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β-cyclodextrin polymers as cholesterol sequestrating agents

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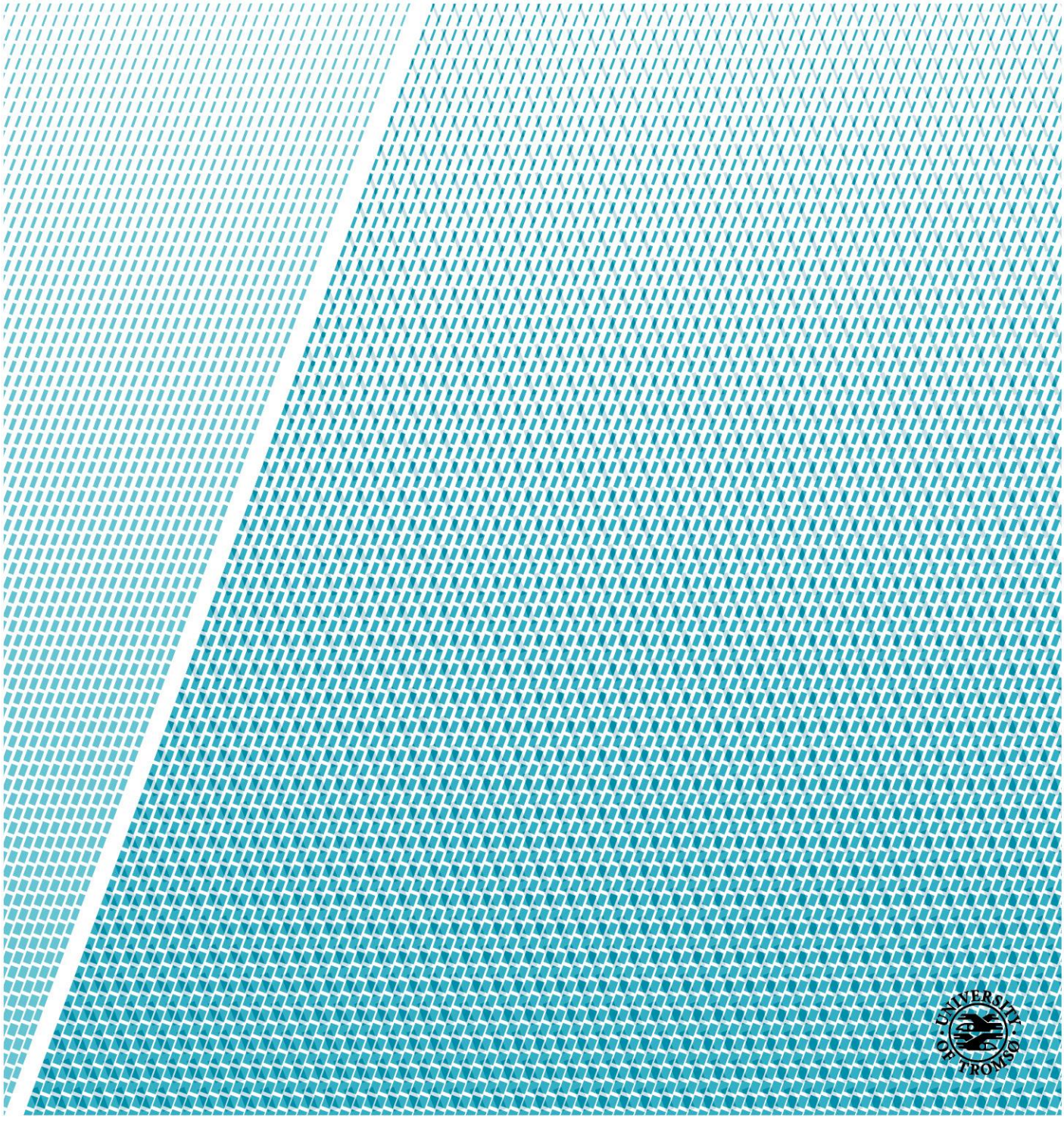

Summary

Background

Elevated serum cholesterol blood levels is one of the central risk factors for cardiovascular diseases. Lowering blood cholesterol have significant impact on lowering cardiovascular disease events. Inhibiting cholesterol absorption in small intestine can significantly lower serum cholesterol levels and thereby - lower cardiovascular events. B-cyclodextrin (βCD)-dextran polymer is expected to form complexes with cholesterol and avoid absorption together with complexed cholesterol. In this thesis, we investigated βCD-dextran polymers ability to complexate cholesterol in simulation of gastric fluids.

AIMS OF THE THESIS

1. To investigate ability of βCD-dextran polymer complexing cholesterol in simulated gastric fluids (Fasted stomach and fed small intestine) and compare it with methyl-β-cyclodextrin (MβCD).
2. Investigate ability of βCD-dextran polymer to be formulated as a tablet.
3. Investigate ability of βCD-dextran polymer to complex dietary cholesterol in a simulation of gastric fluids – fasted stomach and fed small intestine.

Methods

Ability in complexing cholesterol was analyzed by performing a simulation of gastric fluids with added excess cholesterol during digestion process with presence and absence of βCD-dextran polymer and MβCD.

Ability to complex dietary cholesterol was analyzed by performing a simulation of gastric fluids with egg yolk during digestion process with presence and absence of βCD-dextran polymer.

Cholesterol content in samples was analyzed by fluorescence spectroscopy.

Results and discussion

βCD-dextran polymer tablets at βCD conc. of 2.5mM complexed 0.0513 mg/ml crystalline cholesterol after 240 minutes in simulation of gastric fluids, while MβCD at conc. of 10mM complexed 0.45 mg/ml crystalline cholesterol. Extrapolated results to 10mM of βCD cons. shows that βCD-dextran polymer is capable of complexing significant amount of cholesterol.

βCD-dextran polymer was successfully compressed into tablets with no visible defects of friability during handling.

βCD-dextran polymer tablets sequestrate comparable amounts of food cholesterol, compared to experiments with crystalline cholesterol.

Extrapolated results to physiological volumes of gastric fluids shows that βCD-dextran tablets corresponding to a concentration of βCD units of 10 mM were able to solubilize approx. 215 mg of crystalline cholesterol, and approx. 135 mg of food cholesterol after 240 minutes.

Conclusion

In simulation of gastric fluids, βCD-dextran polymer tablet can complex approximately 48% of cholesterol in comparison to cholesterol solubilized by MβCD powder. βCD-dextran polymer tablets show ability to complex similar and significant amounts of both crystalline and food cholesterol in simulation of gastric fluids.

βCD-dextran polymer shows good compaction properties and were successfully compressed into tablets with no visible defects and no visible friability during handling.

Abbreviations

CVD – Cardiovascular diseases

CHD – Coronary heart diseases

CAD – Coronary artery disease

WHO – World health organization

DALY – Disability-adjusted life years

LDL-C – low-density lipoprotein cholesterol

EHN – European heart network

ESC – European society of cardiology

LDL – Low density lipoprotein

VLDL – Very low-density lipoprotein

HDL – High density lipoprotein

NPC1L1 – Nieman-Pick C1like 1 protein

TICE – Trans-intestinal cholesterol excretion

βCD-dextran – β-cyclodextrin-dextran derivate

βCD – β-cyclodextrin

CD – Cyclodextrin

MβCD – Methyl-β-cyclodextrin

HPβCD – Hydroxypropyl-β-cyclodextrin

GI – Gastrointestinal tract

HPLC – High-performance liquid chromatography.

FeSSIF – Fed state simulated intestinal fluid

FaSSGF – Fasted state simulated gastric fluid

FFF01 – Biorelevant dissolution media powder

RT – Retention time

PBS – Phosphate-buffer saline

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1 Introduction

1.1. Cardiovascular diseases

1.1.1 Definition and socio-economic impact of cardiovascular disease

Cardiovascular diseases (CVD) is a generic term for diseases that involve heart and blood vessels, which includes conditions like atherosclerosis, coronary heart disease (CHD), coronary artery disease (CAD), stroke, thrombosis and many other serious conditions.

It is estimated to be the leading cause of death worldwide and accounted for 31% of all global deaths worldwide [1]. In 2004, the World Health Organizations (WHOs) Burden of Disease project have calculated CVD to be the largest disease burden in Europe, using disability-adjusted life years (DALY), a time-based measure that combines years of life lost due to premature death and years of productive life lost due to disability [2].

In addition to human costs, economic impact of CVD is also a considerable part of the burden. European Heart Network (EHN) and European society of cardiology (ESC) presented data in 2012, stating that direct healthcare cost of CVD for EU is €106 billions of which 49% is due to inpatient care, 29% due to medications and 21% due to primary care, outpatient care and "Accident and Emergency". This health care expenditure accounts for 9% of total health care expenditure in European Union [3].

In in addition to healthcare costs, CVD also involves production losses for people in working age as well as cost of informal care of people with the disease. Productivity loss due to CVD is calculated to be €46 billion and informal care cots is calculated to be €44 billion a year according to EHN and ESC [3]. Thus, total cost of CVD for the EU economy is almost €196 billion yearly which is a significant economic burden.

As shown above, CVD is a serious health problem for EU and similar situation is seen in other parts of the world. A substantial part of CVD and its burden can be reduced by implementing and optimizing both prevention and treatments of the disease. This process has been started, positive results are seen and it is still under optimization. Central role in prevention and treatment of CVD involves reduction of risk factors such as tobacco, diet, physical activity, blood pressure, blood cholesterol etc., both with and without medications [4].

1.1.2 Elevated blood cholesterol and CVD relationship

Hypercholesterolemia is a condition defined by elevated levels of blood cholesterol. There are many different reasons for hypercholesterolemia; it may be a result of primary factors, such as genetic factors (e.g. Familiar hypercholesterolemia, polygenic hypercholesterolemia) or because of secondary factors such as other diseases (e.g. Diabetes mellitus, hypothyroidism), diet or even be a side effects of some drugs (e.g. Antipsychotic medications) [5].

Over the past decades, elevated serum cholesterol has been found to be one of the central risk factors for CVD, many studies have been conducted and proved relationship between increasing in CVD events and elevated levels of plasma cholesterol [6-11]. Successful management of low-density lipoprotein cholesterol (LDL-C), which is the main risk contributor to atherosclerosis, have shown to be effective through many studies [12-14]. Previous clinical studies have indicated that *"for every 1% reduction of LDL-C, relative risk for major CHD events is reduced by approximately 1%"* [15]. Main mechanism by which elevated cholesterol increases risk of CVD is believed to be promoting development and progression of atherosclerosis. Atherosclerosis is a complicated, pathologic multi-stage process with multiple factors involved, including: endothelial dysfunction, inflammatory and immunologic factors and hyperlipidemia [16]. Process begins with development of fatty deposits on artery walls which may progress further to more advanced types of plaques that may narrow and even block the arteries leading to complications like angina pectoris, or they may also rapture and cause a blood clot [16-18]. By those mechanisms atherosclerosis leads to CHD and CAD, and it is the main mechanism by which hypercholesterolemia is believed to increase the risk of CVD [5].

