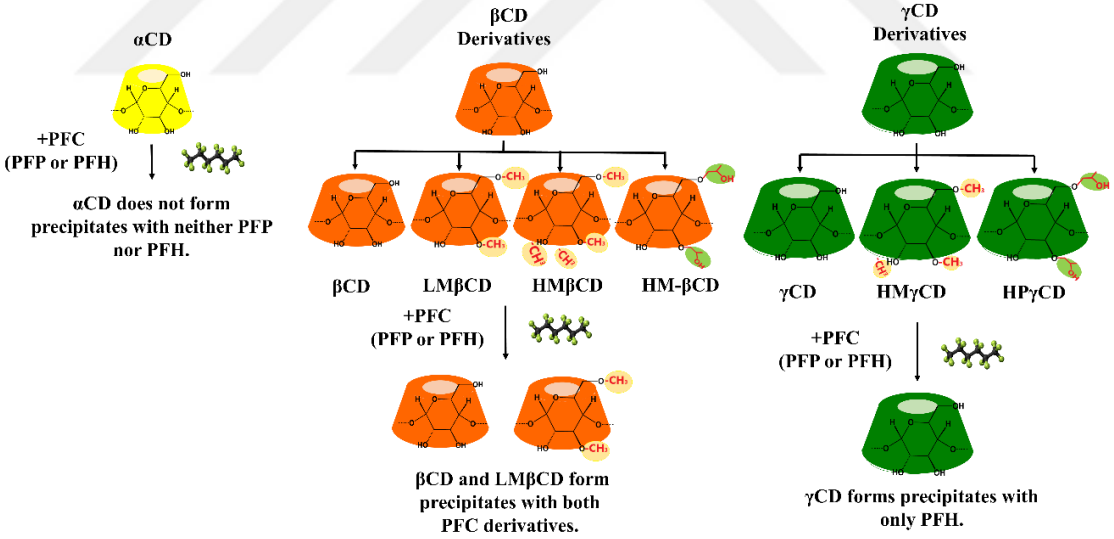


Figure 4.1: All cyclodextrin derivatives that are experimentally tested.

PFC amount in the system is important for the inclusion complex that has potential to form clusters due to the fact that PFC might act as a hydrophobic core for inclusion complex to organize around if it exists in the system more than complexed one. In order to understand the contribution of the amount of PFC, an equal amount of PFC (CD:PFC:1/1) and excess of PFC (CD:PFC:1/5) were experimentally and

computationally used for complexation. It was hypothesized that 1/1 ratio might form only inclusion complex aggregates without free PFC in the system and 1:5 ratio might form inclusion complex organization (clustering) around free PFC droplets. Furthermore, both structures might have different behavior as histotripsy agents because of the presence of free PFC in the system.

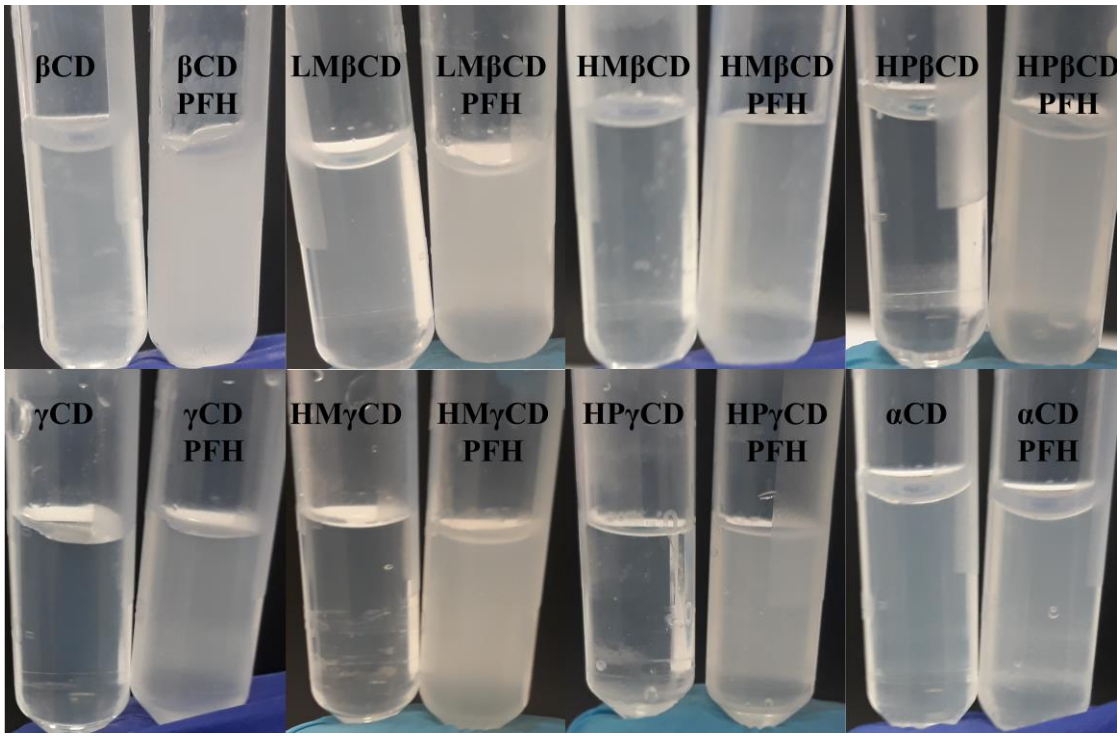


Figure 4.2: Appearance of CD derivatives with and without PFH after overnight stirring at RT.

The solution that did not precipitate, whether they form nanocone clusters (NCC) or not is a question that needs to be answered. It seems like computational data will be more valuable for this question however, a size study using DLS has been experimentally done to show the aggregation potential of different cyclodextrin derivatives in the presence of PFC. Besides the usual size measurement that uses the redispersed NC powder, the formation size of these solutions was measured right after preparation while they are still in solution. This way allows to compare behavior of all cyclodextrin derivatives in the presence of PFC. Size measurements were done before and after PFC addition. A control experiment with the same stirring time with host molecules (without PFC) was included for all derivatives to see behavior of host by itself. **Figure 4.3** and **Figure 4.4** show the size values for inclusion complex formation solution prepared using 1 to 5 and 1 to 1 CD:PFC ratios, respectively. As it can be seen in **Figure 4.3**, cyclodextrin derivative itself has the potential to form aggregates in solution with a size around 200 nm. Once PFH is

added into the solution and let it stir for NCC formation, size of the clusters increases slightly. To solely see the contribution of PFH presence in the system, a control solution without PFH was stirred for the period of NCC preparation, and its size was used for comparison. It showed that further stirring without PFH does not change the cluster size significantly.