1.2 Cholesterol and blood cholesterol

1.2.1 Cholesterol properties and functions

Cholesterol is a lipophilic substance found in every cell of the human body, performing many functions. It is vital for human beings and involves in such functions as biosynthesis of hormones, production of bile acids for digestion and formation of cell membranes. In human body, cholesterol originates from two sources, major part (1g = ~70%) of the cholesterol is being synthesized within the cells and a minor part (~30%) comes from animal food sources like eggs, meat, fish etc. [19, 20]. Every human cell produces cholesterol, but much of its production takes place in liver [21]. Synthesis of cholesterol involves many steps, but simplified version is presented in Fig. 1. The process starts with condensation of tree acetate molecules to form HMG-CoA which is further reduced by enzyme HMG-CoA reductase to produce mevalonate. Mevalonate units are then converted to form activated isoprene units, which are then polymerized to form the 30-carbon linear molecule - squalene. The final step involves cyclization of squalene to form four ring steroid structure and further modifications of the molecule (oxidation, removal of migration of methyl groups) to produce cholesterol [21].

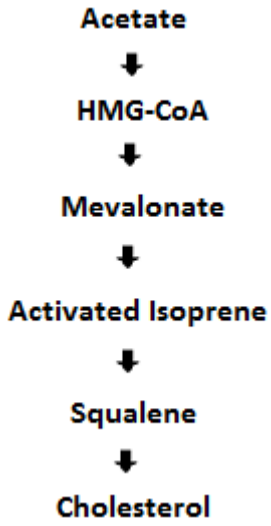

Figure 1: Simplified presentation of cholesterol biosynthesis pathway.

Cholesterol itself is not water soluble and travels in blood incorporated into lipoproteins. Lipoproteins are complexes with lipophilic inner core consisting of cholesterol esters and triglycerides and a hydrophilic outer layer made of phospholipids and unesterified cholesterol. This outer layer also contains at least one protein (alipoprotein) which provides the ligand for interactions with various cells, receptors, enzymes and adds structural integrity. There are

three main types of lipoproteins – Very low-density lipoprotein (VLDL), Low density lipoprotein (LDL) and high density lipoprotein (HDL) [5].

VLDL are particles formed in the liver, it is large particles that transport mostly triglycerides, but also cholesterol and other fats. Its main function is to transport triglycerides from the liver to muscle and adipose tissue. VLDL appear to play a far less significant role in pathogenesis of atherosclerosis compared to LDL and HDL [5].

LDL particles carry most of the total blood cholesterol (60%-70%). It is often referred to as "bad cholesterol" since it plays a major role in pathogenesis of atherosclerosis and its exceeded level dramatically increases the risk of CVD. Low-density lipoprotein cholesterol (LDL-C) due to its strong association with atherosclerosis is there for the main target of cholesterol lowering drugs [5].

HDL particles are small, dense and protein-rich particles that originate from liver and small intestine. HDL-C (cholesterol carried by HDL particles) is often referred to as "good cholesterol" since it has been shown to protect against the development of atherosclerosis, most likely due to three of its functions – stimulation of cholesterol efflux from peripheral cells [22], reverse cholesterol transport and inhibition of LDL-C oxidation [23]. In contrast to LDL-C, higher values of HDL-C concentrations are desirable till a certain extent, because cholesterol is being removed from vascular tissue and is not available to contribute to development of atherosclerotic plaques [5, 24].

1.2.2 Cholesterol secretion and absorption

Absorption of dietary and biliary cholesterol in small intestine and the revers cholesterol transport plays an important role in cholesterol homeostasis (Fig. 2).

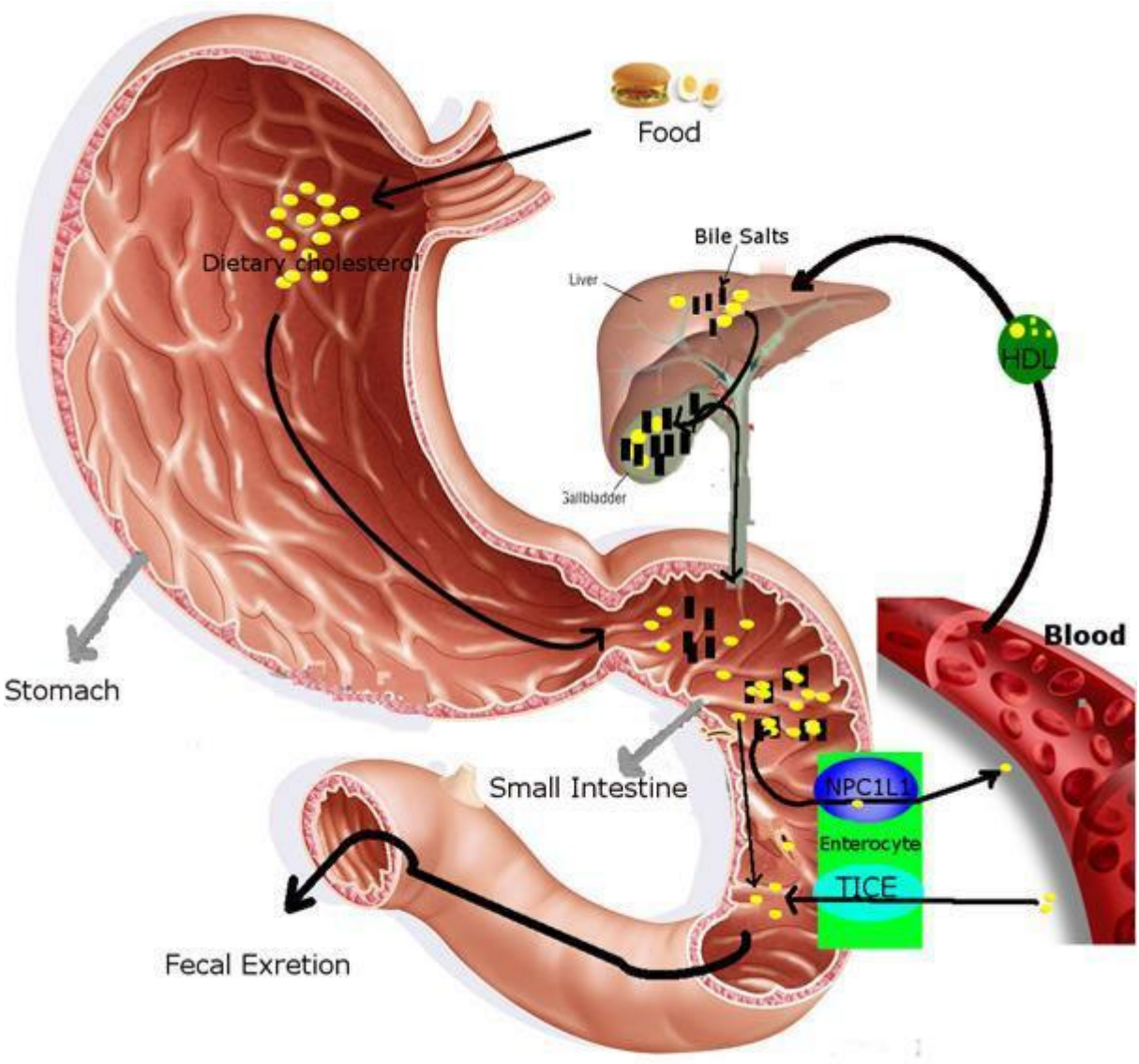

Figure 2: Cholesterol secretion and absorption in the gastrointestinal (GI) tract.
Notes: Cholesterol available for absorption in small intestine originates from diet and biliary secretion. Cholesterol incorporated in biliary micelles is further absorbed by NPC1L1protein. Free cholesterol that is not incorporated in to the micelles in addition to cholesterol secreted by enterocytes from blood by TICE route is eliminated by fecal excretion.

In addition to synthesis of cholesterol and production of different lipoproteins which is introduced to blood stream, hepatocytes also involve in elimination of cholesterol from the body in form of biliary acids and free cholesterol. Process starts as bile salts in hepatocytes are being pumped out across the canalicular plasma membrane by ABCB11, and ATP-driven membrane transporter. This process is accompanied by interaction of bile salts with exterior

surface of the canalicular plasma membrane, witch in turns activate two other ATP-dependent canalicular plasma membrane transporters, ABCB4 and a heterodimer of ABCG5 and ABCG8, which secretes phospholipids and cholesterol, respectively. This process is then followed by formation of micelles between bile salts, phospholipids and cholesterol [25]. Bile is being secreted in amounts of 24g/day, with an average of 1g/hour, accompanied by secretion of approximately 11g of phospholipids and up to 2 g of cholesterol [22, 25]. Between digestion, micellar particles are stored in the gallbladder, but when food is ingested, it is freed. In addition to secretion of cholesterol from liver into the small intestine, bile plays a central role in digestion and absorption of dietary fat, mainly originating from dietary sources. After dietary lipids (e.g. triglicerides, fats, oils) has gone through processing by lipases and being broken down into fatty acids and monoglycerides, they are then as well as cholesterol being incorporated into micelles. These micelles then promote absorption of solubilized lipids to the plasma membrane of intestinal absorption cells, enterocytes. Incorporation of cholesterol into micelles by assist of bile salts is a crucial step in the absorption of cholesterol, studies have shown that almost no cholesterol is absorbed in mice with strongly diminished amount or absence of bile acids [26]. Absorption of cholesterol in enterocytes happens by a separate mechanism from other lipids, it is an active and selective protein-mediated process, facilitated by the protein Nieman-Pick C1like 1(NPC1L1) [27]. A part of the absorbed cholesterol is then secreted back into the lumen by ATB-binding cassette transporters, ABCG5 and ABCG8, a process that increase elimination of cholesterol from body [28]. A portion of the absorbed cholesterol is converted to cholesteryl esters and the remaining cholesterol is absorbed as free cholesterol. Cholesterol esters and cholesterol that have not been secreted into the lumen is then, together with triglycerides and apolipoprotein B48 incorporated in the chylomicrons which are secreted into the lymph to later reach the blood stream.

In addition to secretion of cholesterol by hepatocytes, intestine is also found out to have its own, independent mechanisms for secretion of plasma cholesterol into the small intestine lumen. This mechanism is called trans-intestinal cholesterol excretion (TICE). Some of the cholesterol found in small intestine comes from intestines own secretion of cholesterol, no studies still have shown amount of the cholesterol secreted by the intestine in humans, but studies in mice shows that up to 30% of fecal natural sterols comes from the TICE route [19, 29, 30]. As well as the amount of secreted cholesterol by TICE, understanding of this

mechanism is still missing, but this mechanism can potentially be targeted by cholesterol lowering drugs.

Some of cholesterol is lost due to loss of bile salts that evades absorption and eliminates as fecal loss. As mention above, bile acids are produced with consumption of cholesterol in liver and therefore, fecal loss of bile salts will result in production of it by liver and consumption of cholesterol. Bile salts reabsorption process in small intestine is very efficient, usually close to 98%, and the lost 2% corresponds to approximately 400 mg cholesterol per day [25]. This process is also targeted by cholesterol lowering medication found on the market in class of bile acid sequestrans (e.g. Welchol, Questran, Colestid).

Amount of cholesterol lost as cholesterol molecules depends on how much cholesterol is going through the small intestinal lumen and how much will evade absorption. Amount of cholesterol in lumen of the small intestine as mentioned above depends on amount of cholesterol molecules secreted with bile salts, by TICE and plus dietary cholesterol. Cholesterol esters are a part of dietary cholesterol, they are enzymatically converted into free cholesterol by cholesterol esterase during digestion and presented for absorption. The average western diet contains 300-450 mg/day of cholesterol [31]. In sum with cholesterol secreted into bile acids, it results to approximately 2400 mg/day of cholesterol, where dietary cholesterol being minor, but significant part accounting for approximately one quarter of total cholesterol in lumen of the small intestine. Absorption rates of cholesterol in humans vary a lot starting as low as 25% and going up to around 75% and average approximately 50% [32, 33]. Ezetimibe is a medication working by inhibiting cholesterol absorption in the intestine by blocking a transport protein NPC1L1, and have shown to significantly reduce blood cholesterol [34, 35].

1.3 Medications for treatment of hyperlipidemias

As mentioned above, lowering of blood cholesterol in form of LDL-C is one of the main targets in both primary and secondary prevention of CVD. Several groups of drugs are successfully used for therapeutic reduction of blood cholesterol. Main groups of cholesterol-lowering drugs include inhibitors of biosynthesis of cholesterol - statins, cholesterol absorption inhibitors - ezetimibe and bile acid-binding resins. Today statins are the most potent cholesterol lowering group of drugs and mechanism of action is due to inhibition of the HMG-CoA reductase, and thereby reduction of endogenous cholesterol synthesis – which in turns results in lower levels of blood cholesterol [36]. Statins are the first choice in treatment of hyperlipidemias, but at the same time, significant proportion of patients doesn't respond adequately to statin therapy, being intolerable of statins due to side effects or not achieving targeted level of blood cholesterol. Generally, statins are well tolerated, but serious adverse effects including myalgias, myositis, rhabdomyolysis and diabetes 2 are possible, making statins not suited as a therapy for some patients [37, 38]. An article from 2008 shed light on reality in clinical achievement of cholesterol lowering goal in high and vary high risk patients, and in range from 45% to 82% of patients in different disease groups do not achieve their therapeutic goals in lowering blood cholesterol [39]. Similar results are seen in a more recent study, carried out by Vonbank et al. [40]. Those results point out that treatment of those patients need optimization. It also shows that statins alone often are not enough to bring cholesterol to the therapeutic goal, and additional medications are needed to achieve desirable level of blood cholesterol.

There is therefore a need for new additional medications that may help to reduce cholesterol to its optimal therapeutic goal. This work investigates potential of beta-cyclodextrin-dextran derivatives (βCD-dextran) to be formulated and used as cholesterol absorption inhibitor. We investigated its ability to form complexes with cholesterol in simulated gastric fluids and its potential to be formulated as tablets – therefore as an accurate, convenient and easy to use formulation.

1.4 βCD-dextran polymer as potential cholesterol absorption inhibitor

1.4.1 Introduction to cyclodextrins

Cyclodextrins (CDs) are a group of cyclic oligosaccharides, where the most common derivatives consist of six to eight (α-1,4)-linked -D-glucopyranose units forming a cone structure that is characterized by a hydrophobic central cavity and hydrophilic outer surface [41]. CDs are named based on the amount and chemical modifications of monomers forming the cone structure - where, α corresponds to six, β – corresponds to seven and γ corresponds to eight monomers. Many chemical modifications have been applied to CDs, altering their properties (e.g., increasing its water solubility, optimizing its interaction with guest molecules) some of the most common chemical modified CDs includes methyl-β-cyclodextrin (MβCD) and hydroxypropyl-β-cyclodextrin (HPβCD). Over the last few decades, studying and use of CDs actively increased and especially after more CDs got approved for food and drug application, interest in them increased. Their unique and useful properties have found its place on the markets, currently more than 30 different pharmaceutical products contain CDs and even more products containing CDs are found in the food industry [41, 42].

As mentioned above, CDs are characterized by their cone-like structures, and this structure provides CDs with the ability to spontaneously form inclusion complexes in aqueous media, molecular complexes in which CD molecule entrap a lipophilic molecule or moiety into its hydrophobic inner cavity [41, 43]. During complexation, no covalent bonds are formed, but many noncovalent interactions have been proposed to be responsible for complex formations: hydrophobic interactions, Van der Waals forces, hydrogen bonding and dipole-dipole interactions, the release of “High energy water” from the CD cavity on substrate inclusion, release of confrontational strain in a CD-water adduct [44]. Smaller molecules (size of up to ~8Å) often get fully entrapped during complexation with CD, while larger molecules on the other hand get entrapped partly. Cholesterol molecule (~18 Å) is larger than the cavity of β-cyclodextrins (βCDs) and only half of the molecule may get entrapped by βCD [45]. The most common complex between substrates and βCDs is 1:1 complex where the ratio of complexation CD:Substrate is one to one.

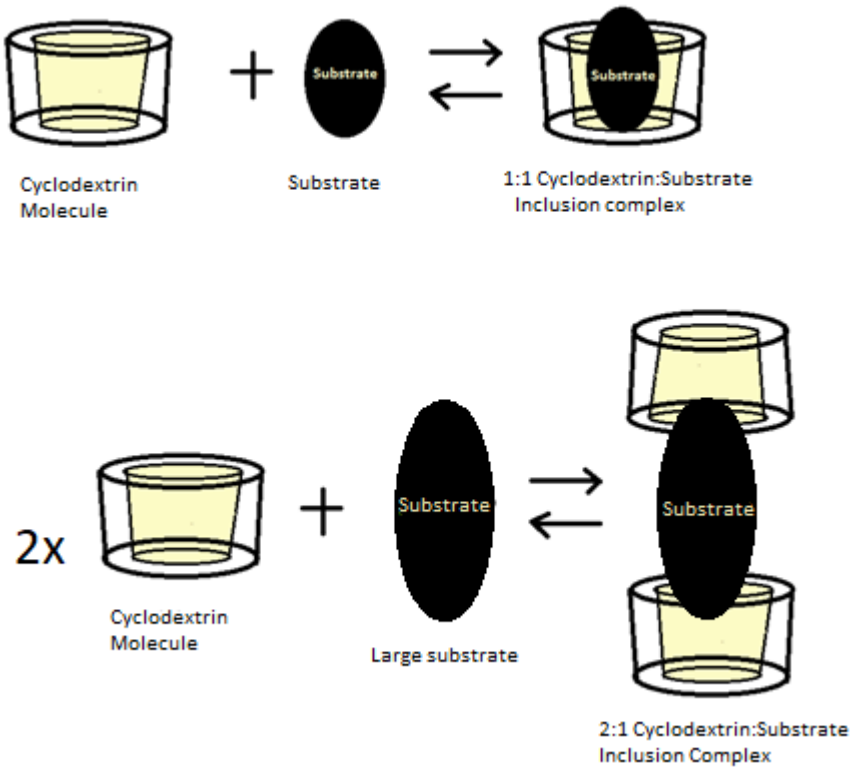

Figure 3: Schematic representation of 1:1 and 2:1 CD:substrate complex formation.

Equilibrium of a complex can be defined by equation (Eq.) (1):

$$m \cdot S + n \cdot CD \overset{K_{m:n}}{\longleftrightarrow} S_m CD_n \quad (1)$$

Where $S_m CD_n$represents the comolerx, of S the substrate and CD the cyclodextrins. The stability constant (K) (also known as binding constant) can be described by Eq. (2):

$$K = \frac{[S_m CD_n]}{[S]^m \cdot [CD]^n} \quad (2)$$

Stability constant represents the affinity or strength of interaction between a compound and a CD. This constant is related to CDs ability to solubilize an insoluble compound in aqueous media and have typically values vary from 0.1 M^{-1} representing weak interactions and up to 1,000,000 M^{-1} for very stable interactions. There are several approaches to investigate stability of complex formation between a CD and a substrate, but one of the most widely used for poorly soluble entities is phase-solubility study [44, 46].

When one molecule of a compound forms a complex with one CD molecule, solubility of a compound in presence of a given CD can be described as Eq. (3):

$$[S]_T = S_0 + S_{CD} \quad (3)$$

Where $[S]_T$ represents the total apparent solubility of the drug, S_0 the thermodynamic solubility (i.e. measured in absence of CD) and S_{CD} the enhancement in solubility due to the complexation. Under the condition that 1:1 S/CD complex is formed; stability constant may be calculated out of plot [S] versus [CD], where stability constant is defined by Eq. (4):

$$K_{1:1} = \frac{slope}{S_0 \cdot (1 - slope)} \quad (4)$$

The ability to form inclusion complexes provides CDs with its unique properties and it has been used for many benefits in food and drug formulations: to enhance solubility, bioavailability, stability and taste masking of a compound [47]. More uses than listed above are taking place or under studying at the time and one of the promising areas is to use CDs as active pharmaceutical ingredient in cholesterol lowering treatments [46].

Unmodified βCD has shown ability to form complexes with cholesterol and solubilize it in aqueous media, but this type of cyclodextrin is poorly soluble in water to be effectively used [48]. Chemical modified CDs like HPβCD, MβCD and highly hydrophilic polymeric derivate of βCD (βCD-dextran polymer) overcome this problem, being water soluble CDs, able to form complexes with cholesterol and solubilize it in aqueous media to a much higher degree [46].

1.4.2 βCD derivates used in this work

Methyl- β-cyclodextrins are cyclodextrins produced by methylation of secondary hydroxyl groups on regular βCD molecules. The amount of methylated secondary hydroxyl groups on CD molecule is called degree of substitution and it may vary. In this work MβCD with 10.5-14.7 degree of substitution is used [49]. Water solubility of MβCD is 50 mg/ml, which is 25-fold higher than regular βCD[49]. In addition to high water solubility – it also has strong cholesterol complexing power and is a widely used as a cholesterol depletion agent [50-52]. In this work, MβCD suits great as a comparison to cholesterol complexation ability of βCD-dextran polymer.

Hydroxypropyl-B-cyclodextrins (HPβCD) have an added 2-hydroxypropyl group on secondary hydroxyl group on regular βCD molecules. Water solubility is greatly increased compered to regular βCD and its interactions with substrate is altered. Moreover, it is safer than MβCD and some possible pharmaceutical applications have been attempted [48, 53]. Currently, HPβCD undergoes multinational Phase 2b/3 clinical efficacy trial and may become first available treatment for the disease [54].