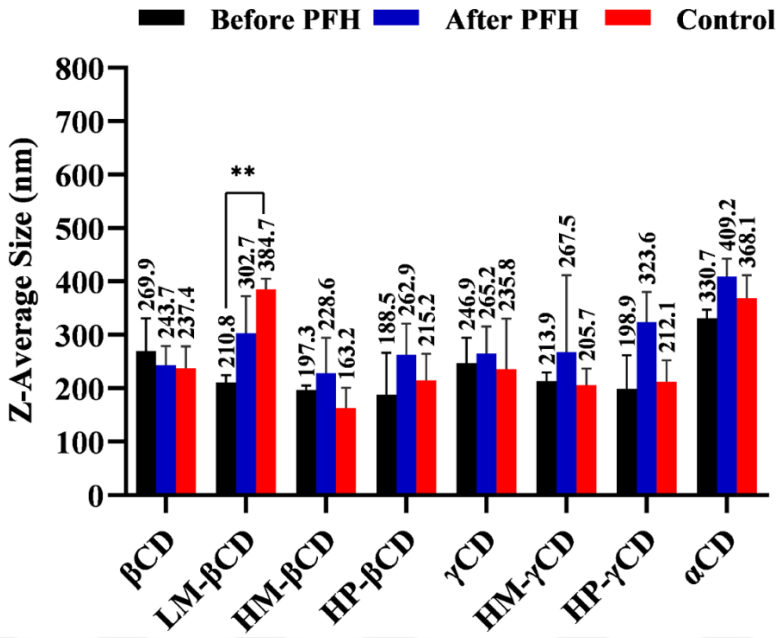


Figure 4.3: Size of different cyclodextrin solution itself and in the presence of PFH of 1/1:CD:PFH ratio. .* donates $P \leq 0.1$, ** donates $P \leq 0.01$, *** donates $P \leq 0.001$. (one-way ANOVA followed by the Tukey test.)

A similar size study was performed to examine the effect of free PFH on size by increasing CD:PFH ratio to 1/5. In this case, contribution of PFH on size was mostly significant showing a clear increase while the control solution stays almost at the same size. Interestingly, hydroxypropyl functional CD derivatives (HPβCD and HPγCD) showed a higher increase on size after PFH addition. Even though this indicates the larger cluster size, they did not precipitate out from solution. This might be because of increasing hydrophobic nature of other surfaces due to the hydrophobic functionality of hydroxypropyl groups [9], [19], [75]. As it has been shown before without PFH, CDs can build a cluster through hydrogen bonds and hydrophobicity [44], [45]. PFH addition also adds an extra force and increases the tendency for cluster formation. Given the fact that CD derivatives have the size of 1-2 nm by themselves, the size obtained from DLS study collectively supports the cluster formations for all CD derivatives.

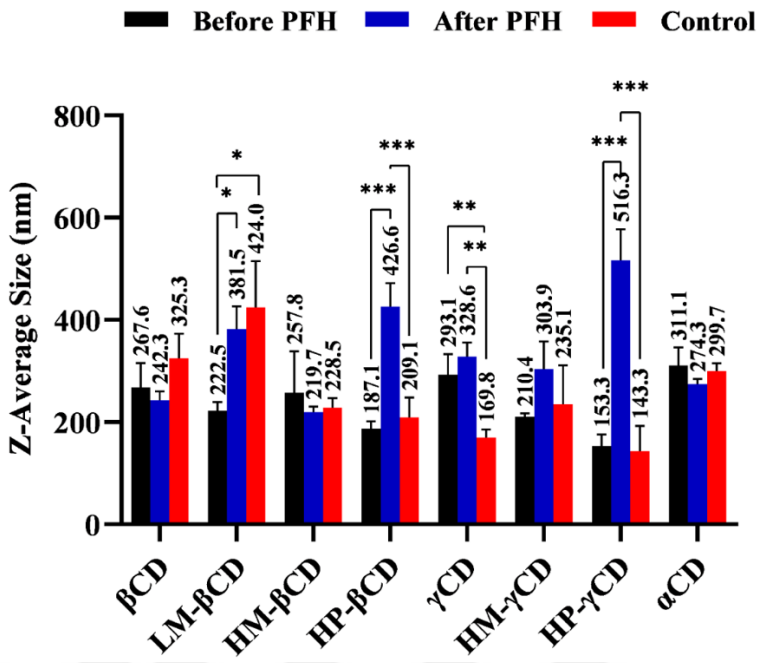


Figure 4.4: Z-Average size comparison of CD derivatives with 1 to 5 molar ratio PFH addition. .* donates P ≤ 0.1, ** donates P ≤ 0.01, *** donates P≤0.001. (one-way ANOVA followed by the Tukey test.)

As it stated before, determination of PFC involved in cluster formation is important to be able to tune the different treatment. NCC has an advantage over nanodroplets in this aspect since low molecular weight of building blocks on NCC allows GC analysis to determine the volatile PFC amount. It is crucial to have a solvent system that solubilizes both PFC and NCC for GC analysis to have reproducible data for small quantity of PFC. Trifluoroacetic acid is the best solvent that provides described conditions. Anisole was used as internal standard and its concentration was constant for all measurements for comparison. PFC amount of NCC was determined using preformed calibration curve of free PFC analysis at different concentrations in the same solvent system. The intensity of PFC peak obtained from the analysis of NCC sample was compared with the peak intensity obtained based on the calibration curve to determine the PFC amount per mg of NCC samples. The obtained GC data was correlated with feeding ratios of NCC. The PFC amount was determined as 1:1.25 and 1:6 for the feeding CD:PFP ratio of 1:1 and 1:5, respectively. This means 0.65µl and 2.62 µl of PFP for every mg of 1:1 and 1:5 CD:PFP ratio of NCC, respectively. Similarly, PFC amount of NCC obtained with 1:1 CD:PFH ratio was calculated as 1:2 and PFC amount of NCC obtained with 1:5 CD:PFH ratio was calculated as 1:5 which is consistent with PFP analogous and feeding ratio. LMβCD form

precipitates only with PFH in case of 1:5 CD:PFH feeding ratio. Experimental results showed that LMβCD and PFP interaction either does not effectively yield NCC for 1:1 ratio or it yields cluster with less PFP content as low as 1:0.3 ratio in case of 1:5 feeding ratio. However, calculated ratio as 1:0.7 and 1:4.6 % for 1:1 and 1:5 ratios, respectively showed that LMβCD has better interaction with PFH and results cluster with higher PFC content. γCD-PFH nanocones did not allow experimental calculations for 1:1 ratio due to insufficient precipitate amount just as LMβCD-PFP nanocones for 1:1 molar ratio. If the comparison was done between the one that can produce enough precipitates, βCD-PFC, and LMβCD-PFC nanocones showed the highest encapsulation rate and they are highly efficient on forming PFC containing clusters.

4.2. Molecular Dynamics Simulations

The inclusion complex of CD derivatives and their aggregates are well studied in the literature [9], [57], [58]. In most of the examples, guest molecules are drugs and have clear optical absorbance to follow up complexation or they allow structural characterization like ^{1}H NMR analysis. PFCs are kind of guest molecules that do not have significant optical properties and they only allow ^{19}F NMR in terms of F atom`s chemical shift which requires special conditions. Even though experimental results support the presence of aggregates, it is not clear whether these aggregates form out of inclusion complexes or it is an organization around hydrophobic PFCs in water. Moreover, it is unknown what happens in the solution of CD derivatives and PFCs that they did not form the precipitate. To investigate the inclusion complex formation of all selected CD derivatives and their aggregation potential in the presence of different PFC, molecular dynamics simulations are performed.