B-cyclodextrin-dextran polymers are dextran polymers with attached CD molecules to it. Dextran itself is a macromolecule consisting of a linear glucose polymer linked by a-1,6-

glycosidic bonds, in conjugation with CDs, it provides CDs with unique properties, including high water solubility and likely lack of gastrointestinal tract (GI) absorption. Previously the polymer has shown great ability to complex hydrocortisone – a steroid with similar molecular structure to cholesterol[55]. Both size of the macromolecule and degree of substitution may be adjusted during synthesis and in this work, βCD-dextran-polymer with average Mw of 25 kDa, molar rate of dextran/βCD 1:10 and 55.8% βCD content is used [56].

1.5 Gastric fluids

1.5.1 Gastrointestinal tract and fluids

Gastrointestinal tract is complex organ system and consists of many different sections which are involved in digestion of food, absorption of nutrients and excretion of waste as feces. The human GI can be divided in four parts: esophagus, stomach, small intestine and column. Esophagus is the part which connects mouth and stomach, food ingested through mouth passes quickly in to stomach. In stomach, solid foods are reduced to a uniform bolus by enzymatic and acidic digestion and passes further into small intestine. In small intestine food is absorbed and further enzymatically digested. Food that passed small intestine reaches the final part – colon, a part of GI where further absorption and exchange of some nutrients happen as well as feces compaction further excretion happen [57].

Stomach in fasted state usually contains no more than 50 mL of fluids, but as food is ingested, its volume increases up to 1.5 L. At fasted state, pH in stomach is between 1 and 3.5, but after food ingestion it rises to values in range of 3-7 depending on the meal. Food is retained in stomach for 5 minutes in fasted and up to 2 hours in fed state [57].

After the stomach, food pass through the small intestine. Small intestine has tubular structure and typically is 6-7-meter long [35]. Liquid volume in small intestine in fasted state is 120-350 mL [58]and up to around 1L in fed state [59]. In fasted state, pH in small intestine ranges from 4.9 and up to 7.4 gradually rising along the length of the small intestine, in fed state pH rises and ranges from 5.2 and up to 7.5 having similar pH rising trend as in fasted state. Food passage time is relatively constant, unlike stomach and is around 3 hours [57].

1.5.2 Simulation of gastric fluids with “biorelevant media”

Biorelevant media is the term used to indicate biologically relevant fluids used in drug dissolution/permeation assays [60]. Biorelevant media are capable of accurately simulating fluids found in different regions of gastrointestinal tract. Simulation of gastric fluids have been showed to be accurate in predicting solubility and absorption of drugs in gastric fluids [61]. It also have been shown that this media are capable of simulating gastric fluids much closer than plane buffer solutions (SGF, SIF) [62]. In our work, we used fasted state simulation of gastric fluid (FaSSGF) and fed state simulated intestinal fluid (FeSSIF) fluids to investigate complexation and solubility of cholesterol in presence of CDs. Composition of the biorelevant media are reported in material and method section.

2 Aims of this thesis

1. Implement method for the quantitation of solubilized cholesterol by high-preformance liquid chromatography (HPLC).
2. Evaluate the solubilization properties of βCD-dextran polymers by the mean of phase-solubility studies.
3. Investigate the ability of βCD-dextran polymer in complexing cholesterol in simulated gastric fluids (fasted stomach and fed small intestine) and compare it with MβCD.
4. Investigate ability of βCD-dextran polymer to be formulated as a tablet.
5. Investigate ability of βCD-dextran polymer to complex dietary cholesterol in a simulation of gastric fluids – fasted stomach and fed small intestine.

3 Methods and materials

3.1 Materials

3.1.1 Chemicals

Table 1: List of chemicals used in this work.

Chemical	Chemical Formula	Mw (g/mol)	Product number Sigma aldrich	Manufacturer
Methanol	CH_3OH	32.04	34860N	Sigma Aldrich
Acetonitrile	C_2H_3N	41.05	34851N	Sigma Aldrich
2-propanol	C_3H_8O	60.10	34965	Sigma Aldrich
Cholesterol	$C_{27}H_{46}O$	386.65	26732	Sigma Aldrich
(2-Hydroxypropyl)- β-cyclodextrin (HPβCD)		~ 1460	-	-
Methyl-β-cyclodextrin (MβCD)		~ 1310	C4555	Sigma Aldrich
Cholesterol quantitation kit			MAK043	Sigma Aldrich
βCD-dextran polymer	55.8% βCD content.	25 000 Da	-	-
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4 * H_2O$	137.99	71504-MM	
Sodium hydroxide	NaOH	39.99	30620-M	Sigma Aldrich
Sodium phosphate dibasic dihydrate	$HNa_2O_4P * 2\ H_2O$	358.14	30435-M	
Biorelevant dissolution media Powder (FFF01)				Biorelevant
Glacial acetic acid	CH_3COOH	60.05	33209-M	Sigma Aldrich
Sodium chloride	NaCl	58.44	71380-M	Sigma Aldrich

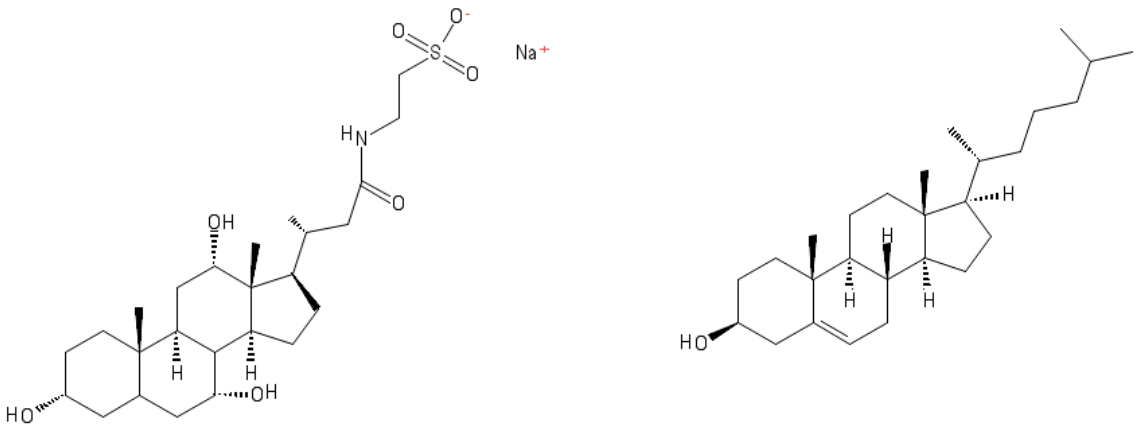

Figure 4: Chemical structure of Taurocholate (to the left) and cholesterol (to the right).

3.1.2 Composition of FaSSGF and FeSSIF solutions

Table 2: FaSSGF composition [63]

Component:	Concentration:
Sodium taurocholate	0.08mM
Lecithin	0.02mM
Sodium Chloride	34.2 mM
Hydrochloric acid	≈25.1mM

Table 3: FeSSIF composition [63]

Component:	Concentration:
Sodium taurocholate	15mM
Lecithin	3.75mM
Sodium Chloride	203.2 mM
Sodium hydroxide	101mM
Acetic acid	144.1 mM

3.2 Methods

To quantify complexed cholesterol by CDs, a method to quantify amount of solubilized cholesterol were required. Stelzl et al. (2015) [64] developed an HPLC method to quantify cholesterol content in aqueous solutions and this method was chosen for analysis of complexed cholesterol in our work.

3.2.1 Cholesterol quantification with HPLC

Quantitative analyses were performed on a Waters 2690 separating module with XBridge C18 column (2.1x150 mm, 5 µm particles) and Waters 996 photodiode array detector. All analysis were performed using Methanol:2-propanol:Acetonitrile (50:25:25) as a mobile phase, isocratic flow with 0.8ml/min flowrate and 25C column temperature. All samples were filtered through a 0.2 um filter prior to analysis.

Standard curve for further analyses were prepared by making standard solutions of cholesterol dissolved in mobile phase. Stock solution with nominal concentration 5 mM has been prepared and further diluted to nominal concentrations of 0.65 mM, 0.5 mM, 0.35 mM, 0.2 mM, 0.01 mM and 0.05 mM. Every sample was analyzed 5 times. Exact concentrations of each standard solution have been plotted against average of integrated peak area of 5 parallels to make calibration curve of cholesterol and used for further quantitation analyses in phase-solubility studies.

3.2.2 Phase solubility studies

Phosphate-buffer saline (PBS) was used as aqueous solution for phase solubility studies. 200 ml of sodium di-hydrogen phosphate monohydrate [$NaH_2PO_4 * H_2O$] solution (2.25% weight/volume (w/v)) were mixed with 800 ml of di-sodium hydrogen phosphate dodecahydrate [$Na_2HPO_4 * 12H_2O$] solution (1.85% w/v). PH was approx. 7.

Solutions consisting of different nominal concentrations (1mM, 2.5mM, 5mM, 7.5mM, and 10mM) of HPβCD and MβCD in PBS buffer were prepared. The solutions were transferred into 5 ml plastic test tubes and excess amounts of cholesterol were added (approx. 3 mg/ml), 2 parallels were made for each solution. The solutions were further placed into a thermostatic shaking water bath for 48 hours (2 days) at 25°C. After 2 days, each sample were filtered through a 0.2 um pore-size filter and further analyzed with HPLC (n=2).

3.2.3 Preparation of biorelevant media solutions (FaSSGF/FeSSIF)

Solution A

One gram of sodium chloride (NaCl) was dissolved in 450 ml of distilled water and pH was adjusted to 1.6 with 1 M hydrochloric acid (HCl). Volumetric flask was filled to exactly 500ml.

FaSSGF/FeSSIF solution preparation

90 ml of solution A were filled into 100 mL volumetric flask. 0.01 g of FFF01 powder was dissolved in the solution and volumetric flask. FaSSGF solution was then transferred into 250ml beaker glass; 2 ml was taken out to test the pH that should be close to 2 (see section 3.1.2 for composition). Test materials were then added into the beaker glass, small magnet was placed (150 rotations per minute), beaker glass covered by parafilm and left for 60 minutes simulating fasted state gastric fluids.

After 60 minutes, conditions were changed to FeSSIF by adding following chemicals: 0, 99 g Sodium Chloride (NaCl), 0,6 g Sodium hydroxide pellets (NaOH), 829 ul Glacial acetic acid and 1,11 g FFF01 powder. 2 mL were taken out after 10 minutes to check pH, which should be close to 5 (see section 3.1.2 for composition). FeSSIF stage lasts over 3 hours, simulating fed state intestinal fluids.

3.2.4 Cholesterol quantitation kit – quantitation of cholesterol in FaSSGF/FeSSIF solutions

Analysis of FaSSGF/FeSSIF cycle samples with HPLC method was unsuccessful due to appearance of a new peak in FeSSIF samples that overlapped cholesterol – making it impossible to quantitate cholesterol precisely. Cholesterol Quantitation kit from Sigma Aldrich was chosen for further analysis, a kit used to quantitate free cholesterol, cholesteryl esters or both (total) present in a sample. In the kit, total cholesterol concentration is determined by a coupled enzyme assay, which results in a colometric(570 nm)/flourmetric ($\lambda ex = 535/\lambda em = 587$ nm) product, proportional to the cholesterol present [65]. Samples being analyzed by this kit, prior to analysis were filtered by 0.2 um pore-size filter to get rid of any non-dissolved particles. We analyzed our samples by fluorescence analysis and measured total cholesterol content in samples by following instruction from the “technical bulletin” followed with the kit, available in the appendix (attachment 1), all samples were run in singlet.