Firstly, the interactions between CD derivatives with PFH and PFP were investigated using a single molecule per each component. **Figure 4.5** shows the simulation data, which explains the tendency of the CD for complexation with PFP and PFH. 1 CD and 1 PFC derivative were put in a water box which was a cube with an edge of 10 Å. As nature of inclusion formation, the best location for highly hydrophobic PFC molecules is a hydrophobic cavity of CD derivatives as long as there is enough space in the cavity for it. Results showed that except αCD, all CD derivatives form inclusion complexes with PFC molecules. **Figure 4.5** shows that both PFC derivatives form NC with βCD and the complexes are stable during the simulation. Similarly, LMβCD and HMβCD derivatives

have strong attraction against PFC due to the elongated hydrophobic cavity [19]. On the other hand, αCD does not form an inclusion complex with any of PFC molecules. It has been previously shown that the cavity of all CD derivatives slightly changes in water and can form a hydrogen bond with water inside the cavity by tilting their glucopyranose units [76]. Thus, the shape change of αCD, which is the smallest CD, in water can block the cavity, and required space for PFC cannot be provided. In fact, glucopyranose unit tilts are not only seen in aqueous αCD solutions, it has been shown for γCD as well. γCD and HPγCD form stable inclusion complexes in contrast to HMγCD. Aree et al. did an investigation for octakis (2,3,6-tri-O-methyl)-γ-cyclodextrin' flexibility and showed that it can tilt the glucopyranose units ≈ 180º easier than smaller CDs and their methylated derivatives. This result explained with its size, there is enough width to tilt without having steric constraints [77].

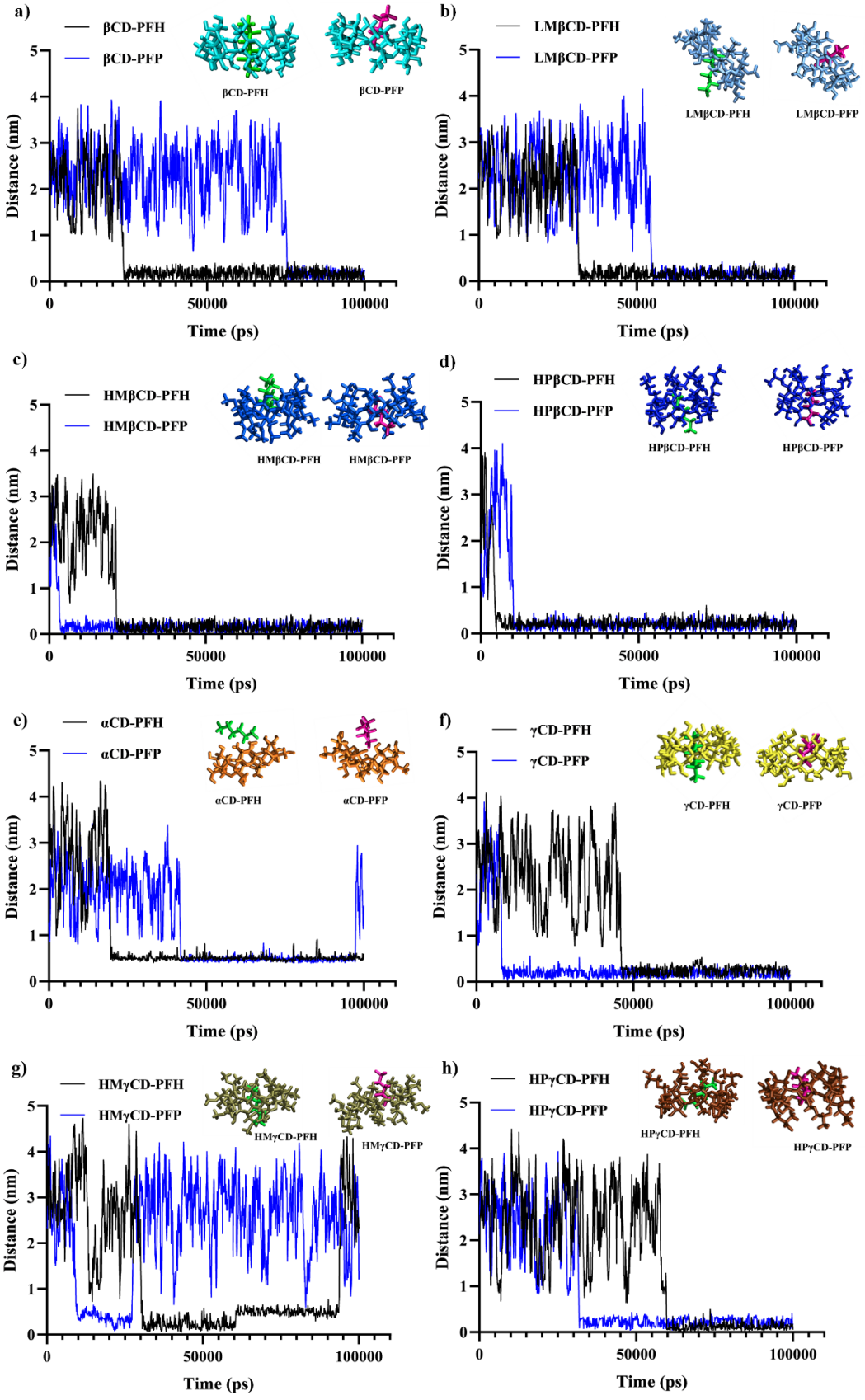


Figure 4.5: Distances between the center of mass of CDs and PFCs.

In addition to the center of mass calculations to determine distance between CDs and PFCs, electrostatic and van der Waals interaction energies of individual inclusion complexes were calculated by using Lennard-Jones and Coulomb potentials. **Figure 4.6** shows the energy comparison of inclusion complexes between CD and PFC derivatives which is consistent with the data given in **Figure 4.** The CD and PFC pair that cannot form an inclusion complex have small energies while favorable one like LMβCD and HMβCD results in higher energies. HPβCD gives the highest energy inside of the test groups as expected since it has been already proved that the addition of hydroxypropyl group increases the inclusion complex capacity of βCD by expending cavity depth [19], [78]. In previous studies, it is also possible to find solubility and stability effect of methyl group addition for inclusion complexes which drastically increases both solubility and stability of inclusion complexes [79].

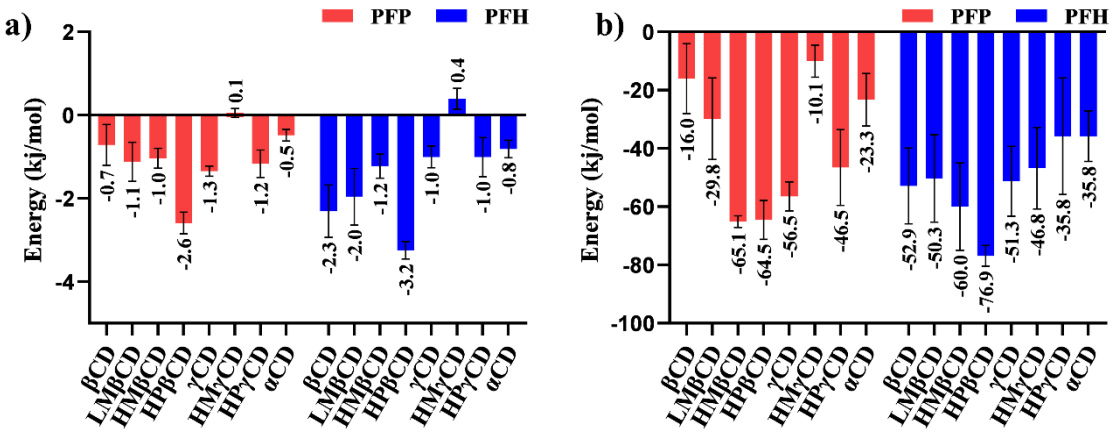


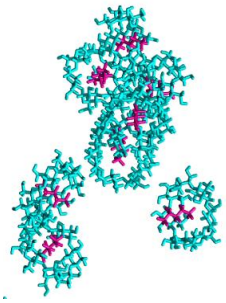
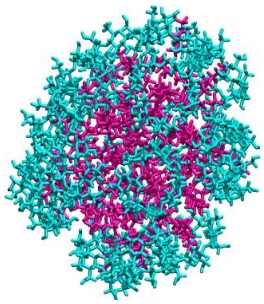
Figure 4.6: Energy values for 1:1 inclusion complexation investigation by **a)** Electrostatic and **b)** van der Waals interactions.