All obtained fluorescence results were calculated like calculation in this example

Fluorescence values were used to set up a standard curve following instruction from "technical bulletin". All samples were corrected for background value values obtained by the blank (cons. 0 ug/well cholesterol). Using the equation obtained from the calibration curve, cholesterol content in each well were calculated. Original concentration in each sample were then calculated with respect to dilution prior to analysis.

This test has also shown to be affected by compounds, most likely by taurocholate, after changing the condition from FaSSGF to FeSSIF, pure FeSSIF sample produces fluorescence equivalent to (approx.0.0034 mg/ml). Therefore, this error contribution was subtracted from further results.

3.2.5 Compression of βCD-dextran polymer into tablets

Compression of βCD-dextran polymer in to tablets was performed on a single punch tableting machine. Four portions of approximately 250mg βCD-dextran polymer powder weighted prior to compression. Tableting machine lower punch were adjusted to make tablets with 12 mm diameter, and 3 mm height. Because of powder consistency being voluminous, tableting process was performed in multiple, repeated steps: die was filled, followed by a partial compression and repeated till all the powder was in the die, then followed by a full final compression.

3.2.6 Cholesterol complexation during FaSSGF/IF cycle in presence of CDs

Analysis was carried out by starting the FaSSGF/IF cycle in presence of excess cholesterol (approximately 2mg/ml) and different CDs: MβCD, βCD-dextran polymer powder, βCD-dextran polymer tablet, with nominal βCD concentrations of 10 mM, 8.5 mM and 2.4 mM (1 tablet of approx. 250mg) βCD respectively. Tablets were placed into a basket used for dissolution test, and then the basket was fixed to be in the middle of the media, so the dissolution process would be closer to digestion process. As a control, FaSSFG/FeSSIF cycle in presence of excess cholesterol without any CDs was used. Value of solubilized cholesterol from control experiment were subtracted from results of all samples with CDs, to quantify correct amount of cholesterol that is solubilized by CDs. Samples of 2ml were taken during the cycle at different time points for further cholesterol quantitation, every sample was filtered with 0.20 um pore-size filter to get rid of any non-dissolved particles. Because of

practical considerations – limited time and amount of analyses in cholesterol quantitation kit, it was decided to analyze 2 samples from each experiment, from 60 and 240 minutes of the cycle. Each sample was analyzed giving 2 parallels for more reliable result. Due to mistakes during analysis only one parallel is made for 2 samples: Chol + FaSSGF/FeSSIF cycle 60 min. and MβCD + Chol. + FaSSGF/FeSSIF cyle 60 minutes.

3.2.7 Ability to complex food cholesterol in simulation of fasted stomach fluids and fed small intestine fluids

Experiments were performed by adding approximately 2g of boiled egg yolk into 50 ml of FaSSGF/FeSSIF cycle in presence and absence of βCD-cyclodextrin polymer tablet, 2 parallels were performed for each experiment. Tablets were placed in to the media by the same method as described above. Samples were collected after 60 and 240 minutes, filtered with 0.2 um pore-size filter and analyzed with cholesterol assay kit.

4 Results

4.1 Standard curve for Cholesterol quantified by HPLC

Retention time (RT) of cholesterol was 3 minutes 18 seconds.

The calibration curve of cholesterol made by HPLC is reported in Fig. 5.

The calibration curve resulted in linear (0.99) in the range from 0.05 mM up to 0.65 mM. This standard curve was used for further quantification of cholesterol by HPLC.

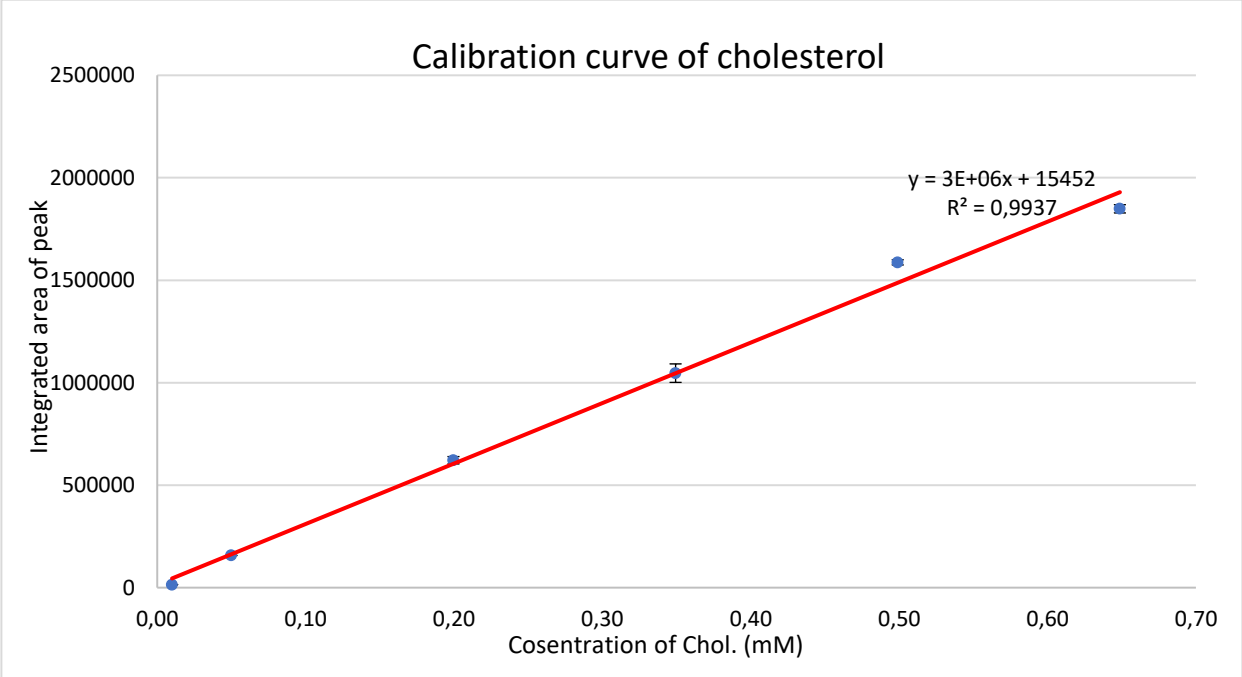

Figure 5: Calibration curve for quantitation of cholesterol by HPLC.
All points on the curve are average values of 5 parallels of HPLC analysis for each sample including standard deviation.

4.2 Cholesterol quantification in presence of FaSSGF/FeSSIF

Analysis of FeSSIF stage samples from FaSSGF/FeSSIF cycle with HPLC was unsuccessful due to overlap of cholesterol peak by another substance, presented as drawing in Fig. 6. Original pictures of those results can be viewed in appendix (attachment 2).

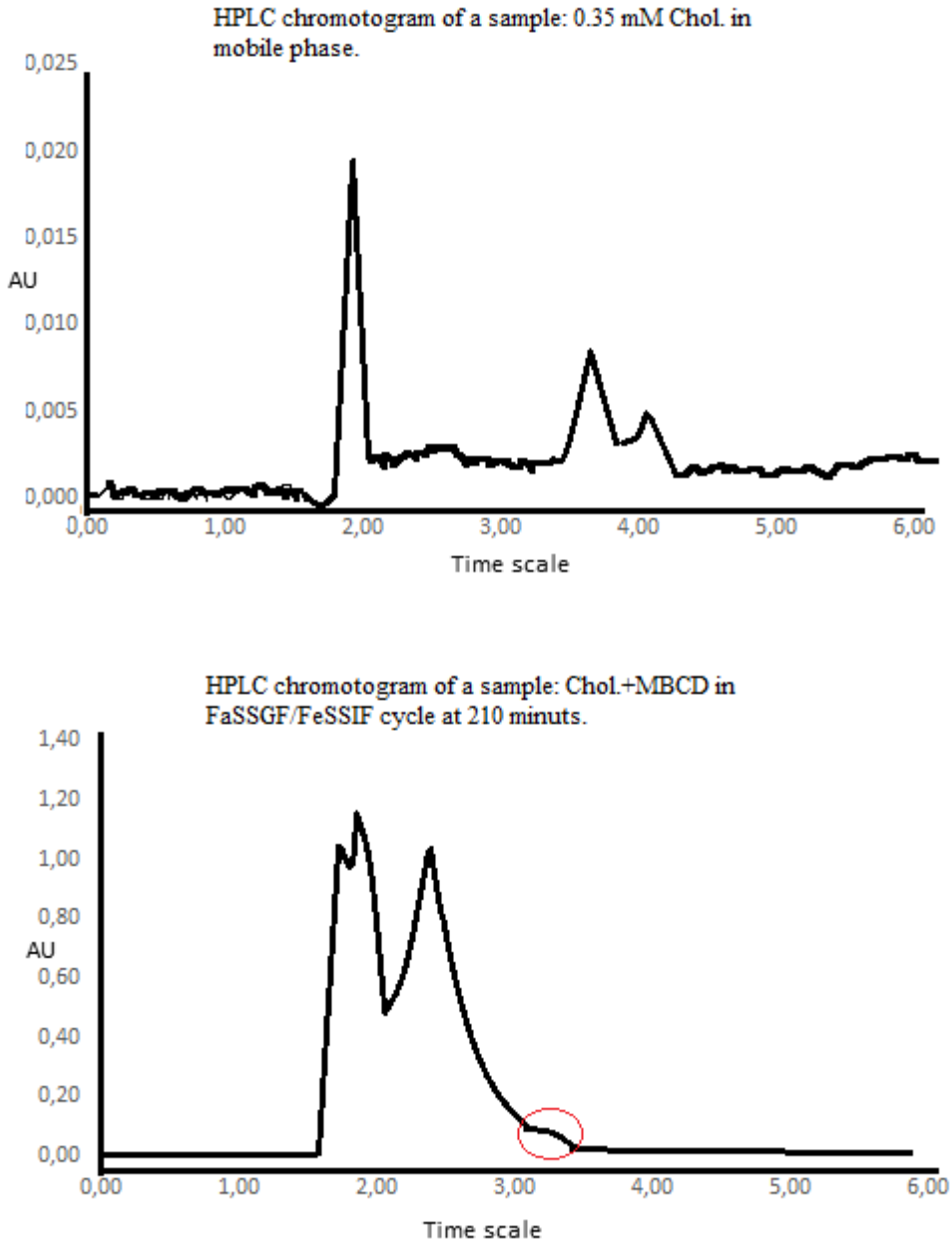

Figure 6: HPLC analysis result: On top – result of HPLC analysis of a sample with 0.35 mM cholesterol concentration in mobile phase, peak with RT of 3.6 is observed, representing total cholesterol (Cholesterol and oxidized cholesterol). Second chromatogram represents a sample containing cholesterol and MβCD in a FaSSGF/FeSSIF cycle, showing big overlapping peak, which most likely represent taurocholate is observed RT of 2.2 minutes and a small overlapped peak at 3.2-3.5 minutes, which most likely represent total cholesterol.

This big overlapping peak is most likely taurocholate, which have similar molecular structure with cholesterol. Overlapping makes it impossible to precisely quantitate cholesterol, due to this difficulty, another method for quantitation of cholesterol was chosen: the “Cholesterol quantitation kit”.

4.3 Phase-solubility study of cholesterol in presence of MβCD and HPβCD

Result of phase solubility study of cholesterol in presence of MβCD and HPβCD at 25C is reported below (Fig. 7). Control of this study was a sample with excess cholesterol in PBS buffer, and no solubility of cholesterol was detectable. Results show that tested CDs form complexes with cholesterol and solubilize it. Cholesterol solubility increases with increased concentration of respective solubilizer. MβCD shows higher cholesterol solubilizing ability (10mM concentration of MβCD results in 1 mM of cholesterol solubility) in contrast to HPβCD (10mM concentration of HPβCD results in 0.0 mM of cholesterol solubility).

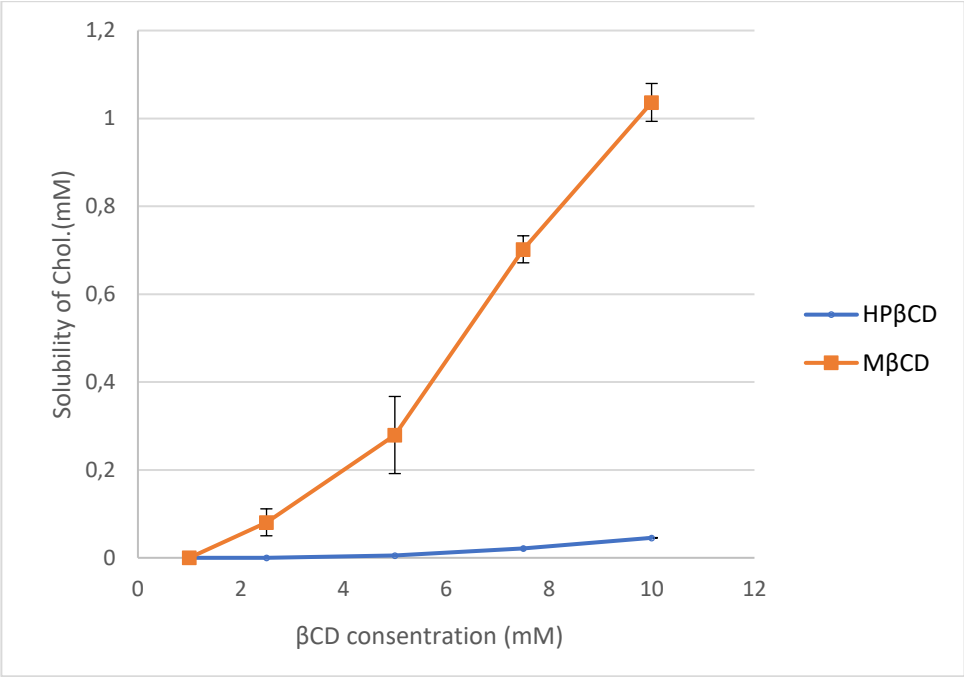

Figure 7: Solubility diagrams of cholesterol in presence of HPβCD and MβCD.
Phase solubility studies of cholesterol were performed in PBS buffer pH 6.9 in presence of HPβCD and MβCD. Samples were incubated at 25C for 48 hours. Results are reported as average and standard deviation of two parallels sample analyses.

4.4 Compression of βCD-dextran polymer into a tablet

Dextran polymer was successfully compressed into tablets by single-punch press. Approximately 250 mg of dextran polymer was compressed into a tablet with diameter of 12mm and thickness of 3mm (Fig. 8). Dextran polymer shows its compression ability suitable for tableting.

When used in experiments through FeSSGF/IF cycle, tablets showed full disintegration and dissolution within 30-40 minutes.

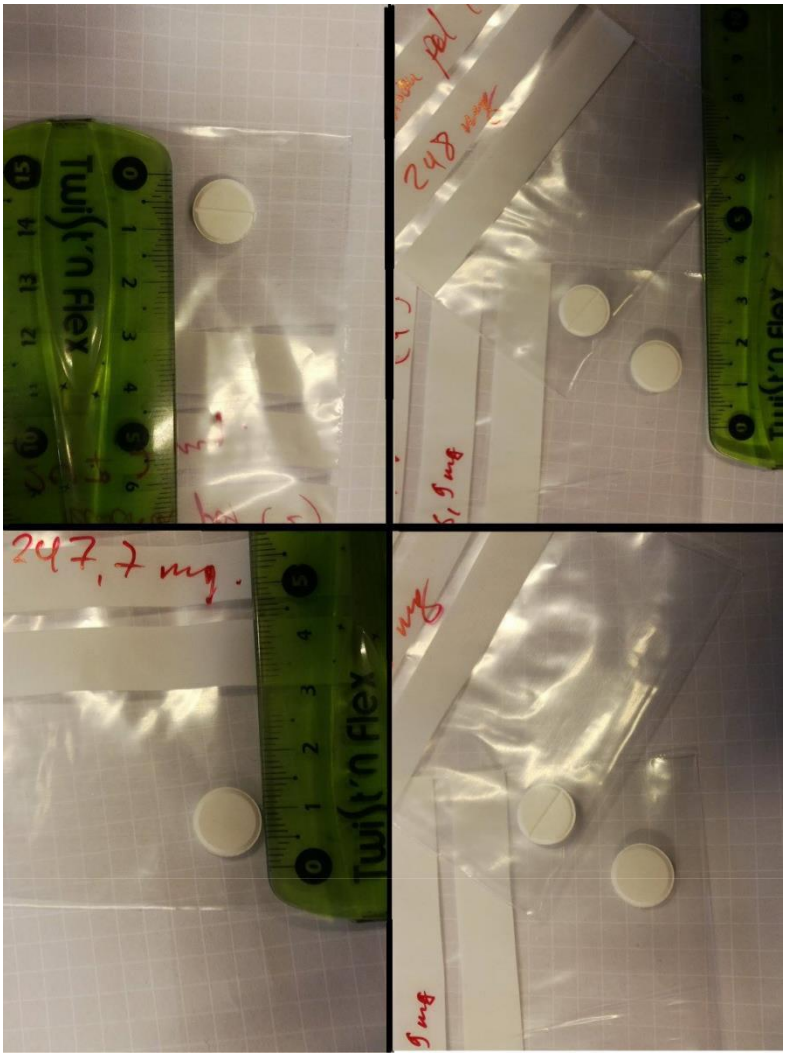

Figure 8: Tablets of βCD-dextran.
Tablets prepared of approximately 250mg βCD-dextran. Diameter of the tablets is 12mm and thickness is 3mm.

4.5 Cholesterol complexation in the presence of FaSSGF/FeSSIF

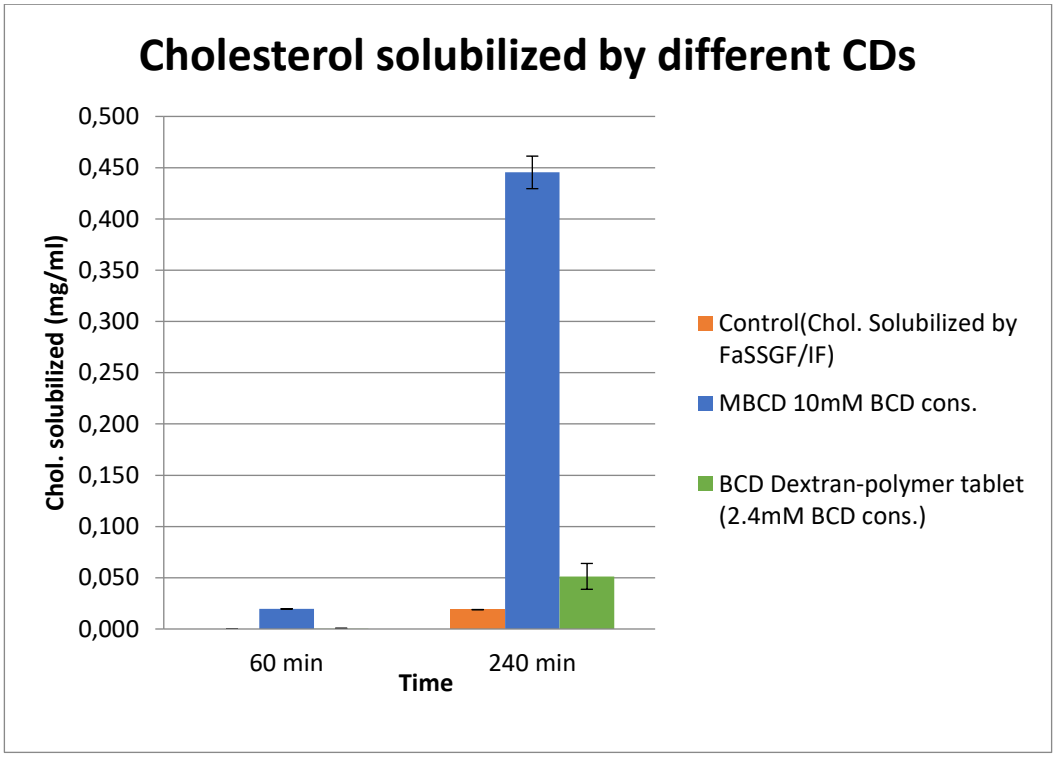

Figure 9: Cholesterol solubilized by different cyclodextrin formulations at different time in mimicked digestive conditions. Figure represents amounts of cholesterol complexed by different CD formulations – MβCD, βCD-dextran polymer powder and βCD-dextran polymer tablets at 60 minutes of FaSSGF/FeSSIF cycle and at 240 minutes of FaSSGF/FeSSIF cycle. The experiments were performed at room temperature (approx. 20C). Results are reported as average and standard deviation of two parallel sample analyses.

Even though different concentrations of CDs have been used, due to limited resources, results clearly indicate ability of investigated CDs to solubilize and form complexes with cholesterol during FeSSGF/IF cycle. After 60 minutes in FaSSGF conditions, highest amount of cholesterol have been solubilized by MβCD powder at 10mM βCD concentration (0.05 mM chol.), followed by βCD-dextran polymer tablet at 2.4 mM βCD concentration (0.001 mM chol.) and no cholesterol solubilization in control experiment (i.e. no CDs). After 240 minutes in FeSSIF, highest amount of cholesterol has been solubilized by MβCD powder at 10mM βCD concentration (1.15 mM, 0.45 mg/ml chol.), followed by βCD-dextran polymer tablet at 2.4 mM βCD concentration (0.13 mM, 0.05 mg/ml chol.). Results obtained from quantitation of cholesterol solubilized by βCD-dextran polymer powder were invalid, due to sample being diluted too much and fluorescence too low for calibration curve.