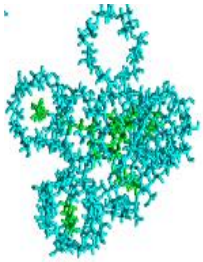
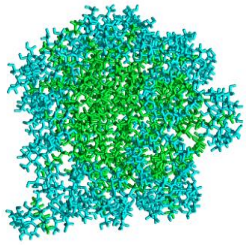
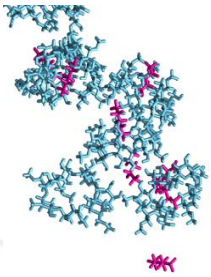
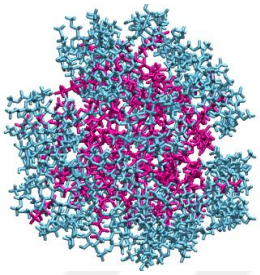
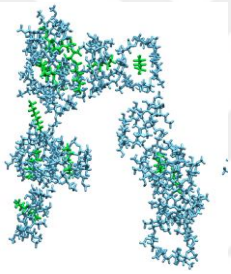
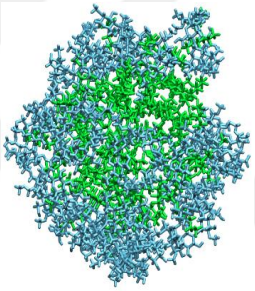
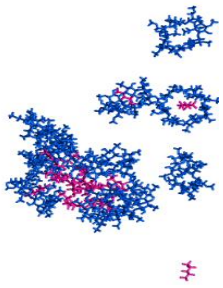
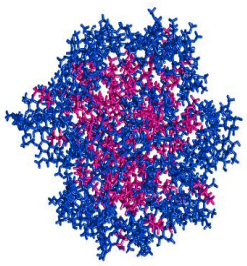
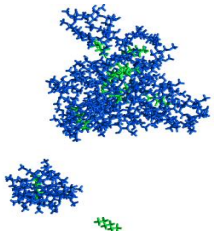
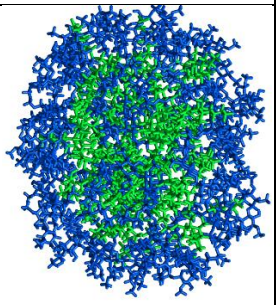
After individual interaction between CD and PFC derivatives investigated using single molecules, the next concern was what would happen if more than one inclusion complex presents in the system. Moreover what would have been happening if excess of PFC presents in the system along with inclusion complexes. To address these questions 20 CDs molecules were placed in the box along with the same amount of PFC for 1:1 ratio and 100 PFC molecules for 1:5 ratio. It is expected that excess of PFC might act like hydrophobic core that CD molecules or already formed inclusion complexes get organized around it to form PFC cored NCC. In the case of equal mol of CD and PFC molecules, inclusion complex of CD and PFC would be the main building block of a

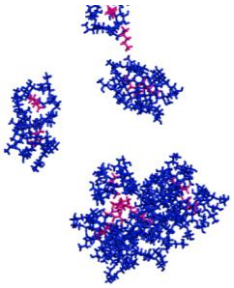
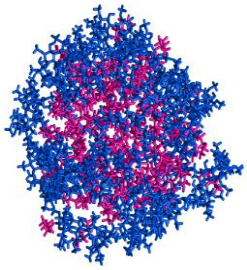
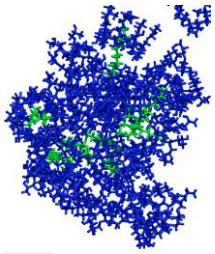
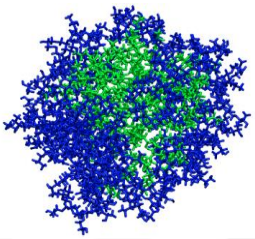
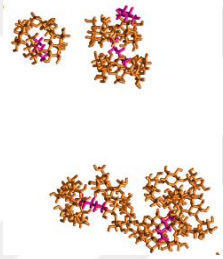
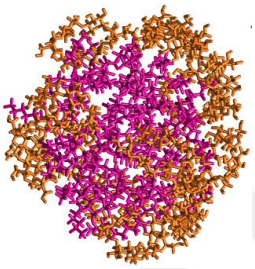
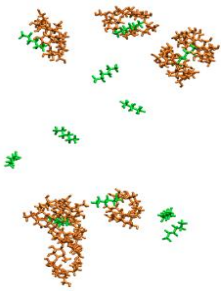
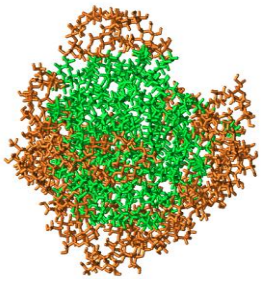
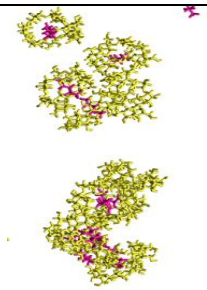
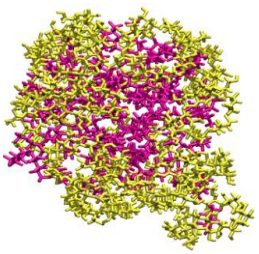
cluster. The organization way might not only effect the stability of NCC, but also it might have a contribution to dose arrangement and efficiency of histotripsy.

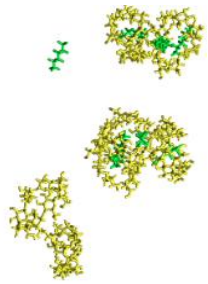
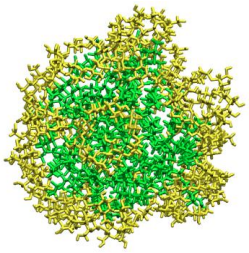
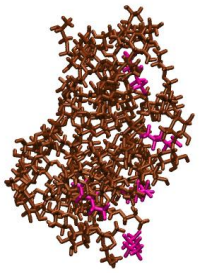
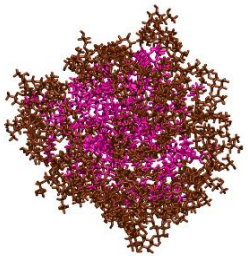
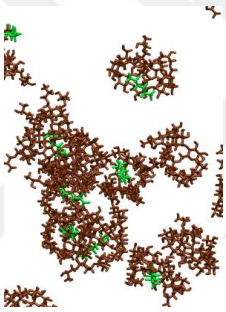
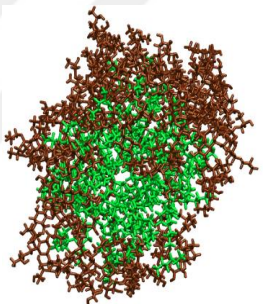
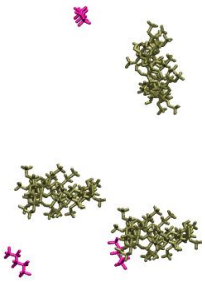
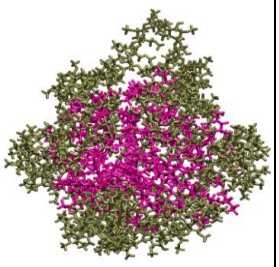
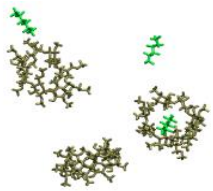
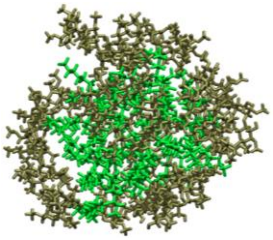
The visual results of all the simulations have been done for CD and PFC derivatives using both 1:1 (20 CDs and 20 PFCs) and 1:5 (20 CDs and 100 PFCs) ratios listed in **Table 4.1**. The data showed that organization of inclusion complexes is different based on presence of excess PFC or not. As it was hypothesized, free PFC serves as a hydrophobic core for surrounded inclusion complexes which might have a small hydrophobic tails because of guest molecules that enhance the interaction and organization around the core. In the case of 1:1 CD-PFC ratio; all cyclodextrins showed the same inclusion complex behavior except for HMγCD and αCD, which cannot build stable inclusion complex (NCs) in 1:1 interaction. αCDs and PFCs stay very close and it is possible to see 2 αCDs share 1 PFC. These dimer self-assembled structures also have been shown in previous studies [53], [80], [81]. Simulated visual data was supported by experimentally showing the ratio of CD:PFC supports the presence of free PFC in the NCC. GC analysis showed that obtained PFC amount for each cluster is consistent with the feeding ratio in simulation.

Table 4.1: Matching of computational and experimental results for 1:1 and 1:5 ratio clusters.

CD	**PFC**	**CD: PFC- 1 to 1 (cluster)**		**CD: PFC-1 to 5 (cluster)**	
		Computational Analysis*	**Experimental Analysis** (CD:PFC Ratio)**	**Computational Analysis***	**Experimental Analysis** (CD:PFC Ratio)**
βCD	**PFP**		**1:1.25**		**1:6**

	PFH		**1:2**		**1:5**
LMβCD	**PFP**		**No precipitates**		**1:0.33**
	PFH		**1:0.67**		**~1:5**
HMβCD	**PFP**		**No precipitates**		**No precipitates**
	PFH		**No precipitates**		**No precipitates**

HPβCD	**P F P**		**No precipitates**		**No precipitates**
	P F H		**No precipitates**		**No precipitates**
αCD	**P F P**		**No precipitates**		**No precipitates**
	P F H		**No precipitates**		**No precipitates**
γCD	**P F P**		**No precipitates**		**No precipitates**

	PFH		**No precipitates**		**1:4**
HPγCD	**PFP**		**No precipitates**		**No precipitates**
	PFH		**No precipitates**		**No precipitates**
HMγCD	**PFP**		**No precipitates**		**No precipitates**
	PFH		**No precipitates**		**No precipitates**

*Obtained from molecular dynamics simulations.
**Obtained from GC.

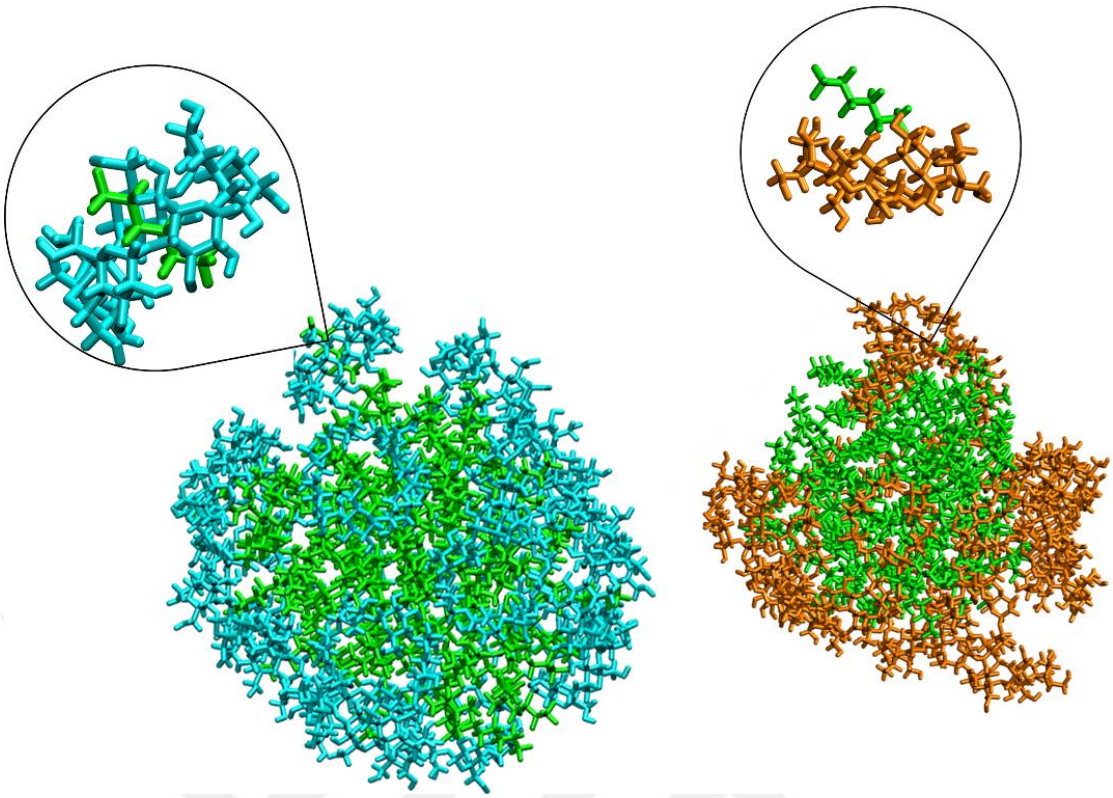


Figure 4.7: βCD (cyan)-PFH (green) and αCD (orange)-PFH (green) complexes comparison. There is 1 to 5 molar ratio between CD and PFH.

The results also showed that inclusion complex formation does not affect the clustering for the sets that have a higher amount of PFC than CD, without any complexation they can stay together, however, it affects the structure of clusters. Molecular dynamics simulation study indicated two types of clustering around free PFC (**Figure 4.7**). CD-PFC pairs which did not form the inclusion complex in 1:1 interaction showed different organization such as αCD. **Figure 4.7** shows the clustering of βCD and αCD in case of 1 to 5 molar ratio between CD and PFH. Inclusion complexes of βCD and PFH get organized around PFH molecules while αCD by itself covers the free PFH. Both the clusters have free PFH in the core however only the one that has inclusion complexes provides molecular protection for PFH which might have effect on histotripsy efficiency. Moreover, rarely these NCs are independent of the big cluster but mostly they are covering the PFCs.

They can stay stable during the simulations but in the wet lab experiments, while trying to get rid of the water, purification of clusters without NC and clusters with NC but contain highly soluble CDs was not possible. This situation was explored by using EDX as a subsequent examination. Likewise, in the simulations, while producing NCC, it was feasible to prove there are clusters for each CD due to the visual difference between

overnight stirred CD-PFC and CD solutions (**Figure 4.2)**. At the end, PFC involved solutions were whitish/blurred meanwhile the control solutions were clear. The turbidity of solutions depends on CDs' solubility and NC formation capability. **Table 4.1** shows there are structural differences between precipitated and non-precipitated NCCs. If βCD-PFC complexes are analyzed in comparison to HPβCD and HPγCD, it is possible to say PFC can stay closer and form a core itself in both derivatives however short hydrophobic tail of PFC located in the cavity of βCD contributes organization around this core while HPβCD and HPγCD just have interaction without contribution of PFC chain in their cavity. Since hydroxypropyl groups make the cavity deeper the interactions between PFCs in the cavity and free core decreases due to better isolation. In case of βCD NCC, PFC in the cavity still can interact with free PFC core. βCD and LMβCD NCCs show almost the same behavior, but LMβCD NCCs have slightly loose PFC interaction since methylation also makes the cavity deeper. When NCC have weak interaction between guest and free PFC, it forms NCC with loose PFC core such as NCC of HPβCD, HMβCD, HPγCD, HMγCD, γCD, and αCD. Increasing cavity size accommodates the PFC in a way that decreases the interaction between guest and free PFC and causes γCD NCCs to have a looser PFC core than LMBCD and as a result, the amount of precipitation was very small compare to βCD and LMβCD. For methylated and hydroxypropylated derivatives of γCD, PFC core gets even looser and as a result, none of them constitute precipitation in the laboratory even though they form NCCs. On the other hand, simulation data showed that αCD-PFH NCC has firm PFC comparing to HPβCD, but it did not precipitate indicating that for precipitation firm PFC core is not enough alone, also inclusion complex (NC) formation is necessary. Taken together, these results provide important insight into precipitation behavior, to obtain precipitates firm PFC core and NC formation must be present at the same time in a NCC.

Furthermore, electrostatic and van der Waals interaction energies were calculated and presented in **Figure 4.9 and 4.10**. **Figure 4.9** indicates that the solutions which do not have NC, form cluster due to the interaction between CDs and PFCs mostly. As previously mentioned, αCD does not form inclusion complexes for all simulation setups and energy values also support the results with the smaller energy in αCD cluster solution calculated for αCD and PFCs. However, taking only these energy calculations for consideration to make a conclusion could be misleading. When the number of molecules is increased in the systems, naturally interaction energy of the system increases. In this case, as it can be seen in **Table 4.1** higher energy does not mean obtained cluster is

stronger and stable. For instance, high energies were calculated for hydroxypropylated derivatives, but their NCC contains inclusion complexes around loose PFC core. In another word, stronger interaction between hydroxypropylated derivatives and PFC to form inclusion complexes decreases the overall core stability. Moreover, energy values for the biggest system (1:5) were naturally calculated as higher values that might not be enough to interpret the data. **Figure 4.10** and **Table 4.1** together can give healthier results.

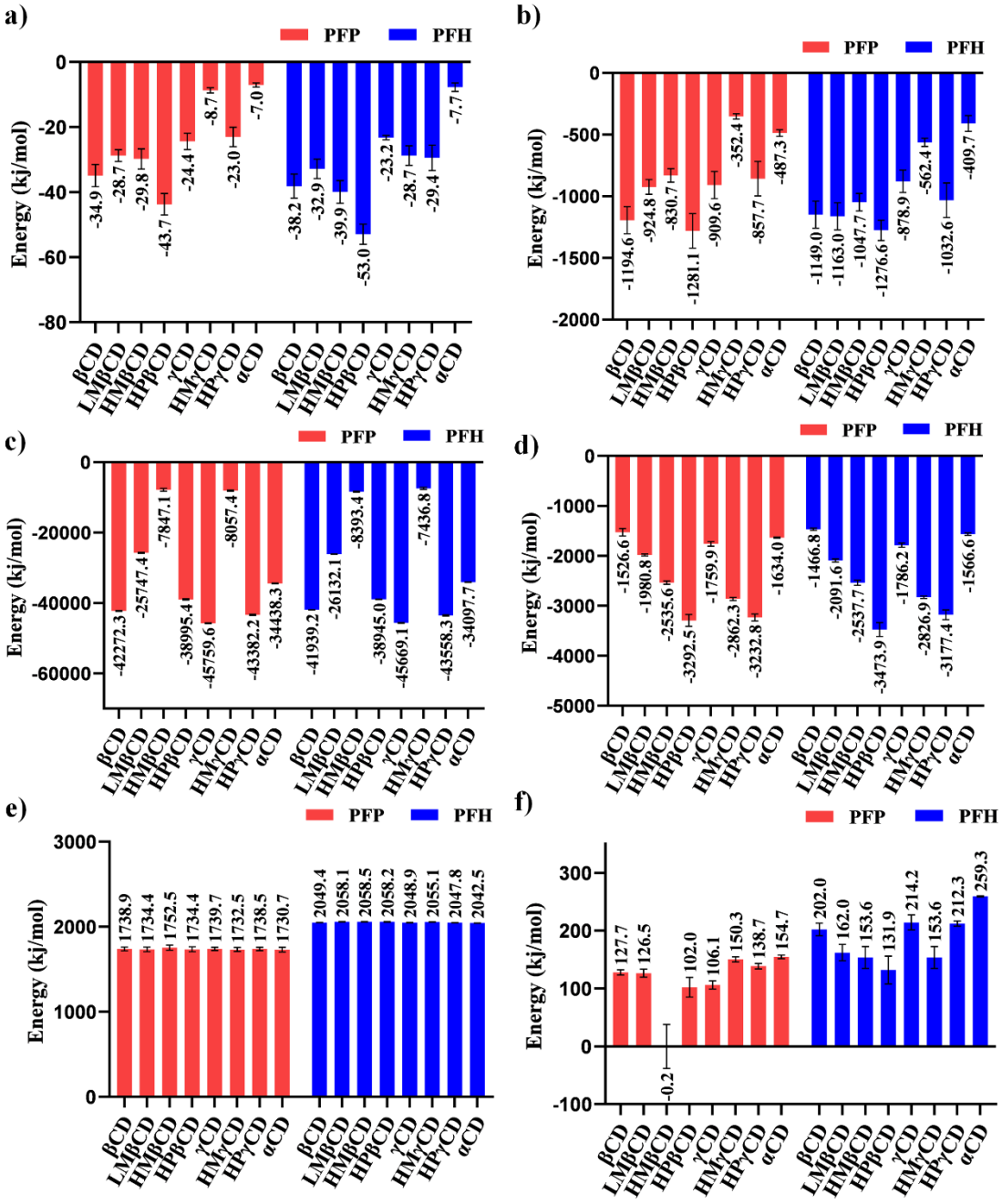


Figure 4.8: Energy values for clusters with 1:1 ratio are shown by **a, c, and e)** Electrostatic interactions and **b, d, and f)** van der Waals interactions. Energy values between CD and PFC, CDs, and PFCs with each other are presented by the first, second, and third rows respectively.

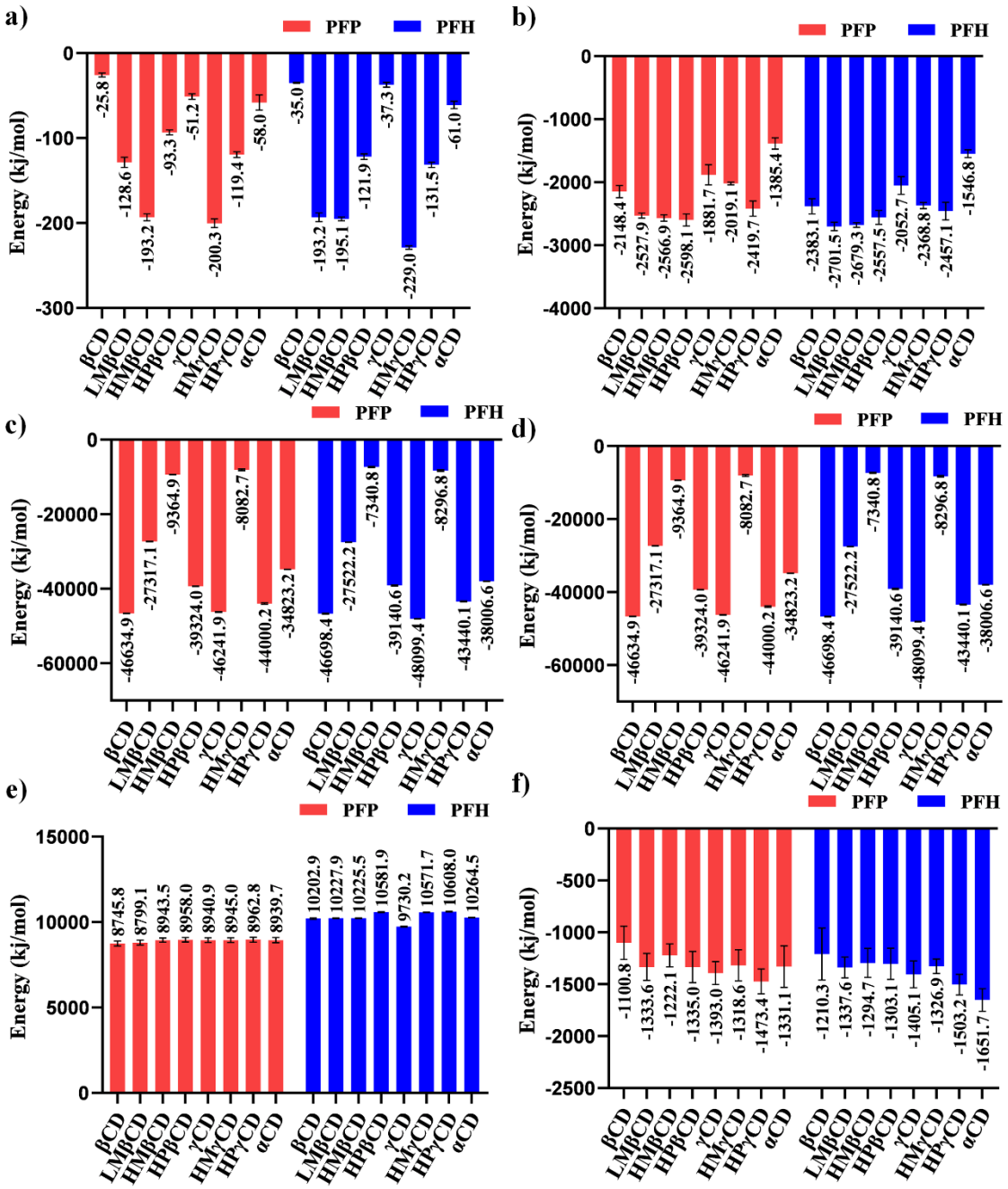


Figure 4.9: Energy values for clusters with 1:5 ratio are shown by **a, c, and e)** Electrostatic interactions and **b, d, and f)** van der Waals interactions. Energy values between CD and PFC, CDs, and PFCs with each other are presented by the first, second, and third rows respectively.

4.3. Recovery of NC Clusters from Solution

As it proved both experimentally and computationally, all derivatives form clusters in solution, however they all do not form precipitate which makes product recovery possible. Only βCD, LMβCD, and γCD were able to collect as precipitate at the end of the routine NC preparation procedure. In order to understand whether it is possible to

recover NCC as powder from solution that they do not precipitate using general laboratory practices. Cluster of βCD and αCD were selected due to their structural differences that can provide further information about stability of these clusters as well. Different drying methods to remove water were applied to NCC solutions and obtained powder was analyzed using EDX to follow up the change in F atom content of NCC. **Table 4.2** shows the obtained F atom content using different laboratory practices such as air dry, rotary evaporation at lower temperature and higher pressure, vacuum evaporation at low pressure and room temperature, lyophilization, precipitation in a solvent that is miscible with water, and collecting via centrifuge to remove water. Air dried, vacuum (600 mPa) dried at room temperature and centrifugation as relatively mild conditions for volatile PFH allow to obtain high F content for the cluster made of inclusion complexes of βCD and PFH around free PFH, but they did not work effectively for the cluster of αCD which is just a host molecules organization around free PFH. On the other hand rotary evaporation at 30 mPA and 37^{o}C resulted in loss of PFH in both cluster types. Similarly, lyophilization did not work due to the evaporation of PFH along with water [82], [83]. Precipitation of NCC in acetone did not work either since PFH prefers not to stay as core and destabilized the cluster although CD prefers to precipitate. These data supported that clusters formed with just the interaction of CD and PFH core without inclusion complex formation are not stable enough that they cannot preserve their formation during the water purification process.

As stated before, there are clusters that include inclusion complexes of CD and PFC but they do not precipitate in solution like HPβCD derivatives. The next point that needs to be clarified is the fate of these clusters during water removal process. To address this point, the most harmless methods which are air drying and low vacuum evaporation (600MPa) methods were tried for HPβCD and F atom content of obtained power were compared with βCD cluster. The results showed that even these mild conditions did not allow the preserve PFC content of these clusters and they have lost it. Collectively, these data showed that general laboratory practices to remove water do not allow to obtain nonprecipitated NCC from solution. Already precipitated form like βCD can also be obtained from air dried and low pressure vacuum evaporation which both of these techniques are not practical and slow evaporation might cause heterogeneity in the sample. Precipitation behavior of clusters mostly depends on the nature of CD, the functionality of cyclodextrin, and interaction of its inclusion complexes with PFC as

discussed before. It is supported that βCD is the best one not only readily form precipitate but also it does not require chemical modifications like LMβCD and cheap comparing to γCD.

Table 4.2: Atomic Content Percentage of βCD-PFH, αCD-PFH, and HPβCD according to EDX.

Methods	Fluorine content of βCD		Fluorine content of αCD		Fluorine content of HPβCD	
	Atomic %	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**	**Weight %**
Air Dried	4.95	6.66	0.94	1.27	0.71	0.99
Rotary Evaporation 30mPA at 37ºC	0.36	0.50	0.32	0.44	-	-
600mPa at RT	7.38	9.86	0.31	0.42	0.80	1.10
Lyophilization	0.60	0.82	0.26	0.38	-	-
Acetone Precipitation	0.43	0.58	0.26	0.50	-	-
Centrifuge	6.55	8.71	0.47	0.64	-	-

These results seem to be consistent with other research which found βCD has a stable cluster formation, even though filtering (0.1μm or 0.02μm) it is possible to get clusters and the same study showed that αCD and γCD lost cluster formation permanently with filtering [84]. Given the fact that only hydrogen bonds and hydrophobic interactions take place during cluster formation, most of them are sensitive to external interferences and not stable enough to survive through water purification [9]. The data of both experiments and simulations showed that except αCD all CD derivatives built an inclusion complex with different stabilities. LMβCD and βCD formed stable clusters with both PFP and PFH which can recover from water for 1:1 and 1:5 ratios and γCD-PFH NCC were recovered with a low yield for just 1:5 ratio. LMβCD and γCD can be eliminated because of the need for methylation as an extra step and low yield, respectively. βCD most commonly used derivatives and commercially available and cheaper than any other CD derivatives. Moreover, it forms precipitated cluster with both PFH and PFP in case different PFC is needed for histotripsy application.

4.4. Histotripsy Study

As experimental and computational investigations indicated the clusters of βCD inclusion complex with PFCs are selected as the most efficient combination that allows practically isolate the sample as powder for further use. Moreover, βCD NCC can be isolated for both 1:1 and 1:5 ratios. This might help to test one of the stated hypotheses about contribution of free PFC in the core of the NCC on histotripsy. Cavitation pressure threshold experiments were planned in agarose gel by tuning the same PFC amount in both 1:1 and 1:5 ratios. It can be seen from **Figure 4.10,** there is no significant difference between the ratios but on the other hand, both of them lowers the cavitation threshold drastically from 26.16 MPa to 9.96. However, quantitatively more 1:1 NNC was used to obtain the same effect with less amount of 1:5 NCC. To be able to clearly indicate the effect of free PFC in the core, another comparison with the same amount of NCC needs to be done.

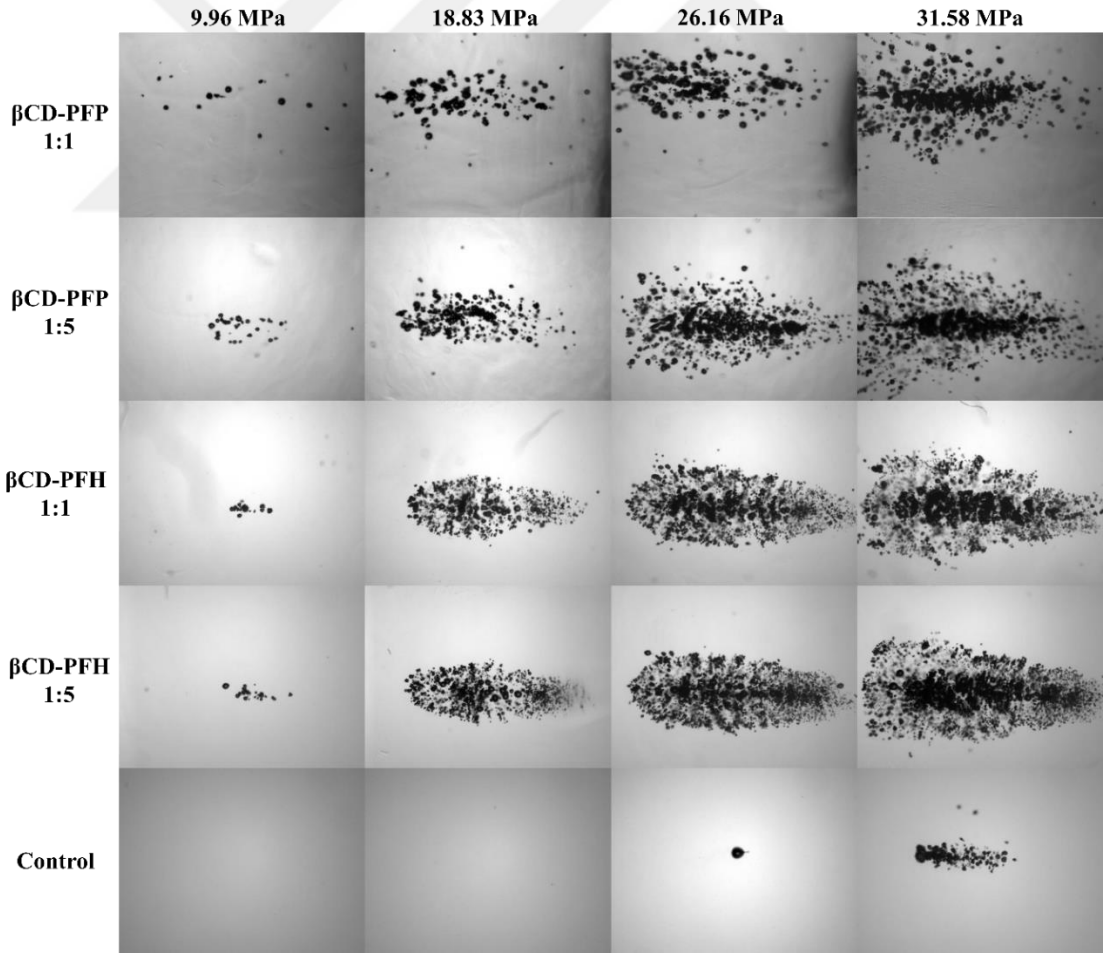


Figure 4.10: Histotripsy experiment results for βCD-PFC NCCs

CHAPTER 5

5. CONCLUSIONS AND FUTURE WORK

This study both experimentally and computationally investigated the interaction of a library of CD with PFC derivatives to define the most efficient histotripsy agent. Initially, inclusion complex formation of each CD derivative with either PFP or PFH was computationally investigated and it was shown that all CD derivatives form inclusion complexes with PFC except smallest cavity size containing αCD. Then, cluster formation of these inclusion complexes both experimentally and computationally was investigated using different CD:PFC ratios to see the contribution of free PFC in the clustering behavior. The results showed that they all form clusters in the presence of excess PFC however they end up with different cluster structures and stability. NCC of CD with higher water solubility did not form precipitated product. It was observed that this is not only because the solubility of CD derivative it is also because of the level of interaction between PFC molecules in the cavity and outside. High water-soluble derivative of CD has either hydroxypropyl or methyl groups that they can expand the cavity of CD and resulted more space for guest PFC in the cavity. Especially hydroxypropyl derivative with more elongated cavity resulted higher interaction with the guest PFC but not free one which leads loose PFC core and resulted NCC structure does not precipitate. It was shown that precipitated NCCs have strong interaction both for inclusion complex and for between guest and free PFC molecules leading firm core. Higher stability of NCs can lead NCC formations with lower stability. Computational findings enlightened the NCC mechanism clearly and support the experimental solubility/stability observation by energy calculations as well. The results showed that βCD NCCs are prominent because production steps are quite easy, do not require advanced chemical experience and water can be removed by centrifuge easily. As a final study, the histotripsy efficiency of βCD-PFH NCC was tested and a drastic decrease in the cavitation pressure threshold was shown successfully.

At this point, a further step for this study should be the investigation of NCC's stability in the bloodstream. Since there are only non-bonded interactions, it needs to be shown how stable they can stay and how their behavior effects the histotripsy applications. Additionally, a computational study can be done to mimic the histotripsy conditions to see the behaviors of NCC during treatment.

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PUBLICATIONS, PRESENTATIONS, AND PATENTS ON THE THESIS:

- W. Mustafa, B. Kaymaz, N.E. Çabuk, E. Vlaisavljevich, Ö.Şensoy, Y. Yuksel Durmaz "Development Of Cyclodextrin Based Histotripsy Agents" *International Natural Science, Engineering and Material Technologies Conference*, Sep 9-10, 2019
- W. Mustafa, B. Kaymaz1, N. E. Çabuk, R. Schmieley , E.Vlaisavljevich, Ö. Şensoy, Y. Yuksel Durmaz "Nanoparçaçık Ortamlı Histotripsi Ajanı Olarak En Etkin Siklodekstrin-Perflorokarbon Konuk-Konak Kompleksinin Belirlenmesi" *31.National Chemistry Congress*, Sep 10-13, 2019

EXPERIMENTAL AND COMPUTATIONAL INVESTIGATION OF CLUSTERING BEHAVIOR OF CYCLODEXTRIN-PERFLUOROCARBON INCLUSION COMPLEXES THROUGH MOLECULAR DYNAMIC SIMULATIONS

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