4.5 Sequestrating of cholesterol form egg yolk in mimicked digestive conditions

The cholesterol sequestrating ability of βCD-dextran polymer tablets were investigated in biorelevant media in presence of standard cholesterol-rich food such as egg yolk. Results are showed in Fig. 10. As it can be seen, food cholesterol is being complexed by βCD-dextran polymer in simulation of digestion process. βCD-dextran polymer tablet (2.5 mM βCD concentration) solubilized 0.08 mM (0.032 mg/ml) of cholesterol from 2g of boiled egg yolk in 50ml of biorelevant media after 240 minutes of exposure to FeSSGF/IF in contrast to control experiment where no cholesterol was solubilized.

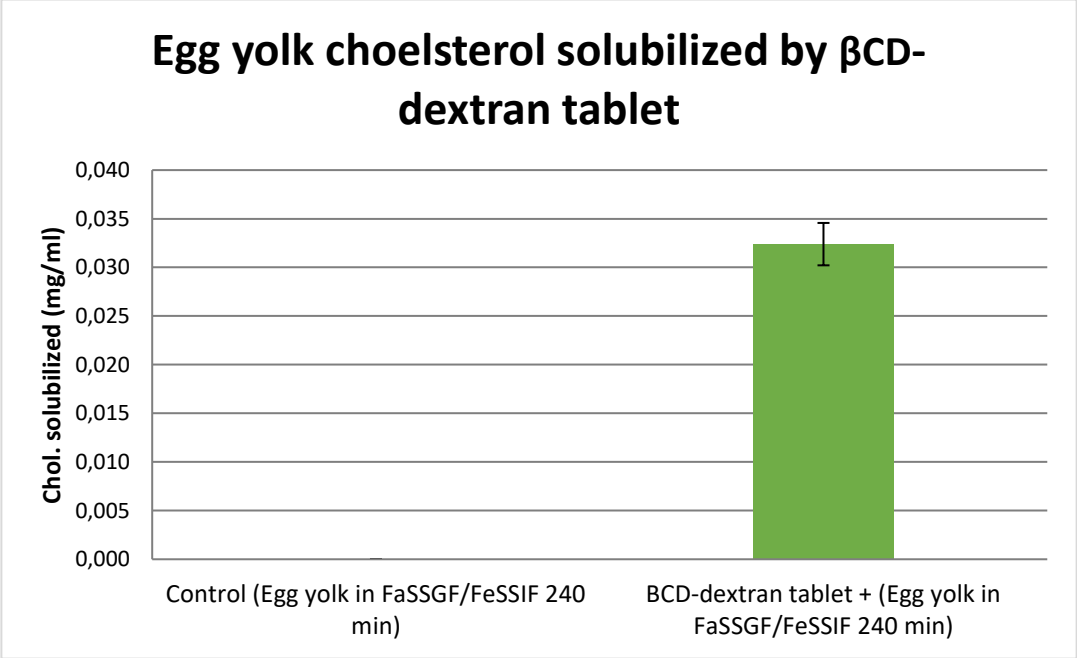

Figure 10: Egg yolk cholesterol solubilized by βCD-dextran tablet at 240 minutes of FaSSGF/FeSSIF cycle. The experiments were performed at room temperature (approx. 20C). Results are reported as average and standard deviation of two parallel sample analyses.

5 Discussion

5.1 Phase solubility study

Phase-solubility studies indicate that MβCD, is a stronger cholesterol complexation agent and solubilized approximately 23 times higher amount of cholesterol compared to hydrophilic cyclodextrins (HPβCD). Results are in agreement with available literature and can be explained with the fact that MβCD has more hydrophobic cavity than HPβCD and this improves the interaction between cholesterol and CDs [66]. Results correlate with the results obtained by Stelzl et al. [64]. indicating that implemented HPLC method worked correctly and ready for further analyses.

In this part of the study HPLC methods resulted suitable for concentration analysis of cholesterol in the presence of cyclodextrins. From those results we can conclude that βCD-dextran solubilizing properties should be compared to MβCD as it a much stronger binder to cholesterol in comparison to HPβCD.

5.2 Cholesterol quantitation in presence of FaSSGF/FeSSIF

Established and confirmed HPLC cholesterol quantitation method was implemented to quantitate cholesterol in FaSSGF/FeSSIF cycle samples. Analysis of cholesterol in the presence of biorelevant media (i.e. FaSSGF/FeSSIF) samples was unsuccessful due to appearance of a large peak on chromatogram, which overlapped cholesterol peak and made precise quantitation impossible. Taurocholate is a component of FeSSIF - a bile acid originated from cholesterol and having a similar chemical structure (see section 3.1.1, Fig. 4). Because of it similarity to cholesterol - it most likely produces this overlapping peak as these two chemicals should have comparable retention time. Possible and appropriate way to overcome this problem would be - optimizing mobile phase to increase the separation, for example so the taurocholates sulfonic acid group become deprotonated, thus providing earlier elution compared to cholesterol. In this work we decided to use cholesterol quantitation kit for further analyses to guarantee a quickly available and reliable quantitation method.

5.3 Compression of βCD-dextran in to a tablet

βCD-dextran polymer showed good compaction ability. Due to lack of time, no tablet testing studies have been performed to assess the quality of the tablets, but tablets have no visible defects, no visible friability is observed during handling. Further tablet is preferable to assess more detailed characteristics of βCD-dextran tablets.

Tablets show rapid disintegration in our experiments, after 30-40 minutes, no visible fragments of the tablet are seen. This is favorable, since the interaction between βCD-dextran and cholesterol will occur sooner.

5.4 Cholesterol complexation during FaSSGF/FeSSIF cycle in presence of MβCD and βCD-Dextran polymer tablet

This experiment shows that both MβCD and βCD-dextran polymer sequester cholesterol in simulation of gastric fluids. MβCD was administrated under the form of powder whereas, βCD-dextran was formulated and administrated under tablet form.
Interestingly, there is a big difference in cholesterol solubilization between FaSSGF condition and FeSSIF in all experiments. Specifically, it appears that in biorelevant gastric fluids there is very poor cholesterol sequestration, whereas, in the intestinal-mimicking fluids the sequestration of cholesterol become very significant for all cyclodextrins species. The reason of this interesting phenomena is probably related to the differences in compositions of those two media. In fact, FESSIF is reach in micellizing agents (e.g. bile salts) that could first solubilize the cholesterol and then transfer the cholesterol to the cyclodextrin (thermodynamic favorable process). To make a better comparison of ability to solubilize cholesterol of MβCD and βCD-dextran, results of βCD-dextran tablet are extrapolated to concentration of 10mM (Fig. 11). This extrapolation is made under the assumption that, increasing the concentration oc CD units in the polymer, this will linearly increase the amount of cholesterol solubilized [55]. Extrapolation were done by proportionally multiplying amount of solubilized cholesterol at a given concentration of CDs to a concentration of 10mM.

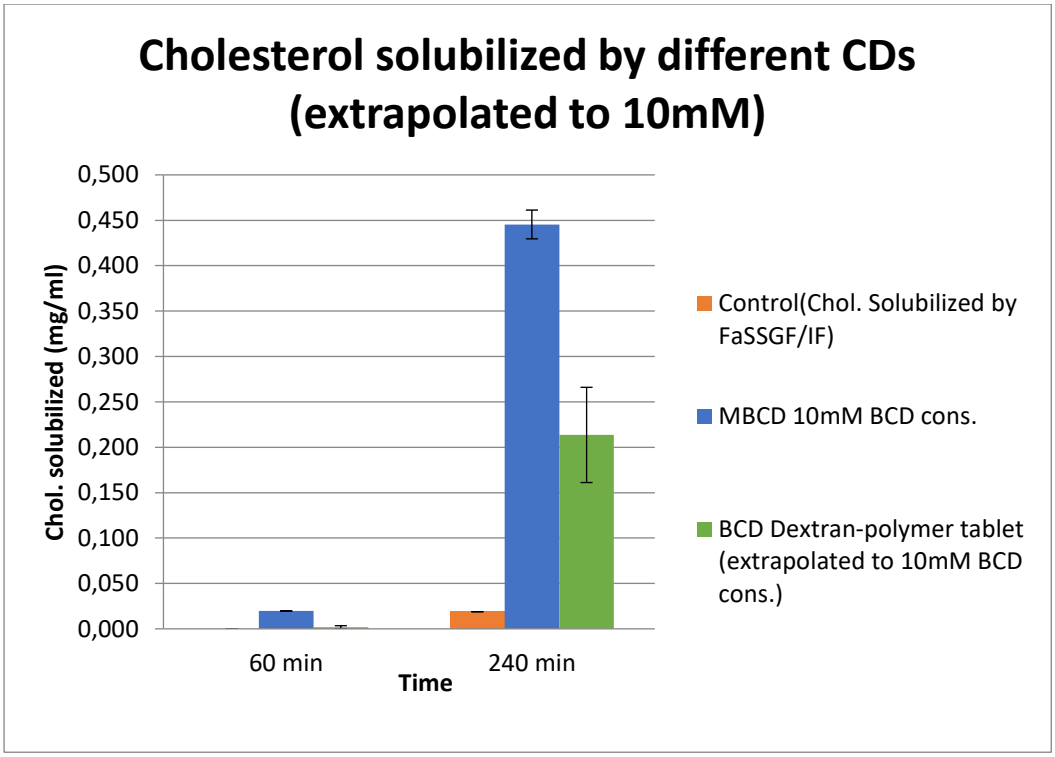

Figure 11: Extrapolated graph - Cholesterol solubilized by different cyclodextrin formulations at different time during FaSSGF/FeSSIF cycle.

Extrapolated results show that at βCD concentration of 10mM, βCD-dextran polymer tablet should complex approximately 48% of the cholesterol solubilized by MβCD. Results also show that both MβCD and βCD-dextran polymer solubilize significantly higher amounts of cholesterol than the bile salts presented in simulation of gastric fluids.

Some factors that possibly could be a part of explanation to the difference in the amount of solubilized cholesterol between MβCD and βCD-dextran polymer tablet:

1. MβCD has a more lipophilic cavity, resulting in more favorable interactions between the CD and cholesterol [66].
2. Since βCD molecules are bond to a dextran polymer backbone - some CD units might not be able to complex due to steric impediment given by the polymer backbone [55].
3. Disintegration of the βCD-dextran tablet takes approximately 30 minutes, resulting in reduced contact time between the CD and cholesterol.

To get physiological perspective, results are further extrapolated to physiological volume of fluids, where fed state gastric fluids has a volume of approx. 500 mL, and small intestine

fluids volume of approx. 1 L [59]. Results are reported in Fig. 12. Extrapolation was made by calculated by normalizing experimental volumes to real physiological volumes (see above).

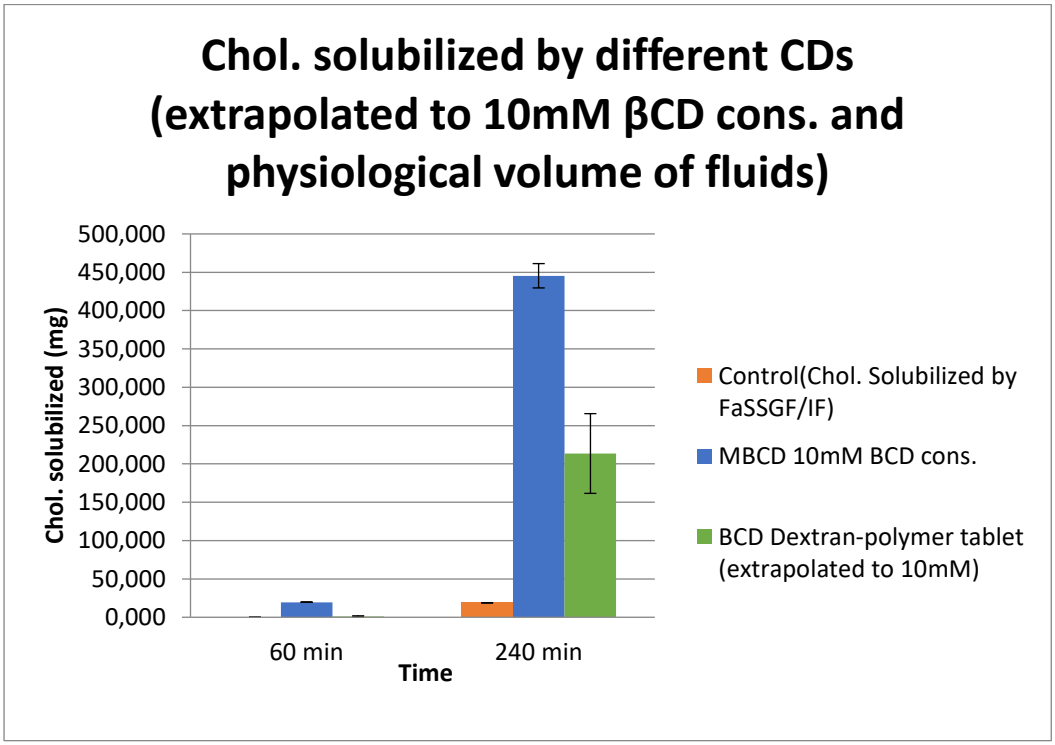

Figure 12: Extrapolated graph: Cholesterol solubilized by different CDs, extrapolated to 10mM βCD cons. and physiological volume of gastric fluids.

Extrapolated results to physiological volumes of fluids, shows that 10mM MβCD should be able to extract approx. 445 mg of cholesterol in intestinal fluids and βCD dextran polymer should be capable of sequestrating up to 214 mg cholesterol. For what concern the MβCD this should be capable of sequestering almost all the cholesterol administered on a normal dietary regime per day (from 300 to -450 mg) [31], whereas approx. 20 g of βCD-dextran should be capable of extracting 57% of dietary cholesterol, which is a significant amount. It should be underline that, unlikely MβCD that can be absorbed by GI tract, βCD-dextran is too big and hydrophilic therefore all the eventual cholesterol sequestered is expected to be excreted by feces. These results highlight the feasibility of an eventual therapy with βCD-dextran polymer needed to achieve concentration of 10mM in 1L is approx. 20g. This weight is within the range of normal daily dose of Questran – a bile sequestrant, established treatment for several conditions including hypercholesterolemia.

5.4 Complexation of cholesterol from egg yolk in biorelevant media

In experiment with egg yolk and βCD-dextran polymer tablet, βCD-dextran has shown ability to sequester significant amount of cholesterol, while no cholesterol was solubilized by FeSSGF/IF. Amount of cholesterol, sequestered form egg yolk is significant compared to experiments with crystalline cholesterol.

Extrapolating the results to βCD-concentration of 10mM and physiological volume of fluids, results in 133 mg cholesterol complexed out of approximately 40g boiled egg yolk containing approx. 43 mg of cholesterol [67]. The results from this experiment shows that βCD-dextran polymer tablets can complexate significant amounts of cholesterol from food during digestion.

5.6 Limitations

5.6.1 Cholesterol oxidation

Prolonged storage eventually results in cholesterol oxidation. Cholesterol used in this experiment was close to expiration date and in addition to that, collected samples were stored up to 14 days at room temperature and protected from light, prior to analysis. Both of those factors could contribute to formation of cholesterol oxidized products.

Cholesterol quantitation kit works by the mechanism of cholesterol being oxidized by cholesterol oxidase to yield H_2O_2 which reacts with a sensitive cholesterol probe to produce color and fluorescence. Therefore, oxidized cholesterol, as also stated by Sigma Aldrich customer support, will not be detected by cholesterol quantitation kit and lead to underestimation of cholesterol quantity in a sample. Therefore, some underestimation in amounts of cholesterol complexation by CDs could have happened.

6 Conclusion

βCD-dextran polymer shows good compaction ability and were successfully compressed into tablets with no visible defects and no visible friability during handling. In simulation of gastric fluids, βCD-dextran polymer, formulated as a tablet can complex approximately 28% of cholesterol in comparison to cholesterol complexed to strong cholesterol binders, such as MβCD.

The polymer also showed it effectiveness in sequestering significant amounts of food cholesterol.

This study shows positive results and adds further evidence for potential of βCD-dextran polymers in treatment of hypercholesterolemia.

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8 Appendix

Attachment 1 page 1/2:

SIGMA-ALDRICH®

sigma-aldrich.com

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Product Information

Cholesterol Quantitation Kit

Catalog Number **MAK043**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Cholesterol is an important component of mammalian cell membranes where it functions in intracellular transport, cell signaling, and maintaining membrane fluidity. Within the blood, cholesterol circulates as both the free acid and as cholesterol esters. Controlling serum cholesterol has an important therapeutic role as elevated cholesterol levels are associated with the development of atherosclerosis and cardiovascular pathologies. Recent evidence suggests a disturbance of cholesterol homeostasis contributes to the development of a chronic inflammatory state.

The Cholesterol Quantitation Kit can also be used to determine the concentration of free cholesterol, cholesteryl esters, or both (total) present in a sample. In this kit, total cholesterol concentration is determined by a coupled enzyme assay, which results in a colorimetric (570 nm)/fluorometric (λ_{ex} = 535/λ_{em} = 587 nm) product, proportional to the cholesterol present. This kit is suitable for use with cell and tissue culture samples, urine, plasma, serum, and other biological samples.

Components
The kit is sufficient for 100 assays in 96 well plates.

Cholesterol Assay Buffer Catalog Number MAK043A	25 mL
Cholesterol Probe in DMSO Catalog Number MAK043B	0.2 mL
Enzyme Mix Catalog Number MAK043C	1 vl
Cholesterol Esterase Catalog Number MAK043D	1 vl
Cholesterol Standard, 2 µg/µL Catalog Number MAK043E	0.1 mL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Fluorescence or spectrophotometric multiwell plate reader
- Chloroform (Catalog Number C2432 or equivalent), Isopropanol (Catalog Number 278475 or equivalent), and NP-40/IGEPAL® CA-630 (Catalog Number I8896 or equivalent)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Cholesterol Assay Buffer – Allow buffer to come to room temperature before use.

Cholesterol Probe – Warm to room temperature to thaw the solution prior to use. Store protected from light and moisture at –20 °C. Upon thawing, the Cholesterol Probe is ready-to-use in the colorimetric assay.

For the fluorescence assay, dilute an aliquot of the colorimetric Cholesterol Probe solution 5 to 10-fold with Cholesterol Assay Buffer, just prior to use. This will reduce the background of the fluorescence assay.

Cholesterol Esterase and Enzyme Mix – Reconstitute each in 220 µL of Cholesterol Assay Buffer. Mix well by pipetting, then aliquot and store at –20 °C. Keep cold while in use and protect from light. Use within two months of reconstitution.

Attachment 1 page 2/3:

Storage/Stability
The kit is shipped on wet ice. Storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

Cholesterol Standards for Colorimetric Detection
Dilute 20 µL of the 2 µg/µL Cholesterol Standard solution with 140 µL of the Cholesterol Assay Buffer to prepare a 0.25 µg/µL standard solution. Add 0, 4, 8, 12, 16, and 20 µL of the 0.25 µg/µL Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 µg/well standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50 µL.

Cholesterol Standards for Fluorometric Detection
Dilute 10 µL of the 2 µg/µL Cholesterol Standard Solution with 790 µL of the Cholesterol Assay Buffer to prepare a 25 ng/µL standard solution. Add 0, 4, 8, 12, 16, and 20 µL of the 25 ng/µL Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 0.1, 0.2, 0.3, 0.4, and 0.5 µg/well standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation
Both the colorimetric and fluorometric assays require 50 µL of sample for each reaction (well).

Tissue (10 mg) or cells (1×10^6) can be extracted with 200 µL of chloroform:isoporpanol:IGEPAL CA-630 (7:11:0.1) in a microhomogenizer. Centrifuge the samples at 13,000 × *g* for 10 minutes to remove insoluble material. Transfer the organic phase to a new tube and air dry at 50 °C to remove chloroform. Discard the pellet. Put the samples under vacuum for 30 minutes to remove any residue organic solvent. Dissolve dried lipids with 200 µL of the Cholesterol Assay Buffer, and sonicate or vortex until mixture is homogenous. The lipid solution may be cloudy.

Serum samples (0.5–5 µL/well) can be added directly to well.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Bring samples to a final volume of 50 µL with Cholesterol Assay Buffer.

Assay Reaction
1. Set up the Reaction Mixes according to the scheme in Table 1. 50 µL of the appropriate Reaction Mix is required for each reaction (well).

Table 1.
Reaction Mixes

Reagent	Total Cholesterol and Standards	Free Cholesterol
Cholesterol Assay Buffer	44 µL	46 µL
Cholesterol Probe	2 µL	2 µL
Cholesterol Enzyme Mix	2 µL	2 µL
Cholesterol Esterase	2 µL	–

Note: Cholesterol Esterase hydrolyzes cholesteryl esters to cholesterol. In the presence of Cholesterol Esterase, the assay detects total cholesterol, both free cholesterol and cholesteryl esters. To detect free cholesterol only, omit the Cholesterol Esterase from the reaction and add 46 µL of the Cholesterol Assay Buffer to the Reaction Mix. To determine cholestryl esters, subtract the free cholesterol value from the total cholesterol value.

The cholesterol standard contains a mixture of free cholesterol and cholesteryl esters. The Reaction Mix containing Cholesterol Esterase must be used in the reactions for the Cholesterol Standards to convert all of each standard to cholesterol.

2. Add 50 µL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 60 minutes at 37 °C. Protect the plate from light during the incubation.

3. For colorimetric assays, measure the absorbance at 570 nm (A_{570}). For fluorometric assays, measure fluorescence intensity (λ_{ex} = 535/λ_{em} = 587 nm).

Attachment 2 page 3/3: