Sodium Caseinate- stabilized Emulsion: A Carrier Matrix to Improve the Delivery of Epigallocatechin-gallate

By
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ABSTRACT

SODIUM CASEINATE- STABILIZED EMULSION: A CARRIER MATRIX TO IMPROVE THE DELIVERY OF EPIGALLOCATECHIN-GALLATE

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Oral bioavailability of tea polyphenols may improve using food matrices as their carrier. Tea polyphenols have a high affinity to bind to proline rich proteins such as caseins. This thesis focuses on the interactions between epigallocatechin-gallate (EGCG) and sodium caseinate at an oil-water interface, the stability and colloidal behavior of the designed emulsion at neutral and low pH, as well as the emulsion digestibility, and efficiency as EGCG carrier.

After addition of EGCG to a model emulsion the stability, size, and charge of the emulsion particles were not affected, and the sodium caseinate oil-water interface showed a high loading capacity for EGCG up to 1 mg/m². Moreover an increased interfacial tension in the presence of EGCG was demonstrated which confirmed the EGCG-sodium caseinate interactions at the interface.

Under acidification, the presence of EGCG at the interface lowered the pH at which emulsion destabilized, and resulted in less droplet coalescence at pH 4.5. While under acidification, sodium caseinate emulsion was stabilized by the addition of 0.3 and 0.45% high methoxyl pectin (HMP), presence of EGCG reduced the electroadsorption of HMP to the interface, affecting emulsion’s colloidal behavior and microstructure.
Designed emulsions were subjected to a static *in vitro* digestion, simulating the three stages of human upper gastrointestinal. Results indicated that sodium caseinate was fully hydrolyzed in the presence of EGCG, while less fatty acids were released.

Futhuremore higher amount of intact EGGC in the digested emulsions containing EGCG was detected compared to equivalent EGGC solutions which suggested a preserved bioactivity for the carried EGCG in the emulsion system. This was also confirmed by digested emulsions containing EGCG showing higher anti proliferative activity than digested EGCG solutions. Intestinal absorption of EGCG after digestion was also studied using an *in vitro* transport experiment. Although it was quite challenging to measure the EGCG recovery after an *in vitro* absorption analysis, the transported fractions showed anti-proliferative activity which was an indicative of presence of EGCG or its bioactive metabolites.

The findings from this thesis propose the application of an emulsion/dairy-based designed structure as a novel vehicle for improving the bioaccessibility of EGCG.
This thesis is dedicated to my much-loved mother and the loving memory of my father
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CHAPTER 1: GENERAL INTRODUCTION

The recognition that certain foods exert health benefits beyond their nutritional value has increased consumer’s interest for functional foods, nutraceuticals and food bioactives, generally. Numerous food components with physiological functionality such as anticarcinogenicity, antimitagenicity, antioxidative and antiaging activities have been characterized. Examples of foods that have been rediscovered because of their positive health effects are fruits and vegetables rich in biologically active phytochemicals or traditionally fermented products containing bioactive molecules or probiotic bacteria.

Plants are a rich source of bioactive components. One of the main groups of phytochemicals which have received significant attention as health-promoting nutrients are polyphenols. While tea is the most consumed beverage after water in the world, consumption of tea polyphenols was linked to several beneficial health effects including prevention of cancer and cardiovascular diseases, neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease), obesity and diabetes (Hollman et al. 1999; Yang et al. 2004; Scalbert et al. 2005; Iso et al. 2006).

Bioactives will be effective only if they maintain their activity in the gut and reach the sites of intended action; to address bioavailability challenges, delivery systems can be designed. Tea polyphenols reportedly have a poor oral bioavailability (Dube et al. 2010a). It is also important to note that in recent years, there has been a growing interest in understanding how the structure and matrix of the food can affect the bioavailability and delivery of bioactive molecules, as the formation of complexes in food may be the cause of changes in the digestibility and availability of important nutrients (McClements et al. 2008).
Complex formation between tea polyphenols and proteins has been suggested as one of the solutions to improve the stability of tea polyphenols, potentially leading to an enhanced bioavailability (Sahu et al. 2008; Shpigelman et al. 2012). Tea polyphenols have been reported to have a high affinity to associate with proline rich proteins such as caseins through hydrophobic interactions and hydrogen bridging (Pascal et al. 2008; Ozdal et al. 2013). Furthermore, the binding of catechins to caseins was suggested to potentially reduce catechin’s sensation of astringency (Lesschaeve and Noble 2005; Hofmann et al. 2006).

The strong interactions between tea polyphenols and caseins (both in monomeric and micellar forms) have been previously shown (Jöbstl et al. 2006; Yuksel et al. 2010; Hasni et al. 2011; Haratifar and Corredig 2014). However, there is no information available on the association of tea polyphenols with caseins once adsorbed at the oil water interface. This is very important from technological and nutritional point of view since emulsions have a broad range of applications in food science while they are a common matrix for delivery of bioactives, and structural design of emulsion-based delivery systems continues to gain attention.

Sodium caseinate, a protein derived from micellar casein, has efficient emulsification properties and therefore has been used widely as a functional ingredient in food emulsions. For many years, a diverse range of food and pharmaceutical products have been created using emulsion science and technology.

Tea catechins have been shown to have antioxidant activities including scavenging of reactive oxygen and nitrogen species, free metal chelation, and inhibitory effect on oxidative enzymes (Green et al. 2007; Almajano et al. 2008; Dubeau et al. 2010). The association of tea polyphenols with protein at emulsion interfaces may not only be beneficial in protecting the bioactive molecules, but also in protecting the oil phase from oxidation. The first objective of this study
(Chapter 2) was to investigate the binding behaviour of epigallocatechin gallate (EGCG), the major and most bioactive tea polyphenol, and sodium caseinate at the oil-water interface and the influence of the presence of EGCG on emulsion’s stability and viscoelastic properties. Most emulsion/dairy-based foods are produced, stored and consumed in an acidic pH range. Therefore, it is important to study the stability, colloidal behaviour and processing properties of the designed emulsion system containing EGCG both at neutral and low pH. Sodium caseinate-stabilized emulsions are destabilized close to protein’s pI as a result of the reduction in electrostatic repulsion between the droplets (Agboola and Dalgleish 1996; Dickinson et al. 1998b). It is possible to limit the destabilization of sodium caseinate-coated oil droplets during acidification by the addition of high methoxyl pectins (Dalgleish and Hollocou 1997; Dickinson et al. 1998b; Bonnet et al. 2005; Liu et al. 2007). At pH values slightly above the isoelectric point of sodium caseinate, pectin molecules interact with caseins through electrostatic interactions between negatively charged pectin molecules and positively charged patches of caseins adsorbed at the interface (Dalgleish and Hollocou 1997; Dickinson et al. 1998b; Bonnet et al. 2005; Surh et al. 2006; Liu et al. 2007). The second objective of this work (Chapter 3) was to determine the effect of the presence of EGCG on sodium caseinate-stabilized emulsions during acidification in the presence and absence of high methoxyl pectin (HMP).

To evaluate the efficiency of the carriers designed to enhance the delivery of bioactive to the target tissue, the behaviour of the food matrix and the extent of resistance of the bioactive molecules to digestion needs to be evaluated after ingestion of food. In vitro digestion models –reproducing, as close as possible, the physiological and physicochemical conditions present in the gastrointestinal tract (GIT)- are tools for rapid screening of delivery systems, and to better understand the mechanisms taking place during food breakdown, bioaccessibility of bioactives and their uptake.
In Chapter 4, the engineered emulsion systems were subjected to an *in vitro* digestion model including oral, gastric and duodenal phases. The effect of entrapment of EGCG at the emulsions’ interfaces on the physical properties of the emulsions, casein proteolysis, and lipolysis of oil phase and bioaccessibility of EGCG were determined. The effect of the presence of pectin in emulsions prepared at both neutral and low pH was also studied and summarized in Chapter 4.

Bioaccessibility determines only the fraction of the bioactive available for absorption to the intestinal cells after digestion process. To predict bioavailability which indicates the fraction that reaches the target site, the absorption of bioactive to the intestinal cells needs to be investigated. By employing enterocyte-like cell cultures, intestinal uptake can be mimicked *in vitro*. In fact, simulating gastrointestinal digestion followed by digest submission to cultured carcinoma cells as a surrogate for intestinal absorptive cells have been reported as the most effective and peer-accepted models to simulate *in vivo* absorption processes (Pinto et al. 1983; Glahn et al. 1996; Yun et al. 2004). In Chapter 5, the objective was to study the absorptive behavior of EGCG from sodium caseinate-stabilized emulsions. For this purpose, transport experiment was conducted through Caco-2 cells, a human colon epithelial cancer cell line. Moreover, anti-proliferative activity of EGCG from both digested and non-digested emulsions and their controls (water solutions containing EGCG) was determined using a cytotoxicity test on Caco-2 cells.

This thesis will provide insight on the association of EGCG to sodium caseinate at an emulsion interface. In addition, the work also evaluated what is the effect of emulsion structure and that of the possible changes in physical properties on digestion behaviour and bioaccessibility of the model bioactive. In particular, this thesis will investigate the possibility of addressing tea polyphenols’ bioavailability challenges through a novel dairy/emulsion-based delivery vehicle.
2.1 Functional Foods

During the last two decades, there has been growing evidence that some food components are linked to health sustaining properties and preventative and/or therapeutic effects on heart disease (Hu and Willett 2002), cancer (Willis and Wians 2003), and degenerative diseases such as diabetes (Silvis 1992), Parkinson and Alzheimer (Mandel et al. 2004). This recognition, coupled with growing lifestyle-driven diseases, increasing health care costs and consumers rising interest to self-medication, shifted the focus of food research and development from considering food only as a source of energy and essential nutrients to employing it as a tool for preventing chronic illnesses and optimizing physical and mental health and well-being. The result of this evolution has been the emergence of specific categories of food products containing physiologically active biomolecules. Examples of such bioactives are vitamins, omega 3 fatty acids, phytochemicals and bioactive peptides (Kaiser and Allen 2002). Despite modern pharmaceuticals which aim for acute relief of symptoms and diseases, diet-based approaches are recognized as imparting the most beneficial physiological effects in long-term. Also in comparison to drugs, natural bioactive molecules can be delivered through foods in a more natural, long term, less toxic fashion, to prevent diseases, and not treat them (Ting et al. 2014).

2.2 Bioaccessibility and Bioavailability

It is important that bioactive compounds maintain their activity throughout their path to reach the sites of intended action. In other words, the effectiveness of functional foods in providing
physiological benefits greatly depends on the availability of key active ingredients at the target site, after digestion. The rate and the extent to which the therapeutic moiety is absorbed and becomes available to the site of action were defined as its bioavailability by Food and Drug Administration (FDA). The overall bioavailability could be a sum of three fractions of the bioactive compound (Figure 2.1); the portion of bioactive molecules present in an accessible form for cell absorption, the fraction of accessible compound which can pass across the small intestinal epithelium, and the portion of the absorbed component that ultimately reaches the systemic circulation not metabolized (McClements and Li 2010a; McClements and Li 2010b).

A biologically relevant concentration of bioactive is required in target tissue to accomplish the meaningful health effects. The challenges faced by a bioactive are physiological and biochemical barriers along the gastrointestinal tract (GIT), absorption into the enterocytes, systematic metabolisms or biotransformation, and absorption into vascular circulation (Ting et al. 2014).

Upon oral administration, the properties of bioactive could change as it passes through the changing physicochemical conditions of GIT with various enzymes, complex mechanistic motilities and change in pH, ionic strength, and compositions of surface-actives and biopolymers. For example, while health benefits of bioactive peptides depend mostly on their characteristic amino acid sequence, they may lose their bioactivity under gastric acid and protease hydrolysis (Banga and Chien 1988; Bernkop-Schnürch 2000; Korhonen and Pihlanto 2006).

Another example is the vulnerability of tea polyphenols to oxidation and polymerization under alkaline condition in the intestine. The oxidation may decrease the bioactivity (Zhu et al. 1997; Su et al. 2003).
Figure 2.1 Bioaccessible and bioavailable fractions of ingested components. (from Guerra et al., 2012)
After being exposed to digestive conditions, the compound must be solubilized or dispersed in aqueous intestinal fluid, to be accessible for absorption by the enterocytes. Bioavailability of lipophilic compounds is reduced by their poor solubility in the aqueous environment of GIT. Although soluble, the transport coefficient of hydrophilic molecules across the phospholipid bilayer of intestinal wall may be low. Since the intestinal uptake of many bioactives is often incomplete, a large percentage of them will be excreted from the body or metabolized by the gut microbiota. The bioavailability of microbial metabolites is even less well understood than that of the original compounds (Ting et al. 2014). Furthermore, the bioactive portion uptaken by intestinal epithelial cells will be subjected to post-absorption metabolism in the enterocytes, altering the chemical structure and functionality of the molecules, and significantly reducing the concentration of intact bioactives reaching the systemic circulation and target sites. In conjunction to intestinal metabolisms, bioactive’s original chemical structure can be also transformed through hepatic metabolism before reaching the target tissue (Wilkinson 2005).

Although human intervention studies are the foundation to understand the link between bioactive molecules and disease treatment or prevention, the complexity of the various stages of digestion and uptake makes it necessary to better understand the critical paths leading to the biological function, to optimize the design of food matrices and ultimately the bioaccessibility and bioactivity of the biologically functional molecules.

2.3 Food Delivery Systems

While digestion and metabolism of food are important mechanisms for the body to exploit the nutrient and remove any potential toxin, they may diminish the bioavailability of functional food and interfere with their therapeutic efficacy. Adapting the pharmacological approach, efforts have
been made in recent years to develop food-based delivery systems that will enhance the bioavailability of the bioactive molecules, through protecting them during processing, storage, gastrointestinal digestion and systemic metabolism or controlling and targeting their release in the sites of action (Benshitrit et al. 2012).

Different types of classification were proposed based on the delivery system production method, size, stability (kinetically, thermodynamically, physically or chemically), intended site of delivery (mouth, stomach, small intestine or colon), the matrix’s components (protein-based, carbohydrate-based, lipid-based, or mixed systems) (Benshitrit et al. 2012). Figure 2.2 illustrates some of the most commonly used food based matrices.

Food carriers capable of controlling the digestive degradation of bioactives have been proposed (Benshitrit et al. 2012). For instance, liposomal encapsulation has been shown to be protective against gastrointestinal enzymatic degradation, thereby increasing the bioactive’s absorption levels in plasma and brain of rats (Huang et al. 2011). An *in vivo* study on loading α-tocopherol in calcium-pectinate microcapsules showed sustained release pattern and enhanced bioavailability of entrapped tocopherol (Song et al. 2009). Encapsulation of vitamin D₃ in β-lactoglobulin-based coagulum with a high encapsulation efficiency showed improved bioavailability for vitamin D3 in rats. Encapsulated vitamin was protected against intestinal degradation leading to a slower release, longer residence time in the intestine and in turn higher uptake (Diarrassouba et al. 2015).

The application of positively-charged chitosan in a nanocomplex delivery system affected the intestinal membrane integrity by opening the tight junctions of cell monolayers leading to elevated absorption of the encapsulated bioactive into systemic circulation via the paracellular pathway (Hu et al. 2012a; Hu et al. 2012b). Moreover, an enhanced plasma delivery of curcumin was reported
Figure 2.2  An outline of some food-grade delivery systems (from (Benshitrit et al. 2012)).
in rats through a curcumin-phospholipids complex attributed to better absorption of curcumin when combined with phospholipids (Maiti et al. 2007). In summary, the available evidence continues to indicate that food-based delivery systems are effective tools in enhancing oral efficacies of biologically active molecules.

2.4 Plant Foods Bioactives

Diets rich in plant foods have been shown by epidemiological studies to contribute significantly in maintaining human health and protecting against chronic/degenerative diseases. Phytochemicals are one of the food components to which health improving properties of plant food have been attributed (Manach et al. 2005). Figure 2.3 shows different categories of phytochemicals. Carotenoids, polyphenols, alkaloids, nitrogen-containing compounds, and organosulfur compounds are amongst the most important (Liu 2004). With 8000 different phenolic structures, polyphenols are one of the most numerous and ubiquitously distributed groups of phytochemicals and an integral part of both human and animal diets. They are the most investigated group of compounds in nutritional research, due to their proven relationship to health properties. They are secondary metabolites of plants playing essential part in the plant growth and reproduction; they also function in plant defense mechanisms and impart color (Liu 2004; Bravo 2009).

Polyphenol molecules contain one or more aromatic rings, with one or more hydroxyl groups. They are generally divided into phenolics (simple molecules), flavonoids, stilbenes, coumarins, and tannins (highly polymerized compounds) classes (Figure 2.3) (Liu 2004).

Among the above classes, flavonoids are the most ingested and the most widely distributed, with over 4000 unique structures found in different plant sources such as fruits, vegetables, tea, nuts,
Figure 2.3 Classification of dietary phytochemicals (from (Liu 2004)).
seeds, herbs and spices. These low molecular weight substances share a chemical skeleton of diphenylpropane (C6-C3-C6) consist of two aromatic/benzene rings (A and B) linked through three carbons usually in an oxygenated heterocycle form (ring C) (Middleton et al. 2000; Bravo 2009) (Figure 2.4). Flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins and isoflavones are subclasses of flavonoids (Figure 2.3). The most common group of flavonoids in the diet are flavan-3-ols (catechins) abundantly found in tea, grapes, berries, apples, and chocolate (Aron and Kennedy 2008).

### 2.4.1 Tea Polyphenols

Tea is one of the most significant and concentrated dietary sources of flavonoids in Americans’ diet (Chun et al. 2007; Song and Chun 2008). Fresh tea leaf is extraordinarily rich in the flavan-3-ols (catechins) with smaller quantities of caffeine, theanine, theobromine, theophylline and phenolic acids, all comprising up to 30% of the leaf weight on a dry basis (Balentine et al. 1997; Johnson et al. 2012). Tea catechins are water soluble, astringent, and colorless. They act as free radical scavengers and have been employed as food antioxidants (Henning et al. 2003). As of 2006, more than 8000 citations about the chemistry, bioactivity, production, and potential health benefits of tea has been reported (Nagle et al. 2006).

The tea plant, *Camellia sinensis*, is cultivated in about 30 countries and is the most widely consumed beverage around the world after water. From fresh tea leaves, three common types of tea are obtained through different manufacturing processes and extent of fermentation. Green tea (unfermented) is prepared by steaming and drying tea leaves to prevent the oxidation of polyphenols. To produce black tea (fully fermented), the tea leaves are crushed to promote
Figure 2.4 Chemical structure of flavonoids (from (Bravo 2009)).
compounds which impart black tea the characteristic taste and color. Consequently, green tea usually contains higher concentrations of catechins than black tea. Oolong tea is partially fermented and intermediate in catechin concentrations between green and black teas (Graham 1992; Balentine et al. 1997; Song and Chun 2008). Green tea’s numerous health effects made products based on them the fourth most commonly consumed dietary supplements in the United States (Sarma et al. 2008).

Figure 2.5 represents the chemical structure of main tea catechins namely (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), and (-)-epigallocatechin gallate (EGCG). EGCG and ECG contain a gallic acid moiety at position 3 on the C ring. EGCG is considered the most abundant tea catechins, constituting more than half of the tea catechins’ mass (Singh and Sarkar 2011; Braicu et al. 2013).

ECGG showed the highest intrinsic antioxidant capacity among other tea catechins (Kilmartin and Hsu 2003) and was known as the most bioactive and the main contributor to green tea health-promoting effects (Khan and Mukhtar 2007; Velayutham et al. 2008). Traditionally, green tea has been consumed to improve blood and urine flow, eliminate toxins and clear urine, relieve joint pain, and improve disease resistance (Balentine et al. 1997).

An expanding body of scientific research has found a link between consumption of tea catechins and several health-promoting effects such as prevention of cancer, obesity, diabetes, cardiovascular and neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease), in addition to antithrombogenic, anti-inflammatory, antihistaminic, anti-arthritis, antioxidant, antibacterial and lipid-lowering properties (Hollman et al. 1999; Yang et al. 2004; Scalbert et al. 2005; Iso et al. 2006; Khan and Mukhtar 2007).
Figure 2.5 Structure of major green tea catechins (from Green et al. 2007)
2.4.2 Bioactivity of Tea Polyphenols

The occurrence of aging and degenerative diseases such as cancer, Alzheimer’s and cardiovascular diseases has been mainly attributed to the oxidative damage to DNA, lipid membrane and proteins caused by the production of free radicals by living systems (Harman 1992; Bagchi et al. 2003; Aron and Kennedy 2008).

Electronic configuration of catechins allows electron delocalization, thereby conferring to them a high reactivity to quench free radicals. They easily donate electrons to reactive oxygen, nitrogen and free radical species (R’) such as superoxide radical, hydroxyl and peroxyl radical, nitric oxide, and peroxynitrite (Aron and Kennedy 2008) (Figure 2.6). The derived radicals are more stable and less harmful than the initial radical species; therefore, catechins are considered ideal antioxidants. Ascorbic acid has been shown to be protected through preferable oxidation of catechins (Kitao et al. 2006; Khan and Mukhtar 2007). Their antioxidant activity depends on the presence of galloyl groups, also the number and position of hydroxyl groups. Glycosylation and methoxylation at carbon 3 may inhibit their activity (Nanjo et al. 1996; Rice-Evans et al. 1996). In addition, catechins’ ability to chelate transition metals enable them to reduce the oxidative stress imposed by the free iron and copper which are generally present in biological systems and catalyze free radical reactions. Enzyme mediation and inhibition has been also shown to be a specific effect of catechins, and key to many of their biological functions (Beecher 2004; Aron and Kennedy 2008).

It has been reported that tea catechins exert protective effects against coronary heart disease through inhibitory effects on the oxidation of low-density lipoprotein (LDL), lowering plasma LDL and cholesterol levels, and inhibition of platelet adhesion and aggregation (Miyazawa 2000; Yang and Landau 2000; Aron and Kennedy 2008). Also, tea catechins’ inhibitory effect on
Figure 2.6 Phenolic oxidation and radical formation (form (Aron & Kennedy 2008)).
enzymes such as phospholipases, cyclooxygenase, and lipoxygenases is suggested to contribute to their anti-inflammatory effects (Miyazawa 2000).

The broad cancer-preventive effects of tea catechins reported in a variety of cell lines and in different organs of animal models has been attributed to multiple molecular mechanisms. Their inhibitory activity on cancer cells during both the initiation and progression stages of carcinogenesis was suggested to be through decreasing cell proliferation or promoting cell growth arrest and inducing apoptosis. Moreover, EGCG was reported to modulate tumor response to chemotherapy and act as an antitumor agent (Vaidyanathan and Walle 2001; Chen et al. 2004; Chen and Zhang 2007; Singh et al. 2011). The cancer preventive effects of tea catechins seems to be a result of both direct involvement of EGCG, such as binding to molecular targets, and indirect involvement, such as free radicals scavenging or enzyme inhibition of certain cell pathways (Yang et al. 1998; Yang et al. 2006; Yang et al. 2009; Visioli et al. 2011). For example, in cases such as colon cancer development, which involves membrane phospholipid peroxidation, the antioxidant and lipoxygenase inhibitory effect of tea catechins could be key to their preventative role (Nakagawa and Miyazawa 1997). However, there is still much unknown about the mechanisms related to the anticarcinogenic properties of tea catechins.

### 2.4.3 Bioavailability of Tea Polyphenols

Suggested roles of tea catechins in prevention of chronic diseases and promotion of health and well-being have drawn much attention to issues of their bioavailability. A number of confounding factors including individual variation in metabolism and gut microflora, age, exercise and smoking habits, diet and alcohol consumption may significantly affect tea catechin’s uptake and bioavailability (Johnson et al. 2012). Furthermore, catechins undergo a complex catabolism in the
human body and analytical limitations such as lack of pure standards makes the identification and quantification of their metabolites difficult. Therefore there have been inconsistent reports in the literature regarding the bioavailability of tea polyphenols (Del Rio et al. 2010).

Human studies found 0.1-1% of the consumed amount of EGCG in the systemic circulation (Chow et al. 2001; Chow et al. 2003). A study in rats reported 0.0003–0.45% of the ingested EGCG dose in the tissues and blood (Nakagawa and Miyazawa 1997). Other studies indicated 5% of orally administered dose of EGCG to be found in the systemic circulation of rats (Chen et al. 1997; Dube et al. 2010b). Urinary excretion from small traces to around 10% of the ingested green tea and its metabolites was also reported (Chow et al. 2005; Stalmach et al. 2009). The data on the incorporation of tea catechins in target tissues, which is a direct indicator of bioavailability, is limited. In a green and black tea intervention study after 1 week of daily consumption of 5 cups of tea both gallated and nongallated catechins was found in the human prostate with EGC showing the highest concentration (Henning et al. 2006). In another study, relatively high levels of EGCG were detected in the mucosa of small intestine which suggests the potential of EGCG in preventing intestinal carcinogenesis (Nakagawa and Miyazawa 1997).

Tea catechins have been generally regarded as poorly bioavailable after oral administration. While an EGGC concentration of at least 10 μM has been estimated to be required for therapeutic effects (Scalbert and Williamson 2000; Yang et al. 2008), plasma concentrations in the nM range were obtained following oral administration of EGCG to rodents (Chen et al. 1997; Dube et al. 2011a). Tea catechin’s low bioavailability has mainly been attributed to their instability to digestive conditions (Zhu et al. 1997; Record and Lane 2001; Green et al. 2007), poor permeability across the intestine resulting in poor absorption, their efflux back to the intestinal lumen after absorption.
(Vaidyanathan and Walle 2001; Cai et al. 2002; Dube et al. 2011b), extensive rapid metabolism and high systemic clearance (Cai et al. 2002; Fang et al. 2006).

Catechins degrade easily under oxidative conditions and are unstable in neutral and alkaline solutions (Zimeri and Tong 1999; Mochizuki et al. 2002). The duodenal pH range of 6-8, along with the presence of reactive oxygen species from normal digestive function favor degradation reactions such as auto-oxidation and epimerization (Green et al. 2007). EGCG and EGC were reported as the most sensitive to in vitro digestive conditions among tea catechins (Green et al. 2007). A study suggested around 80% degradation of EGCG in simulated intestinal fluid with pH of 7.4 at 37 °C within 1 h (Dube et al. 2010b). Another study suggested that at pH 7, EGCG and EGC degrade rapidly with only 50% remaining after 2–3 h. EC and ECG were stable for 8 h and decrease by 10 and 25%, respectively, after 17 h (Henning et al. 2008).

EGCG shows an apparent permeability co-efficient (P_{app}) for Caco-2 cells of 0.83 \times 10^{-7} \text{ cm/s}. This value is quite low, as a P_{app} of a poorly-permeable marker, mannitol, is 6.8 \times 10^{-6} \text{ cm/s} (Polentarutti et al. 1999; Stephens et al. 2002; Zhang et al. 2004). The diffusion across the lipid-bilayer membrane of intestinal epithelial cells is a challenging, critical step for absorption for many bioactives.

Most polyphenols, being too hydrophilic, hardly transmit through the gut wall by passive diffusion (Manach et al. 2004; Hu et al. 2012b). The “Rule of five” described by Lipinski states that a compound containing 5 or more hydrogen-bond donors is likely to be poorly bioavailable. Interactions of hydrogen-bond donors with water molecules probably form a large hydration shell increasing the apparent size of the molecules and challenging their transport through the lipid bilayer (Lambert and Yang 2003; Lipinski et al. 2012). This mechanism can explain the reported low bioavailability of gallated catechins (EGCG and ECG) compared to the non-gallated (EGC
and EC) catechins, the later being characterized by a smaller molecular weight and fewer hydrogen-bonds (Lambert and Yang 2003; Peters et al. 2010).

The small percentage of catechins absorbed from the small intestine undergoes extensive biotransformations to conjugated sulfate, glucuronide, or methylate metabolites in the intestinal epithelial cell (Lambert et al. 2007; Henning et al. 2008). While most of nongallated catechins (EC, EGC) are found conjugated in plasma and urine, gallated catechins such as EGCG and ECG are mainly found in their free form (Chow et al. 2001; Lee et al. 2002). The conjugated metabolites are then transported into vascular circulation, further metabolized in the liver, and finally excreted in the urine or bile (Nakagawa and Miyazawa 1997). Since conjugation of catechins take up their hydroxyl groups, the conjugated metabolites seem to be less effective antioxidants than their parental compounds. Glucuronide and sulfate conjugates excrete rapidly from the blood system because of their high polarity (Nakagawa and Miyazawa 1997; Miyazawa 2000). Efflux of some conjugated metabolites back to the intestinal lumen has also been reported which in turn limits catechin’s absorption into system circulation (Lambert et al. 2007).

The residual, unabsorbed catechins and the metabolites effluxed back to the intestine are transported to the colon; there, colon microorganisms degrade them to ring-fission metabolites and phenolic acids, which are then absorbed to system circulation, and finally excreted in the urine. The bioactivity of phenolic acids is not well understood, but some antiproliferative activities have been found (Gao et al. 2006; Henning et al. 2008).

Various food delivery systems have been reported to improve tea catechins’ bioavailability. For example, chitosan nanoparticles can be used to encapsulate EGCG to increase the stability of the bioactive against simulated gastrointestinal fluids, and to enhance cell absorption, as shown by an in vitro intestinal permeability model (Dube et al. 2010a; Dube et al. 2011b). A water-in-oil-in-
water emulsion has been designed to co-deliver curcumin and catechins and the stability and bioaccessibility of both bioactives was improved (Aditya et al. 2015). An *in vivo* study suggested an increase in catechin bioavailability when consumed in combination with sucrose and ascorbic acid (Peters et al. 2010). Nanoemulsification improved the bioavailability of green tea extract, as shown by the improved hypocholesterolemic effect in mice (Kim et al. 2012). Furthermore, self-assembled nanoparticles composed of chitosan and polypeptides increased the bioavailability of tea catechins, via an increased intestinal transport (Tang et al. 2013). Intragastric co-administration of EGCG and piperine (an alkaloid derived from black pepper) showed a rise in EGCG concentration of mice plasma. This increase was attributed to piperine inhibition of the glucuronidation activity (Lambert et al. 2004). Increased bioavailability for curcumin through co-administration of piperine and curcumin has been also reported in humans (Shoba et al. 1998). Chitosan–whey protein conjugate particles with positive charge below pH 7 showed stability to aggregation under digestive conditions, potential for bioadhesion in the intestinal tract, and improved controlled release for the entrapped catechins (Zhang et al. 2009). Mixing fruit juices (grapefruit, orange, lemon, or lime) with tea catechins also showed an enhanced digestive recovery for tea catechins (Green et al. 2007). Other approaches for EGCG delivery include chitosan-tripolyphosphate nanoparticles (Hu et al. 2008), Zein fibers (Li et al. 2009) and pectinate gel beads entraping catechin-loaded liposomes (Lee et al. 2008).

### 2.5 Complex Formation Between Polyphenols and Proteins

The ability of polyphenols to associate with proteins is well documented, and it mainly involves hydrophobic interactions and hydrogen bridging (Murray et al. 1994; Charlton et al. 2002; Ferruzzi et al. 2012; Ozdal et al. 2013). The phenolic rings of polyphenols associate with the hydrophobic
patches of protein chains. Polyphenols have a strong affinity for non-globular proteins such as salivary proteins and caseins. These proteins have extended conformations and contain high number of proline aminoacids (Siebert et al. 1996; Baxter et al. 1997; Fox 2001; Jöbstl et al. 2004; Richard et al. 2006). The association of polyphenols with proteins depends on the number of aromatic rings in the polyphenols, temperature and presence of other components such as carbohydrates (von Staszewski et al. 2012). Catechin-protein binding was reported to increase in the presence of the galloyl and hydroxyl groups (Song et al. 2015).

Complex formation between polyphenols and proteins has been suggested as one of the solutions to improve the stability of polyphenols. Given that milk proteins have been recognized as a natural carrier for bioactives (Livney 2010), their interaction with phenolics has been used to develop vehicles for targeted delivery of these molecules (Sahu et al. 2008; Bohin et al. 2012). Some examples are as follows. Encapsulation of curcumin in beta-casein nanostructures improved the antioxidant activity and cytotoxicity of curcumin in vitro (Esmaili et al. 2011). Casein micelle-curcumin complexes were introduced as an alternative drug formulation for cancer therapy (Sahu et al. 2008). Whey proteins have also been shown to interact. For example, a β-lactoglobulin-naringenin complex was employed as a nano-vehicle for promoting the bioavailability of naringin (Shpigelman et al. 2014).

2.5.1 Catechin-Casein Complexes

Tea catechins have a high affinity for caseins (Ferruzzi and Green 2006; Jöbstl et al. 2006; Haratifar and Corredig 2014). With complex formation, there is a decrease in the proteins’ surface hydrophobicity (Yuksel et al. 2010). The authors suggested that this decrease is an evidence for hydrophobic interactions between proteins and EGCG. The proline groups of caseins have shown
a strong affinity for catechins’ hydroxyl groups (Arts et al. 2002). Furthermore, the addition of a gallate moiety to catechins increased their binding affinity to protein (Bohin et al. 2012).

The formation of complexes between milk proteins and catechins may be utilized to create the ideal platform for delivery of catechins (Livney 2010). Thermally-induced β-lactoglobulin-EGCG co-assemblies showed promising results for nanoencapsulation and sustained release of tea catechins (Shpigelman et al. 2010; Shpigelman et al. 2012). Addition of milk to tea increased total catechin recovery after *in vitro* digestion (Green et al. 2007). The intestinal absorption of green tea catechins was enhanced by addition of milk to tea, in an *in vitro* digestion/Caco-2 cell model (Xie et al. 2013). Moreover, the affinity of tea catechins to milk proteins was proposed to boost catechins’ stability against thermal treatment (Song et al. 2015). In another study, β-casein, showing one of the highest affinities for EGCG, was suggested to be the most promising carrier for catechin among common food proteins such as α-lactalbumin, serum albumin, β-lactoglobulin, gelatin, lysozyme, ovalbumin and phosvitin (Bohin et al. 2012). Furthermore, the inclusion of tea catechins in milk-tea complexes was shown to reduce their bitter off-taste and sensation of astringency (Lesschaeve and Noble 2005; Hofmann et al. 2006; Schwarz and Hofmann 2008; Bohin et al. 2013).

As summarized above, the interactions between caseins and tea catechins have been described both for monomeric caseins (Jöbstl et al. 2006; Hasni et al. 2011; Bohin et al. 2012) and micellar caseins (Ferruzzi and Green 2006; Yuksel et al. 2010; Xie et al. 2013; Haratifar and Corredig 2014). However, catechin-casein interactions, once caseins are adsorbed at an oil water interface, have not been investigated before. Sodium caseinate is commonly used as an emulsifier for emulsions with a broad range of applications in food, cosmetics, and pharmaceuticals (McClements et al. 2007). Since tea catechins are strong natural antioxidants, the association of EGCG with protein
at emulsion interface may not only be beneficial in protecting the bioactives, but also in protecting the oil phase from oxidation (Dubeau et al. 2010). Therefore, studying the binding behaviour of tea catechins at the sodium caseinate stabilized oil-water interface could be of interest from technological and nutritional point of view.

2.6 Caseins and Sodium Caseinate

Caseins constitute about 80% of the total proteins of bovine milk. They are phosphoproteins, and precipitate at pH 4.6 at 20°C (Jenness et al. 1956). The main caseins in bovine milk are αs1-, αs2-, β- and κ-casein, respectively present as 45, 12, 34, and 10%, of caseins. Their molecular weight range is 20-25 kDa (Dalgleish 1998). Caseins contain a high proline content, which impedes the formation of ordered structures such as α-helix or β-sheets, making the structure very open, often described as rheomorphic, for their ability to adapt the structure to the environmental conditions (Holt and Sawyer 1993).

Since acidic amino acids, hydrophobic amino acids, and serine phosphate groups are distributed in a disproportionate order in the polypeptide chain, both hydrophilic and hydrophobic regions are found on the same peptide chain, as well as phosphate rich regions. Because of their amphiphilic nature, caseins adsorbs at air/water or oil/water interfaces easily and orient their hydrophobic residues towards the interface, while the hydrophilic regions protrude into the aqueous phase (Mohanty et al. 1988). Caseins, usually in the form of soluble sodium caseinate, are widely employed as a functional surface active protein in the food product applications (Modler 1985; Mohanty et al. 1988).

Sodium caseinate is derived from micellar casein, and it is produced through direct acidification by hydrochloric or sulfuric acid leading to co precipitation of the four principal casein proteins at
pH 4.6. The acid coagulated curd is then washed, neutralized by sodium hydroxide to pH 6.8, usually pasteurized and then spray-dried (Modler 1985; Dickinson and Golding 1997b). Sodium caseinate is a soluble mixture of four casein proteins excluding the phosphate and calcium components, and it has no structural resemblance to the native casein micelles in milk (Lucey et al. 2000; Surh et al. 2006). In solution, sodium caseinate is a mixture of monomeric casein molecules (≈ 25 kDa) and small aggregated particles typically composed of 12-15 monomer units (≈ 250-300kDa) (Dickinson and Golding 1997a).

2.7 Emulsions

Most foods are dispersed systems. For many years, emulsion science and technology has been employed to create a diverse range of commercial products, including pharmaceuticals, foods, personal care products, and cosmetics. The majority of these products are in the form of conventional emulsions which consist of dispersed droplets of one liquid in the other immiscible liquid, e.g., oil-in-water emulsions. Milk and milk products, sauces, dressings, and soups are example of oil-in-water emulsions.

For decades, colloidal delivery systems have been utilized in the food and pharmaceutical area. Structured emulsions were employed for certain applications, such as improvement of physical or chemical stability, encapsulation, controlled release, and retarding the digestion of lipids (Li and McClements 2011). The major factors governing the emulsion properties are their type; oil in water or water in oil, the size distribution of dispersed droplets, the volume fraction of the dispersed phase, the composition of the continuous phase and the composition and thickness of the interfacial layer (Fennema 1996).
Proteins are ideal oil in water emulsifiers because they are surface active, and provide excellent resistance to coalescence via electrostatic and steric repulsion forces (Fennema 1996). For example, the caseins present in sodium caseinate adsorb on the surface of the oil droplets and create a polyelectrolyte layer at the interface leading to electrostatic and steric repulsion at neutral pH. The interfacial layer of sodium caseinate formed around dispersed oil droplets is relatively thicker than that of globular proteins (i.e. 10 nm versus 1–2 nm for whey proteins) (Dalgleish et al. 1995). Sodium caseinate molecules also impart high viscosity to the emulsion system which lowers the rate at which adjacent oil droplets can approach (Modler 1985; Dickinson et al. 1998b). Furthermore, sodium caseinate’s unique iron chelating properties and their ability to produce thick interfacial layers around the droplets has been suggested to contribute to their protection against oil oxidation (Hu et al. 2003). The net charge of sodium caseinate-emulsified droplets becomes neutral at pH values close to the proteins’ isoelectric point (pH=4.6), whereas at pH below and above the pI, the droplet holds positive and negative charge, respectively. Close to protein’s pI, the reduction in electrostatic repulsion between the droplets leads to instability of sodium caseinate-stabilized emulsions resulting in droplet flocculation (Agboola and Dalgleish 1996; Dickinson et al. 1998b). It is possible to stabilize sodium caseinate-coated droplets during acidification by the addition of high methoxyl pectin (Dalgleish and Hollocou 1997; Dickinson et al. 1998b; Bonnet et al. 2005; Liu et al. 2007).

2.8 Pectin

Pectins are polysaccharides composed of 1, 4 D-galacturonic acid, with different level of methyl esterification. Depending on their degree of esterification, pectins are categorized as either high methoxyl pectin (HMP, with a DE greater than 50%) or low methoxyl pectin (with a DE less than
50%). Owing to the ionization of the free carboxylic groups, pectin has a negative charge in mildly acidic conditions, and its pKa is around pH 3.5 (Sinha and Kumria 2001; McClements 2005). At pH values slightly above the isoelectric point of sodium caseinate, pectin molecules interact with caseins through electrostatic interactions between negatively charged HMP molecules and positively charged patches of protein. These interactions have been shown to protect emulsion droplets from destabilization through a combination of electrostatic and steric repulsion (Dalgleish and Hollocou 1997; Dickinson et al. 1998b; Bonnet et al. 2005; Surh et al. 2006; Liu et al. 2007). The degree of esterification and the distribution of the charges on the pectin chain will affect the colloidal stabilization behaviour, as different portions of the molecules will be available to interact with the solvent and provide steric stabilization. HMP possess fewer carboxylic acid sites than low methoxyl pectin, hence, the portion not interacting with the protein may be much larger causing a larger fraction of the polymer protruding into solution, while the rest of the polymer is anchored to the protein (Pereyra et al. 1997).

Pectins are classified as indigestible dietary fibres and, although present in relatively low amounts in an emulsion, their presence in the outer coating of an emulsion may delay lipid digestion (Li et al. 2010; Viuda-Martos et al. 2010). However, as the interactions at the interface are non-covalent in nature, it is yet not possible to fully predict their behavior during gastrointestinal transit.

2.9 Investigating the Efficacy of a Carrier Matrix

To assess the efficacy of delivery systems in improving the bioavailability, it is necessary to track down the bioactive’s fate after ingestion, all the way through digestion, intestinal assimilation, and systematic metabolisms up to reaching the systemic circulation and target sites.
While the ideal approach with the most accurate results would be in vivo studies such as human or animal feeding studies, they are time consuming, resource intensive, technically difficult and very expensive to conduct, and also involved with ethical and practical concerns (Singh and Sarkar 2011). In particular, human studies are impractical for large-scale applications, almost impossible for mechanistic studies, and are disadvantaged by variability in individuals’ physical and inherent states. While animal models could address some of the human study limitations, their response may differ from that of humans (Reddy and Cook 1991).

Consequently, there is a real need for close reproduction of physiological processes out of the body. In vitro models provide a rapid, low cost, and less labor intensive option for bioavailability evaluation. Although in vitro models cannot totally simulate in vivo human conditions, they can be utilized for initial screening of a large number of samples in parallel and are very suitable for mechanistic and structural studies. In addition, owing to their reproducibility as a result of no biological variations, choice of controlled conditions, and easy sampling at the site of interest, in vitro models provide useful information in order to build stronger hypothesis for the subsequent costly and timely, yet more accurate in vivo (Hur et al. 2011; Minekus et al. 2014).

The first component of an in vitro models is the simulation of digestion process in order to mimic the complex physicochemical events occurring in the human gastrointestinal tract (GIT) (Boisen and Eggum 1991). Based on the purpose of the study, relevant analysis can be then conducted on the digestates at any required time point during and after in vitro digestion process to investigate composition, structural changes, digestibility, solubility, encapsulation efficiency, and percent recovery or degradation of a component (bioaccessibility).

After simulated digestion, only the bioaccessible fraction of the bioactive which is available for absorption can be determined. In order to get closer to predict the bioavailable fraction, the
intestinal transport of the bioactive needs to be simulated. In recent decades, intestinal cell uptake has been attempted to be reproduced by culturing enterocyte-like cells in vitro. In fact, simulated gastrointestinal digestion combined with cultured carcinoma cells as a surrogate for intestinal absorptive cells have been reported as the most effective and peer-accepted models to simulate in vivo digestion and absorption processes (Pinto et al. 1983; Glahn et al. 1996; Yun et al. 2004).

2.9.1 In Vitro Digestion

Human digestion is a multistage dynamic process wherein ingested food is broken into nutrients to be used for energy, growth and cell maintenance (Figure 2.7). Mastication of food in the mouth is the beginning of the process which is a short step with a significant effect particularly on gastric emptying rate (Woda et al. 2010). The bolus, i.e. the product of mechanical and enzymatic degradations of food in the mouth, is transported to the stomach through the esophagus by the mechanism of peristalsis.

Gastric digestion is a dynamic step where bolus is placed in contact with gastric juices including pepsin, lipase, hydrochloric acid, and mucus respectively responsible for protein and lipid digestion, lowering pH thus promoting protein hydrolysis, and protecting mucosal surfaces. At the antrum, the opening of the stomach, peristaltic waves help grinding and mixing gastric contents and breaking down large particles. The pylorus at the exit of the stomach acts as a sieve with a selective emptying of small particles while keeping the large ones in, to be further degraded. Gastric emptying which is determined by factors such as food composition, food structure and biological factors is a crucial parameter of digestion (Kong and Singh 2008; Guerra et al. 2012). The acidic product of the stomach is called chyme. Chyme is delivered to the small intestine where both macromolecules breakdown and absorption of nutrients happen. To provide an appropriate
Figure 2.7 Human gastrointestinal tract. (from (Guerra et al., 2012)).


pH for the activity of the enzymes, sodium bicarbonate is secreted from pancreas to the small intestine. A complex mixture of proteases, amylases, lipases and other digestive enzymes called pancreatic enzymes are secreted by the small intestine’s inner wall. Bile salts and phospholipids, secreted from the liver, plays a specific role in lipid digestion by emulsifying dietary fats into small droplets. They also form mixed micelles and vesicles capable of incorporating lipid digestion products thereby removing them from the lipid droplet surfaces.

Ca$^{2+}$ is necessary for pancreatic lipase activity, it reacts with liberated free fatty acids by means of ionic complexation, and removes them from the lipid droplet’s surface, hence preventing lipase inhibition (Carey et al. 1983; Fatouros and Mullertz 2008). Segmental movements help mix chyme with the intestinal juices, and peristalsis activity propels the digestate through the small intestine. Absorption of water and nutrients by intestinal absorptive cells, enterocytes, happens via simple diffusion, facilitated diffusion, or active transport. The huge absorptive surface area of small intestine prevents accumulation of digestion products in the small intestine’s lumen which could inhibit enzyme activities. Absorbed nutrients are biotransformed in the enterocytes and the metabolites will be then absorbed to the systemic circulation. Non-absorbed material travels down to the large intestine where more water and electrolytes are absorbed, bile salts are reabsorbed and colonic microbiota ferment the polysaccharides and proteins (Guerra et al. 2012). Hormonal and neural mechanisms regulate the main digestive practices such as secretion of digestive fluids, gastric emptying, intestinal transit, and the motilities (Schubert 2008; Mayer 2011). It was reported that the amount of each enzyme is regulated by the amount of its substrate in the ingested food through feedback mechanisms (Boisen and Eggum 1991). It was also reported that the presence of the lipid in ingested food generally increases gastric transit time by inducing delays in gastric emptying (Van Citters and Lin 1999).
Given the brief description above, digestion is an inherent complex process. In designing an in vitro model, several factors such as the enzymes’ concentration and activity, ionic composition, pH, transit time, temperature, mechanical forces, and also the absorption of digestion products should be taken into consideration and be adjusted according to the sample characteristics (Hur et al. 2011; Guerra et al. 2012). Yet, many in vitro models lack the hormonal/nervous controls, feedback mechanisms, continuous changes in pH and flow rates of secretions, product removal/absorption, complex GIT movements, microbial flora and immune system. Therefore, the dynamic digestion processes cannot be completely mimicked under in vitro conditions and a careful interpretation of results is always required. Correlating in vitro and in vivo data can also help to assess and improve in vitro models (Boisen and Eggum 1991; Hur et al. 2011; Guerra et al. 2012).

During the past two decades, a number of in vitro digestion models from static monocompartmental to dynamic multicompartmental models with significant variations in the use of digestion parameters have been utilized to study the structural and chemical changes of food during digestion process and the bioaccessibility of bioactives. Some of commonly used in vitro models merely include one particular region of the GIT based on the study question, or apply a single enzyme useful for predicting the digestibility of a single nutrient, whereas in others, several sequential steps such as oral, gastric, small intestinal phases, and occasionally large intestinal fermentation are taken into account. In addition, dynamic aspects of digestion, such as enzyme concentration changes over time, and transport of digested food from one step to the next one, have been simulated in some computerized sophisticated models such as TIM model (Blanquet et al. 2004). Yet, the majority of models reported in literature are static models where constant ratios
of food to enzymes and other components of digestive juices were applied (Hur et al. 2011; Minekus et al. 2014).

Significant variations in the digestion parameters used in different digestion models impeded the ability to compare results across research groups and to deduce general findings. Recently more than 200 scientists working in the field of digestion from 32 countries created an international network (COST Infogest) with the purpose of finding a consensus for simulated digestion of food. A standardised digestion method using widely available instrumentation and chemicals and based on the current state of knowledge of human digestion conditions was proposed by this team (Minekus et al. 2014). This in vitro digestion model was employed in this thesis.

2.9.2 In Vitro Absorption

The development of human cell culture systems has been restricted by several factors. Normal intestinal epithelial cells are difficult to culture and have limited viability thereby their application for in vitro models has been limited (Moyer 1983). Some human tumour cell lines showed lack of differentiated properties in culture. Human adenocarcinoma cell lines, when cultured in vitro, showed reproducible properties distinctive of mature human intestinal epithelium. This allowed their usage as surrogates for enterocytes and a suitable model to study cellular permeability and absorption (Pinto et al. 1982; Rousset 1986).

Caco-2 cell, a human colon carcinoma cell line, has become one of the most popular in vitro models, because this cell line exhibited many functional and morphological properties of the normal enterocytes under standard culture conditions (Pinto et al. 1983). When grown on semipermeable filters (Figure 2.8), Caco-2 cells spontaneously differentiate and form confluent
monolayers with several features characteristic of differentiated enterocytes. The monolayers are morphologically polar with well-developed brush borders at their apical surface and enzymes and

Figure 2.8 Schematic of Caco-2 cell culture for transport experiment (from (Meunier et al. 1995)).
Caco-2 cell monolayers morphologically resemble small intestine absorptive cells, their ability to form dense intercellular junctional complexes resulted in a tight epithelium and a paracellular as well as transcellular barrier (Hilgendorf et al. 2000). Several studies showed their possession of functionally active transport systems, receptor-mediated endocytic pathways and carrier-mediated transports for the uptake of different nutrients (Wilson et al. 1990). The reproducibility and long-term viability make Caco-2 cells advantageous over other in vitro models used for absorption studies (Hilgers et al. 1990; Meunier et al. 1995).

Furthermore, the anti-carcinogenic activity of a bioactive can be assessed through determining the level of proliferation of carcinoma cells to which the bioactive were subjected (Frontela-Saseta et al. 2011; Cilla et al. 2015). As the intestinal absorption of tea catechins is low, the non-absorbed fraction will be in direct contact with the digestive tract, potentially exerting their cancer-preventive activity at those sites. For instance, after ingestion of tea polyphenols, the intestine may be exposed to high levels of tea polyphenols. Hence, cancer cells from the digestive tract such as Caco-2 cell line have been employed in many studies where the anti-cancer effect of polyphenols has been studied (Yang et al. 1998; de Mejía et al. 2010).

In this study the interaction of tea polyphenols with sodium caseinate at an emulsion interface was investigated and the emulsions incorporating EGCG were characterized at neutral pH and low pH in the presence of pectin. Then the digestive behavior of such designed matrices and their efficiency in improving the bioaccessibility and bioavailability of EGCG were determined.
CHAPTER 3
TEA POLYPHENOLS ASSOCIATION TO CASEINATE-STABILIZED OIL-WATER INTERFACES

3.1 Abstract

Tea catechins associate with proline rich proteins such as caseins. In this work, the interactions between epigallocatechin-gallate (EGCG), one of the main tea catechins, and caseins at an oil water interface were studied. The association of EGCG with sodium caseinate was quantified by measuring the amount of EGCG adsorbed on the emulsion droplets. In addition, the viscoelastic properties of the protein and EGCG-protein layers formed at the interface were studied using drop tensiometry. Different concentrations of EGCG were added to a model emulsion to obtain final concentrations of 10% soybean oil, 0.5% sodium caseinate and 0-9 mg/mL EGCG. At concentrations < 2 mg/mL EGCG, more than 90% was adsorbed at the interface. At higher concentrations, below 5 mg/mL, about 70% of EGCG was adsorbed. The surface load reached about 1 mg/m² at 9 mg/mL of EGCG. The dissociation constant for the complex formed was estimated using Scatchard plot, and was $3.7 \times 10^{-5}$ M for less than 20 mol bound EGCG/protein. The interactions of EGCG with sodium caseinate did not affect the interfacial tension but increased the dilational modulus of the EGCG-protein layer at the interface. This study demonstrated that sodium caseinate emulsions could be employed as carriers for EGCG, and that the complexes formed at the interface are affecting the physical chemical properties of the emulsions. The results are of significance in the development of dairy based beverages containing tea polyphenols.

3.2 Introduction

In recent years polyphenols have received significant attention as health-promoting nutrients. They are found in different foods, including vegetables, fruits, and tea. Catechins (flavon 3-ols) are the most abundant group of water-soluble polyphenols in tea. Epicatechin (EC), epicatechin-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-gallate (EGCG) are main tea catechins within which EGCG is the major component (Braicu et al. 2013). Beneficial health effects have been linked to the consumption of tea polyphenols, including prevention of cancer and cardiovascular diseases, neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease), obesity and diabetes (Hollman et al. 1997; Yang et al. 2004; Scalbert et al. 2005; Iso et al. 2006). The potential role of dietary catechins in the prevention of chronic diseases has intensified the interest in their bioavailability (Peters et al. 2010). Reportedly catechins from tea have a low oral bioavailability; in human studies only 0.1–1% of the consumed amount seems to reach the systemic circulation (Dube et al. 2010a). Some factors limiting their bioavailability may include poor stability to digestive conditions (Record and Lane 2001; Green et al. 2007) poor intestinal absorption and rapid metabolism (Vaidyanathan and Walle 2001; Zhang et al. 2004).

The ability of polyphenols to associate with proteins is well documented (Ferruzzi et al. 2012), and it involves hydrophobic interactions and hydrogen bridging (Pascal et al. 2008; Ozdal et al. 2013). Complex formation between tea polyphenols and proteins has been suggested as one of the solutions to improve the stability of these molecules. Of particular relevance to the current work are studies on formulations of green tea with milk (van het Hof et al. 1998; Green et al. 2007; Xie et al. 2013) and the studies on binding of tea catechins with casein micelles (van het Hof et al. 1998; Shukla et al. 2009; Haratifar et al. 2013). Tea catechins associate with proline rich proteins such as caseins (Jöbstl et al. 2006; Yuksel et al. 2010; Haratifar and Corredig 2014).
Milk proteins have been suggested as an ideal system for the delivery of these bioactive molecules (Livney 2010; Xie et al. 2013). Furthermore, the binding of catechins to caseins reduces their sensation of astringency in the mouth (Lesschaeve and Noble 2005; Hofmann et al. 2006). The interactions between caseins and tea polyphenols have been previously studied, both for monomeric caseins as well as for micellar caseins (Jöbstl et al. 2006; Yuksel et al. 2010; Hasni et al. 2011; Haratifar and Corredig 2014). However, there is no information available on the association of tea polyphenols with caseins once adsorbed at the oil water interface.

Sodium caseinate derives from micellar casein, and it is composed of a soluble mixture of $\alpha_{s1}$, $\alpha_{s2}$, $\beta$, and $\kappa$-caseins. This protein mixture is commonly used as an emulsifier in foods. Sodium caseinate adsorbs on the surface of oil droplets and creates a polyelectrolyte layer at the interface leading to electrostatic and steric repulsion at neutral pH. This thick protein layer stabilizes the oil droplets against coalescence (Dickinson et al. 1998a). Emulsions are also a common delivery platform for hydrophobic bioactives (McClements et al. 2007).

Tea catechins have been shown to have antioxidant activities including scavenging of reactive oxygen and nitrogen species, free metal chelation, and inhibitory effect on oxidative enzymes (Green et al. 2007; Almajano et al. 2008; Dubeau et al. 2010). The association of EGCG with protein at emulsion interfaces may not only be beneficial in protecting the bioactive molecules, but also in protecting the oil phase from oxidation. However, very little work has been carried out on this topic. Studying the binding/association behaviour of tea polyphenols and protein at the oil-water interface is therefore of great significance from the technological and nutritional point of view. Objective of this work was to study the association of EGCG to sodium caseinate in an oil/water emulsion and its effect on the viscoelastic properties of the interface.
3.3 Materials and Methods

3.3.1 Emulsion Preparation

Sodium caseinate (1.25 % w/w) (New Zealand Milk Products, Mississauga, Ontario, Canada) was dispersed in ultrapure water (Millipore Corp., Bedford, MA) containing 0.02 % w/v sodium azide (Catalog No: S227I, Fisher Scientific, Fair Lawn, NJ), added to prevent bacterial growth. The mixture was continuously stirred for 2.5 h at room temperature. After overnight storage at 4 °C, the solution was filtered through 0.8 μm filter (Millex-HV, Millipore Co., Billerica, MA), and then added to soybean oil (Sigma, St. Louis, MO) to obtain an emulsion of 20 % w/w oil and 1% w/w protein. The mixture was pre-homogenized for 2 min at 10000 rpm using an Ultra Turrax stand homogenizer (IKA T18 Basic, Germany) and immediately processed with a microfluidizer (M-110EH, Microfluidics, MA) with four passes and a pressure of 69 MPa.

A commercial green tea polyphenol extract containing a high concentration of EGCG (min 94%) was kindly provided by DSM Nutritional Products Inc. (Ayr, Ontario, Canada). A stock solution of EGCG in ultrapure water was made just before use in order to avoid oxidation and then diluted appropriately to be added to the emulsion to obtain final mixtures containing 0.5% sodium caseinate, 10% oil, and EGCG concentrations ranging between 0.5-9 mg/mL EGCG. The mixtures were then vortexed for 1 min and kept at room temperature (22°C) for 15 min before the next experiments.

3.3.2 Particle Size Measurements

Particle size distribution, surface-weighted mean diameter \[d_{3,2}=(\Sigma n_i d_i^2 / \Sigma n_i d_i)\], volume-weighted mean diameters \[d_{4,3}=(\Sigma n_i d_i^3 / \Sigma n_i d_i^4)\] and total oil surface area of the emulsions were determined through static light scattering (Mastersizer 2000S, Malvern Instruments Inc.,
Southborough, MA). Samples were diluted with water. Refractive indices of 1.46 and 1.33 were used for the emulsion droplets and dispersant (i.e. water), respectively.

### 3.3.3 Composition of the Interfacial Layer

Emulsions containing 0.5-9 mg/mL EGCG were centrifuged at 60000 g for 1 h (Beckman Coulter Optima™ LE-80K ultracentrifuge, rotor type 70.1Ti, Beckman Coulter Canada Inc., Mississauga, Canada). The clear subnatant was carefully withdrawn using a Pasteur pipette with a very fine tip, filtered through a 0.45 µm filter and immediately analyzed for EGCG by reverse phase HPLC as previously published (Ferruzzi and Green 2006). A corresponding set of solutions with known concentrations of EGCG dissolved in ultrapure water were also prepared and analyzed by HPLC to be used as standards. A Nova-Pak C18 column (4 µm, 3.9×150 mm, Waters Corporation, Milford, MA, USA) connected to a guard column with identical chemistry (Waters) was used. The mobile phase consisted of water/acetic acid (98:2, v/v) (buffer A) and acetonitrile (buffer B). A 20 min linear gradient from initial conditions of 99:1 A/B to 70:30 A/B was followed by a linear gradient back to 99:1 A/B over 5 min and at last 5 min equilibration at 99:1 A/B. The analysis was conducted at 35 °C and a flow rate of 1 mL min⁻¹. The detection was carried out at 280 nm. HPLC grade water, acetonitrile, methanol, and glacial acetic acid were obtained from Fisher Scientific.

The amount of protein present in the subnatant was also determined using the Lowry assay method (DC Protein Assay, Bio-Rad, Hercules, CA, USA). The protein surface load (mg protein/m²) was calculated from the difference between the initial protein content and that measured in the subnatant along with the total oil surface area determined by static light scattering. The concentration of EGCG adsorbed at the interface (mg EGCG/m²) was also calculated the same way. Sodium caseinate/EGCG (w/w) ratios at the oil-water interface were determined.
The binding strength between proteins and their ligands in terms of how easy it is to separate the complex can be determined through dissociation constant ($K_d$) of the complex. Using the Scatchard equation, the $K_d$ of EGCG-sodium caseinate complex can be measured.

\[ \frac{v}{[L]} = \frac{1}{K_d} - \frac{v}{K_d} \]  

(1)

Here $v$ is the molar ratio of bound ligand/total protein and $[L]$ is the free ligand molar concentration. Curved Scatchard plots can be decomposed into successive linear plots, and the dissociation constant $K_d$ can be derived from each of the slopes (Relkin and Vermersh 2001). The average molecular weight of sodium caseinate is 24 kDa and EGCG is 458.4 Da.

### 3.3.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The emulsion mixtures were centrifuged at 60000 g for 1h (Beckman Coulter) and the subnatant was carefully separated from the cream and analyzed for protein by SDS–PAGE under reducing conditions. The resolving gel (15%) was composed of 4.8 mL of water, 5 mL of resolving buffer pH 8.9 (1.5M Tris, 0.2% SDS), 10 mL of acrylamide bis (30%), 100 µL of ammonium persulfate (10%), and 7 µL of TEMED. The stacking gel (4%) was composed of 3.56 mL of water, 5 mL of stacking gel buffer pH 6.7 (0.1M Tris-PO$_4$, 0.2% SDS), 1.34 mL of acrylamide bis (30%), 100 µL of ammonium persulfate, and 7 µL of TEMED. Aliquots (100 µL) of the emulsion’s subnatant were mixed with 200 µL of sample buffer composed of 3.9 mL of 1 M Tris-HCL (pH 6.8), 2.0 mL of 75% glycerol, 1.6 mL of 10% w/v SDS, 0.4 mL of 2-mercaptoethanol, and 0.1 mL of 1% bromophenol blue in water. The solutions were then heated at 95 °C for 5 min while shaking using the Eppendorf Thermomixer 5436 (Eppendorf, Hamburg, Germany). After cooling for 15 min, the
samples were centrifuged at 10,600 rpm for 10 min using an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY). 5 μL of the subnatant was then loaded onto the gels and run at a constant voltage of 200 V in a Bio-Rad mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A sample of whole emulsion with no EGCG was also mixed with the sample buffer and used as control. After each run, the gels were immediately stained for 30 min using Coomassie blue R-250 and destained for 2 h with a destaining solution of 45% ultrapure water, 45% methanol and 10% acetic acid, with two changes, then destained overnight with a destaining solution containing 22.5% methanol and 5% acetic acid. SHARP JX-330 scanner (Amersham Biosciences, Quebec) was employed to scan the gels.

### 3.3.5 ζ-Potential Measurements

The overall surface potential of the emulsions was determined using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Malvern, UK) at a constant temperature of 25 °C. Samples were diluted in ultrapure water by 1:300 ratio. Their pH values were also measured after preparation using a pH meter (Accumet Research AR 15, Fisher Scientific).

### 3.3.6 Interfacial Properties

Sodium caseinate and EGCG solutions were prepared in HPLC water (Fisher Scientific, Fair Lawn, NJ, USA). Soy bean oil was pretreated with Florisil (Sigma-Aldrich, St. Louis, MO, USA) at a ratio of 10:1 for 2 h. Florisil was then removed by filtration. This cleaning procedure was repeated for three times (Gülseren and Corredig 2011).

Using drop shape tensiometer (Tracker, IT Concept, Longessaigne, France), a syringe needle containing soy bean oil was immersed in a glass cuvette containing 5 mL of 0.001% w/w sodium
caseinate solution. An oil droplet was formed at the tip of the needle and while the droplet’s volume was consistently controlled at 6 µL using volume control regulation program, the shape of the droplet was recorded by a CCD camera connected to a computer. Interfacial tension was automatically determined by analyzing the recorded profiles according to the Young-Laplace equation. The interfacial tension reached a plateau after approximately 10000 s. Then, 10µL of 0.2% w/w EGCG solution was added into the cuvette. The surface tension was measured for another 10000 s to observe the possible changes in the presence of EGCG. Each measurement was performed in duplicate. The range of concentrations selected for EGCG and sodium caseinate solutions were based on the ratios obtained from the adsorption experiments on the emulsions (see section 3.2). The interfacial dilational elastic modulus was also measured by drop shape tensiometer. The interfacial dilational elastic modulus is defined by the ratio of change in interfacial tension (dγ) to the change in droplet surface area (dA) (Lucassen-Reynders et al. 2001).

\[ \varepsilon = \frac{d\gamma}{d \ln A} \]  

(2)

The interfacial elasticity measurements were conducted after 10000 s of equilibration when a sinusoidal compression–expansion of 50 mHz and 0.1 strain amplitude was applied to the oil droplet in the presence of sodium caseinate + EGCG over a time scale of 200 s. The amplitude used was within the linear viscoelastic range.

### 3.3.7 Statistical Analysis

All results were analyzed using ANOVA testing with excel 2010. Significance was considered at p<0.05.
3.4 Results and Discussion

3.4.1 Emulsion Characterization

The values of size, charge, and pH of emulsions with or without EGCG are summarized in Table 3.1. Figure 3.1 shows the distribution of the droplet diameter for three representative samples containing EGCG. There were no significant differences in the droplet size distribution of emulsions containing EGCG up to 9 mg/mL; all emulsions had an average \( d_{4,3} \) of 0.24 \( \pm \) 0.01 \( \mu m \). It was concluded that the addition of EGCG, even at highest concentrations used in this study did not cause emulsion destabilization.

It is important to note that the addition of EGCG caused a significant decrease in the emulsion pH from 7.5 to 7.1 and 6.7 for 2 and 6 mg/mL EGCG, respectively; however, the \( \zeta \)-potential of the oil droplets did not show any statistical difference, with emulsions containing 0, 2 and 6 mg/mL EGCG having values of \(-41 \pm 2\), \(-43 \pm 1\) and \(-41 \pm 2\) mV respectively.
Table 3.1 Particle diameter, pH and ζ-potential of 10% oil in water emulsions stabilized with 0.5% sodium caseinate and containing 0, 2 and 6 mg/mL EGCG. Values are the means of three replicates. Within a column, different superscript letters indicate statistical difference at $p < 0.05$.

<table>
<thead>
<tr>
<th>EGCG concentration (mg/ml)</th>
<th>0</th>
<th>2</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{4,3}$ (µm)</td>
<td>0.25 ± 0.01 $^a$</td>
<td>0.23 ± 0.02 $^a$</td>
<td>0.23 ± 0.01 $^a$</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 ± 0.2 $^a$</td>
<td>7.1 ± 0.1 $^b$</td>
<td>6.7 ± 0 $^c$</td>
</tr>
<tr>
<td>ζ-potential (mV)</td>
<td>-41 ± 2 $^a$</td>
<td>-43 ± 1 $^a$</td>
<td>-41 ± 2 $^a$</td>
</tr>
</tbody>
</table>
**Figure 3.1** Particle size distribution of sodium caseinate stabilized emulsions at three concentrations of EGCG (0 (●), 2 (○) and 6 (▼) mg/mL). Results are representative samples of three replicate experiments.
3.4.2 Composition of the Interfacial Layer

To quantify the binding properties of EGCG to sodium caseinate at the oil-water interface, the amount of protein and EGCG associated with the oil phase of the emulsions was determined by estimating the protein and EGCG present in the centrifugal serum. It is important to note that high speed centrifugation may lead to a slight underestimation of surface load (Hunt and Dalgleish 1994).

Figure 3.2 illustrates the percentage of EGCG adsorbed and the EGCG/protein ratio at the interface as well as the EGCG surface load (mg/m²) for emulsions containing 10% soy oil and 0.5% sodium caseinate as a function of initial EGCG concentration. At concentrations of EGCG < 2 mg/mL, 85% of the EGCG was recovered with the oil droplets. At higher concentrations, more EGCG was found unadsorbed in the aqueous phase, but at 5 mg/mL, still 70% of total initial EGCG was adsorbed at the interface. The surface load of EGCG also continued to increase and reached a value of approximately 1 mg/m² at the highest concentrations of EGCG added.

Protein analysis of the subnatant revealed that about 80% of the total sodium caseinate was adsorbed at the interface, with a surface load of 1.5 mg/m². The EGCG/protein ratio increased with increasing EGCG concentration. At 2 mg/mL initial EGCG, when 90% of EGCG was adsorbed to the interface, the EGCG/protein ratio was about 0.4 (w/w) corresponding to an EGCG/sodium caseinate molar ratio of 20, and it reached values of 1.2 w/w at the highest concentrations used in this research. In a study on the interactions between EGCG and native milk proteins, a ratio of 0.08 mg EGCG/mg casein was reported when caseins are present in micellar form with a molecular weight of 22 kDa (Haratifar and Corredig 2014).
Figure 3.2  Adsorbed EGCG (●), EGCG surface load (□) and EGCG/sodium caseinate ratio (w/w) (▲) in emulsions containing 10 % soy oil and 0.5% sodium caseinate, as a function of total EGCG. Error bars indicate standard deviation. Values are the means of three replicates.
Furthermore, recent fluorescence spectroscopy studies reported molar ratios of about 1.5 for solutions of EGCG/β-casein and EGCG/α-casein, molecular weight of caseins being 24 kDa (Hasni et al. 2011). In another study, molar ratios as high as 6 have been reported for EGCG/β-casein in solution (Sausse et al. 2003). The higher amount of EGCG associated with the caseins in this study may suggest higher binding ratios of EGCG at the interface, because of the open and flexible structure of casein proteins once adsorbed onto the oil droplets. The results also led to the conclusion that caseinate-stabilized emulsions could be employed as carriers for EGCG because of their ability to capture these molecules at the oil-water interface at high ratios.

A Scatchard plot for the binding of EGCG to sodium caseinate at the interface of oil in water emulsions is shown in Figure 3.3. The dissociation constants (K_d) of EGCG-sodium caseinate complexes at the oil-water interface were estimated from equation (1). The Scatchard plot and three K_d values derived from splitted curve is shown in Figure 3.3. The K_d values increased from 3.7×10^{-5}, to 8.7×10^{-5}, and 2×10^{-3} M with increasing concentration of bound EGCG. These results are well in agreement with other studies showing more than one type of binding and a decrease in specificity of the binding with increasing concentration of EGCG bound (Haratifar and Corredig 2014). The Kd value calculated for a molar ratio of EC GG/protein < 20, was lower than that reported for EGCG-casein micelles (Haratifar and Corredig 2014) demonstrating that there was a higher binding specificity in the case of sodium caseinate adsorbed at the interface. This was most probably due to the monomeric nature of the layer of protein adsorbed at the interface, and the open structure of the caseins, when compared to native caseins assembled in a casein micelle in skim milk.
Figure 3.3 Scatchard plot for EGCG-caseins adsorbed at the oil water interface. The amount of bound EGCG was estimated from the amount present in the emulsions subnatant. Data are the average of three independent experiments. Lines are drawn to guide the eye.
SDS-PAGE electrophoresis was performed on the aqueous phase (subnatant) of the emulsions containing increasing concentrations of EGCG. There were no differences in the amount of sodium caseinate adsorbed at the interface (data not shown) and it was concluded that the presence of EGCG did not affect protein surface load.

### 3.4.3 Interfacial Properties

The interfacial tension of soy oil in contact with water, and a sodium caseinate solution (0.001%) with and without EGCG (0.4 w/w or 20 moles/moles EGCG/casein) was determined by drop shape tensiometry. This ratio corresponded to about 90% EGCG adsorption (Figure 3.2). The interfacial tension (\(\gamma_0\)) of the oil-water interface was 30.3 ± 0.2 mN/m, well in line with what was previously reported (Gülseren and Corredig 2011). Figure 3.4 depicts the surface tension of the oil drop in contact with a 0.001% sodium caseinate solution before and after the EGCG injection. The plateau values of interfacial tension and interfacial pressure (\(\Pi = \gamma_0 - \gamma\)) were 13.3 ± 0.2 and 16.94 ± 0.2 mN/m, respectively. These values were consistent with the literature (Benjamins and Lucassen-Reynders 1998). There was no significant change in the interfacial tension or surface pressure once EGCG was present in the solution.

In addition to the surface tension, the viscoelastic properties of the EGCG-protein layers at the interface were also studied. The interfacial dilational modulus describes the resistance of the interface against surface tension gradients or homogeneous dilatation. The structure of the molecules at the interface and their interactions determine the dilational elasticity (Gülseren and Corredig 2012). The results are summarized in Table 3.2 and Fig. 3.5. There was no significant effect of oscillation frequencies and amplitudes on the elastic modulus either with or without EGCG present.
Figure 3.4 Changes in the surface tension at the water/soy oil interface covered with 0.001% of sodium caseinate as a function of time. At 10,000 s, EGCG, at an EGCG/sodium caseinate molar ratio of 20 was added in the cuvette and the changes in surface tension were further measured as a function of time. Run is representative of two replicates.
Table 3.2  Equilibrium interfacial tension, interfacial pressure, and dilational elastic modulus of oil surface in contact with a solution of 0.001 % sodium caseinate before and after addition of EGCG at a molar ratio of 20 EGCG/protein. Values are the means of two replicates.

<table>
<thead>
<tr>
<th></th>
<th>Sodium caseinate</th>
<th>+ EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Tension (mN/m)</td>
<td>$13.3 \pm 0.2$ ^a</td>
<td>$13.9 \pm 0.4$ ^a</td>
</tr>
<tr>
<td>Surface pressure (mN/m)</td>
<td>$16.9 \pm 0.2$ ^a</td>
<td>$16.4 \pm 0.4$ ^a</td>
</tr>
<tr>
<td>Modulus (at 50 mHz)</td>
<td>$18 \pm 3$ ^a</td>
<td>$55 \pm 9$ ^b</td>
</tr>
</tbody>
</table>
Figure 3.5  Dilational modulus of sodium caseinate water/oil interface. Solutions contained sodium caseinate (O) or sodium caseinate in the presence EGCG (●). Measurements were conducted as a function of frequency (0.1 constant amplitude) (A), and as a function of amplitudes (50 mHz constant frequency) (B). Values are the means of two replicates.
At all oscillation frequencies and amplitudes, the elastic modulus in the presence of EGCG was higher than the one of only sodium caseinate. The elastic modulus of the oil-water interface covered with sodium caseinate was 18 mN/m. After addition of EGCG, the elastic modulus sharply increased to 55 mN/m. The results confirmed the association of EGCG with sodium caseinate at the interface and the complex created a surface more resistant to dilation.

Very little has been reported on the interfacial interactions between caseins and EGCG. However, the results are consistent with a recent study on the interactions between other proline rich proteins, human saliva proteins, and tea polyphenols, where it was noted that EGCG increased the dilational modulus, without causing any changes to the surface tension (Rossetti et al. 2013). Similarly, an increase in the dilational modulus of an air/water interface in the presence of EGCG was noted for a β-casein stabilized interface (Sausse et al. 2003).

### 3.5 Conclusions

This work clearly demonstrated that EGCG molecules associate with caseins when adsorbed at an oil-water interface, and such interfaces can load high ratios of EGCG to protein. Caseinate-stabilized emulsion can therefore be used as a platform for the delivery of tea polyphenols. The stability, size, and charge of the emulsion particles were not affected by the presence of EGCG. In addition, there were no differences in the amount of sodium caseinate adsorbed at the interface, although the complex formed affected the viscoelastic properties of the protein layer at the interface. The effect of complex formation on physical properties of the emulsion have never been reported in the past, and the findings could be of interest from technological and nutritional point of view.
CHAPTER 4:
ACID INDUCED DESTABILIZATION OF EMULSIONS PREPARED WITH SODIUM CASEINATE-EPIGALLOCATECHIN-GALLATE COMPLEXES

4.1 Abstract

Complexes of epigallocatechin-gallate (EGCG) and sodium caseinate adsorbed at the interface of oil in water emulsions may affect the stability and processing functionality of the emulsion droplets. The objective of this work was to determine the effect of the presence of these complexes at the oil-water interface on the colloidal behaviour of the emulsion during acidification. Emulsions were prepared with 7 % soybean oil, 0.35 % sodium caseinate, and 0, 2, or 6 mg/ml EGCG. The acid induced aggregation was studied in the presence of high methoxyl pectin at concentrations ranging from 0 to 0.45 %. In situ light scattering measurements demonstrated that the presence of EGCG at the interface lowered the pH of destabilization of sodium caseinate covered droplets, and caused changes in the processing properties of the emulsions. The complex formed at the interface also reduced the electroadsorption of high methoxyl pectin, and caused differences in emulsion microstructure at acid pH. In spite of the widespread utilization of EGCG as a bioactive ingredient in emulsion dairy drinks, this work illustrates how the presence of protein-EGCG complexes at the interface affects the processing behaviour of emulsion droplets.

Keywords: EGCG, sodium caseinate emulsions, HMP, stability, acid beverages.

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4.2 Introduction

For many years, a diverse range of food and pharmaceutical products with different nutritional and functional properties have been created using emulsion science and technology. Emulsions are a common type of carrier for the delivery of bioactives, and the structural design of emulsion delivery systems continues to gain attention.

A protein commonly used as emulsifier is sodium caseinate (Nac). Nac is derived from micellar casein and composed of a soluble mixture of four proteins (αs1, αs2, β and κ-caseins), excluding the inorganic phosphate and calcium components. In solution, Nac is a mixture of monomeric or small aggregated particles (Lucey et al. 2000; Surh et al. 2006). At neutral pH, Nac adsors on the surface of oil droplets and creates a polyelectrolyte layer at the interface leading to electrostatic and steric repulsion, which stabilizes the droplets (Dickinson 1989; Dickinson 1992; Dalgleish 1997). The net charge of Nac-emulsified droplets is neutral at pH values close to the proteins’ isoelectric point (pH=4.6), whereas at pH values below and above the pI, the droplets hold a positive and negative charge, respectively. Therefore, when the emulsion’s pH is lowered towards the pI of the protein, electrostatic and steric repulsions between the emulsion droplets are reduced. Close to the proteins’ isoelectric point, droplets destabilize, come closer together, and finally aggregate into a gel (Dalgleish 1997).

It is possible to stabilize Nac-coated droplets during acidification by the addition of pectins (Dalgleish and Hollocou 1997; Dickinson et al. 1998a; Bonnet et al. 2005; Liu et al. 2007). Pectins are polymers of 1, 4 D-galacturonic acid with different extents of esterification with methanol. Depending on their degree of esterification, pectins are categorized as either high or low methoxyl pectin. Owing to the ionization of the free carboxylic groups, pectin has a negative charge in mildly acidic conditions, and its pKa is around pH 3.5 (Sinha and Kumria 2001; McClements 2005). At
pH values slightly above the isoelectric point of Nacas, pectin molecules interact with caseins through electrostatic interactions between negatively charged high methoxyl pectin molecules and positively charged patches of protein, aiding to colloidal stabilization (Dalgleish and Hollocou 1997; Dickinson et al. 1998a; Bonnet et al. 2005; Liu et al. 2007). The degree of esterification and the distribution of the charges on the pectin chain will affect the colloidal stabilization behaviour, as different portions of the molecules will be available to interact with the solvent and provide steric stabilization.

Tea polyphenols have been reported to easily interact with proline rich proteins such as caseins (Papadopoulou and Frazier 2004; Jöbstl et al. 2006; Frazier et al. 2010; Haratifar and Corredig 2014). In a recent study we showed that epigallocatechin-gallate (EGCG), the major and most bioactive tea catechin, has a high affinity for Nacas at the oil/water interface and that EGCG can be loaded at the Nacas-stabilized interface (Sabouri et al. 2015). Hence, Nacas-stabilized emulsions may be used as a delivery vehicle for polyphenols (Sabouri et al. 2015). However, the stability and processing properties of Nacas-stabilized emulsions containing tea polyphenols at acidic pH needs to be investigated. In addition, the colloidal and physicochemical behaviour of the emulsions containing EGCG and Nacas in the presence of high methoxyl pectin (HMP) is not known.

The objective of this work was to determine the effect of the presence of EGCG on emulsions stabilized by Nacas during acidification, in the presence and absence of HMP. In this study advanced spectroscopic techniques were employed to determine emulsion’s colloidal and physicochemical changes during acidification in situ without dilution.
4.3 Materials and Methods

4.3.1 Emulsion Preparation

A Nacas solution was prepared by dispersing 1.25 % w/w Nacas (New Zealand Milk Products, Mississauga, Ontario, Canada) in ultrapure water. Sodium azide (0.02 % w/v) (Catalog No: S227I, Fisher Scientific, Fair Lawn, NJ, USA) was added to prevent bacterial growth. The mixture was continuously stirred for 2.5 h at room temperature. After an overnight storage at 4 °C, the solution was filtered through 0.8 μm filters (Millex-HV, Millipore Co., Billerica, MA, USA). 80 % w/w filtered Nacas solution was added to 20 % w/w soybean oil (purchased from Sigma Chemical Co. St. Louis, MO, USA) to prepare a final emulsion of 20 % w/w oil and 1 % w/w protein. The mixture was pre-homogenized for 2 min at 10000 rpm using an Ultra Turrax stand homogenizer (IKA T18 Basic, Germany) and were immediately processed thorough a microfluidizer (M-110EH, Microfluidics, MA, USA) with four passes and an operating pressure of 69 MPa. The particle size distribution of the oil droplets was measured by static light scattering (Mastersizer 2000S, Malvern Instruments Inc., Southborough, MA, USA), and the diameter (D3,2) obtained after homogenization of the NaCas emulsion was 140 ± 5 nm.

A commercial green tea polyphenol extract containing a high concentration of EGCG (min 94 %) was obtained from DSM Nutritional Products (Ayr, Ontario, Canada). EGCG solutions were made in ultrapure water fresh and added to Nacas-stabilized emulsion. The mixtures were then vortexed for 1 min and incubated at room temperature (22 °C) for 15 min before addition of pectin solution or ultrapure water for the treatments with no pectin. Pectin solutions were prepared by dispersing HMP (DE 71.4, unstandardized, CpKelco, San Diego, CA, USA) in 70 °C Milli-Q water followed by continuous stirring for 3 h and overnight storage at 4 °C. The pH of the pectin solutions was adjusted to 6.8 right before mixing with the emulsions with and without EGCG and the final
mixtures were vortexed for 1 min. Final emulsion formulations contained 7 % oil, 0.35 % Nacas, 0, 2 and 6 mg EGCG/ml emulsion and 0, 0.15, 0.3 and 0.45 % HMP. The two concentrations of EGCG (2 and 6 mg/ml) were chosen based on recent results, and corresponded to 90 and 66 % of total added EGCG adsorbed to the o/w interface (Sabouri et al. 2015).

4.3.2 Dynamic Light Scattering (DLS)

The change in the overall surface potential of the emulsions was measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Malvern, UK) equipped with a MPT-2 Autotitrator (Malvern Instruments Ltd., Malvern, UK) which enabled ζ-potential determination as a function of pH. Emulsions were diluted in 1:300 ratio using Milli-Q water to minimize multiple scattering effects. The measurements were conducted at 25 °C in disposable capillary cells (Malvern Instruments, UK). The autotitrator was programmed to lower the pH of samples in the capillary cell from their initial pH of preparation to pH 3.5 with 0.5 intervals using 0.01 and 0.001M HCl.

4.3.3 Diffusing Wave Spectroscopy (DWS)

DWS is well suited to study turbid systems such as emulsions in situ (Weitz et al. 1993; Alexander and Dalgleish 2006). By means of DWS, early stages of colloidal destabilization can be noted (Bonnet et al. 2005; Gancz et al. 2005; Ruis et al. 2006). DWS was utilized to characterize the stability and in situ colloidal properties of the Nacas-stabilized emulsions during acidification in the presence of different concentrations of EGCG and/or pectin.

The experimental set up used for DWS experiments in this study has been previously described (Alexander and Dalgleish 2004; Gancz et al. 2005). In brief, the samples were placed in a 5 mm optical glass cuvette (Hellma Canada Limited, Concord, Canada) immersed in a thermostatted
water bath at 30 °C. A 100 mW solid-state laser (532 nm) (Coherent, Santa Clara, CA, USA) illuminated the samples. To gradually lower the pH of emulsions, 0.3 % (w/v) glucono-δ-lactone (GDL) was added. GDL hydrolyses in solution to gluconic acid and gradually decreases the pH. Immediately after GDL addition, DWS measurements in parallel with continuous pH monitor using a pH meter (Accumet Research AR 15, Fisher Sci.) started and measurements were carried out at 22 °C until pH 4.5 was reached. During acidification, intensity fluctuations of the scattered light and the corresponding temporal autocorrelation function were collected continuously for each 2 min. Characteristic decay time ($\tau$) and diffusion coefficient were derived using DWSfit (Mediavention Inc., Guelph, ON, Canada). By following these parameters in situ, it was possible to determine the effect of EGCG on the acidification behaviour of Nacas emulsions with and without pectin.

4.3.4 Confocal Laser Scanning Microscopy (CLSM)

Confocal microscopy was employed to characterize the microstructure of the emulsions at acidic pH conditions. An upright Leica DM 6000B microscope connected to a Leica TCS SP5 system (Heidelberg, Germany) coupled with the Leica LAS AF Imaging software was used. 0.1 wt % Nile red was added to the soy bean oil and stirred at room temperature for 1 h to selectively stain the oil droplets during microscopy. The oil was then filtered through 0.45 μm nylon filters (Fisher Scientific) and used to prepare the emulsions. 0.11 % w/v GDL was added to the emulsions and the mixtures were vortexed for 30 s, then 100 μl of each emulsion formulation was transferred onto a concave microscopy slide and covered with a cover slip. The slides were incubated in room temperature for 16 h to reach pH 4.5 and then cooled on ice. Images were immediately taken using a 63× magnification oil immersion objective at 543 nm.
4.4 Results and Discussion

4.4.1 Charge Measurement Using DLS

During acidification, the overall charge of the protein drops, until close to the isoelectric point, when the molecules present at a net zero charge. This decrease in charge will affect electrostatic repulsion between the droplets as well as steric repulsion, as the proteins act as a hydrated polyelectrolyte layer around the oil droplets. The association of EGCG with sodium caseinate (Sabouri et al. 2015) may affect the charge distribution on the surface of the emulsion droplets, causing changes to the stability of the emulsions during acidification or changes to the charge driven interactions between casein proteins and HMP molecules. Differences in the behaviour of emulsions with EGCG compared to control will suggest the presence of EGCG-casein complex also at acidic pH.

Figure 4.1 illustrates the changes in ζ-potential of the oil droplets as a function of pH. All Nacas-stabilized emulsions with no pectin (filled circles) showed an initial negative potential near neutral pH (>6.0), and a decrease in the overall potential with decreasing pH, until pH of about 4.4, where the emulsion droplets reached a neutral potential. At pH <4.3 the control Nacas emulsions were positively charged. There was no significant effect of EGCG on the overall charge behaviour of Nacas-covered oil droplets over the entire pH range.

The presence of HMP caused a change in the charge behaviour with acidification; as the droplets maintained a negative potential (around – 20 mV) at pH values close to pI of the protein.
Figure 4.1 Values of $\zeta$-potential for emulsions containing A) No EGCG, B) 2 mg/ml EGCG, C) 6 mg/ml EGCG and 0 pectin (●), 0.15 % pectin (▽), 0.3 % pectin (■), and 0.45 % pectin (◇) during acidification. Data is the average of three independent experiments and error bars represent standard deviation.
This was consistent with the known electro-adsorption of pectin on the surface of the Nacas-stabilized oil droplets (Dalgleish and Hollocou 1997). It is known that the adsorption of negatively charged pectin to the interface causes the stabilization of the droplets during acidified conditions, via electrostatic and steric repulsion (Dalgleish and Hollocou 1997; Dickinson et al. 1998a; Bonnet et al. 2005; Surh et al. 2006). In the emulsions containing pectin, also, the presence of EGCG did not affect the overall charge behaviour.

4.4.2 Diffusing Wave Spectroscopy (DWS)

Using transmission DWS, it was possible to measure the changes in the diffusion coefficient of the emulsion droplets as a function of pH. The system’s diffusivity is affected by the physical properties of the oil droplets, such as the size and shape, the positional correlation of the droplets, which is influenced by their interactions, and also the viscosity of the continuous phase. Any changes in the dilution of the emulsion droplets may affect the aggregation behaviour of the oil droplets, therefore, acidification experiments were carried out in situ, without dilution. Previous work demonstrated that the presence of EGCG in these emulsions did not affect the particle size distribution measured, after dilution, by light scattering. The emulsions at neutral pH were stable, and did not show aggregation over time (Sabouri et al. 2015). To better identify the destabilization point, and compare all the treatments, normalized diffusion coefficients were evaluated as shown in Figure 4.2. A drastic change in the diffusion coefficient is indeed expected at the point where the electrostatic and steric repulsion is no longer sufficient to keep the droplets from aggregating.
Figure 4.2 Normalized diffusion coefficient values as a function of pH for NaCas-stabilized emulsions containing 0 (○), 2 (●), and 6 (●) mg/ml EGCG. Results are representative data for 4 separate experiments.
Control emulsion with no EGCG and pectin showed a constant diffusion coefficient down to pH 5.3, confirming the stability of such emulsions at pH near neutral (Gancz et al. 2005). At pH 5.3, there was a decrease in the diffusion coefficient, indicative of instability in the emulsion system. Instability and droplet aggregation of Nacas-stabilized emulsions at pH values close to the isoelectric point is attributed to the eliminated repulsive colloidal interactions between particles due to charge neutralization (Agboola and Dalgleish 1996; Dalgleish 1997). A similar trend was shown for emulsions containing EGCG, demonstrating that at pH values close to the proteins’ isoelectric point, emulsions containing both 2 and 6 mg/mL tea polyphenols also underwent surface charge neutralization and consequent aggregation.

Table 4.1 summarizes the differences in pH of aggregation. The pH value at which diffusion coefficient started to decrease shifted significantly from 5.2 ± 0.04 for control emulsions to 5.0 ± 0.03 and 4.9 ± 0.02 for emulsions containing 2 and 6 mg/mL EGCG, respectively. The presence of EGCG at the interface shifted the destabilization of the emulsion droplets to lower pH values. Since the charge distribution did not change in the presence of EGCG (Figure 4.1), these results suggest that even at acidic pH, EGCG is present at the interface; as the shift in stability to lower pH values was not caused by a change in the overall surface charge of the emulsion droplets, but by increasing steric repulsion. This may have occurred as a result of a change in the structure of the emulsion droplets’ interface, due to the association of EGCG with the caseins. It has been previously demonstrated that albeit the interfacial tension of a Nacas interface does not change in the presence of EGCG complexation, the viscoelastic properties are affected, and the interface becomes more elastic (Sabouri et al. 2015).
Table 4.1 pH of destabilization measured *in situ* during acidification for emulsions stabilized with sodium caseinate and containing different concentrations of EGCG. Values are the means of three replicates. Within a row, different superscript letters indicate statistical difference at p < 0.05.

<table>
<thead>
<tr>
<th>EGCG (mg/ml)</th>
<th>0</th>
<th>2</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.2 ± 0.04 $^a$</td>
<td>5.0 ± 0.03 $^b$</td>
<td>4.9 ± 0.02 $^b$</td>
</tr>
</tbody>
</table>
As HMP is known to electrostatically interact with Nacas at the interface at a pH near the isoelectric point of the protein, the presence of EGCG-Nacas complexes may influence the electroadsorption, by changing the structure of the protein layer at the interface. It is therefore expected that the ability of HMP to associate with the emulsion droplets may be affected by the addition of EGCG, causing a change in the ability of HMP to increase the steric repulsion between the oil droplets at the critical destabilization pH of the emulsions. Hence, the effect of EGCG at the interface was also studied in the presence of HMP.

The normalized diffusion coefficient values for emulsions containing 0.45 % HMP in the presence of 0, 2, and 6 mg/mL EGCG, are shown in Figure 4.3. The emulsion containing pectin with no EGCG started with a constant value which gradually increased at lower pH values and finally reached a plateau. At neutral pH, both the polysaccharide molecules and the protein-stabilized oil droplets were negatively charged and thus the unadsorbed pectin polymers caused depletion flocculation (Dickinson et al. 1991; Gancz et al. 2005; Surh et al. 2006). By lowering the pH towards the isoelectric point of the protein, positively charged patches start to appear on the Nacas layer at the interface enabling the negatively charged pectin molecules to adsorb to the interface. Thus the pH drop leads to an apparent increase in the system’s diffusivity (Gancz et al. 2005). These results were fully consistent with what shown in the literature (Dalgleish and Hollocou 1997; Bonnet et al. 2005; Gancz et al. 2005; Surh et al. 2006; Liu et al. 2007). A similar trend was observed for emulsions containing both pectin and EGCG, but the increase in the emulsions’ diffusivity was delayed significantly from pH 5.8 ± 0.05 in the emulsion with pectin in the absence of EGCG to 5.4 ± 0.02 and 5.3 ± 0.01 in the pectin containing emulsions containing 2 and 6 mg/mL EGCG, respectively (Table 4.2).
Figure 4.3 Normalized diffusion coefficient values as a function of pH for NaCas-stabilized emulsions containing 0.45 % HMP in the presence of 0 (☆), 2 (○), and 6 (●) mg/mL EGCG. Results are representative data for 4 separate experiments.
Table 4.2  pH onset of change of diffusion coefficient measured *in situ* during acidification for emulsions stabilized with sodium caseinate, containing various concentrations of EGCG and 0.45 % high methoxyl pectin. Values are the means of three replicates. Within a row, different superscript letters indicate statistical difference at p < 0.05.

<table>
<thead>
<tr>
<th>EGCG (mg/ml)</th>
<th>0</th>
<th>2</th>
<th>6</th>
</tr>
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<tr>
<td>pH</td>
<td>5.8 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>
Also the diffusivity increased at slower rates and the plateau was reached at lower diffusion coefficient values in a direct proportion to EGCG concentration (Figure 4.3). The results indicated that the EGCG-Nacas complexes at the oil/water interface interfered with HMP adsorption to the casein molecules thereby reducing HMP’s stabilizing/protective effect at acidic conditions. The exact same trends were seen for the emulsions containing 0.15 and 0.3 % HMP in the presence of 0, 2, and 6 mg/mL EGCG (data not shown). These results clearly demonstrated that EGCG was present at the interface at low pH and that it affected the ability of Nacas to interact with HMP.

4.4.3 Confocal Laser Scanning Microscopy (CLSM)

Microstructural analysis was carried out using confocal microscopy, to determine the effect of EGCG and pectin on the emulsions at acidic pH and to confirm the results obtained by DWS. A lipid specific fluorescent dye was used for the analysis; the bright areas correspond to the signal derived from the oil droplets. Figure 4.4 depicts microstructure images of Nacas-stabilized emulsions containing 0, 2 and 6 mg/mL EGCG as well as 0, 0.15 and 0.45 % HMP. Samples were imaged at pH 4.5. Emulsions at neutral pH were stable, and showed very little signal when observed by confocal imaging, due to the small size of the emulsion droplets (data not shown). On the other hand, large oil droplets were observed for control emulsion at pH 4.5, because of droplet aggregation and coalescence (Figure 4.4.A). The same emulsion in the presence of different amounts of EGCG without pectin showed smaller droplets, and more flocculation (Figure 4.4.B and 4.4.C).

Therefore, although all three Nacas-stabilized emulsions (0-0, 2-0 and 6-0) were destabilized and formed a gel at this pH (evident from macroscopic observation as well), the presence of EGCG caused a change in the microstructure: there were fewer large droplets, with less coalescence, and
**Figure 4.4** Representative images of emulsions at pH 4.5 taken by Confocal Laser Scanning Microscopy. Scale bar corresponds to 50 μm. Emulsions contain 0 (A–C), 0.15% (D–F), 0.45% (G–I) HMP, and 0 (A,D,G), 2 mg/ml (B,E,H), and 6 mg/ml (C,F,I) EGCG.
the samples showed a packed network of aggregated particles. It is important to note that previous studies on the interfacial properties of EGCG-Nacas interfaces showed a significant increase in the elastic modulus of the emulsions compared to the same interfaces without EGCG. This would lead to interfaces more resistant to coalescence (Sabouri et al. 2015).

The presence of 0.15 % HMP still showed extensive coalescence in the emulsions with no EGCG at pH 4.5 (Figure 4.4.D) and a higher concentration of HMP showed improved protection against acid-induced aggregation (Figure 4.4.G). Emulsions containing both EGCG and 0.15 % pectin (Figure 4.4.E and 4.4.F) still showed extensive flocculation at pH 4.5. With 0.45 % HMP, emulsions containing EGCG showed increased stability at low pH (Figures 4.4.H and 4.4.I). The same trends were seen in the confocal images of emulsions containing 0, 2, and 6 mg/ml EGCG in the presence of 0.3 % HMP (data not shown).

Microstructural observations also seemed to suggest that the presence of EGCG increased the extent of destabilization of emulsions containing pectin at pH 4.5, and this would confirm the light scattering measurements shown in Figures 4.3, which suggested an effect of EGCG on the stabilization behaviour of HMP. These results were also consistent with visual observations.

4.5 Conclusions

This study demonstrated that the formation of complexes at the interface between EGCG and Nacas affect the colloidal behaviour of the oil droplets. The presence of EGCG caused a shift in the pH of destabilization of the emulsions to lower pH values, and caused less droplet coalescence at pH 4.5. However, there were no differences in the ζ-potential profile of the emulsion droplets as a function of pH, in the presence of EGCG. Hence, it was concluded that the increased stability was caused by an increased steric repulsion between the droplets caused by the presence of the
EGCG-Nacas complexes. EGCG adsorption at the interface of the Nacas emulsions also affected the electrosorption of the HMP during acidification, further demonstrating that EGCG-sodium caseinate complexes were present at the interface at the critical pH of destabilization. However, HMP caused an increased stabilization of the emulsion.

Since Nacas-stabilized emulsions could be potentially engineered as a delivery vehicle for tea catechins, these novel findings are of importance for the development of new structurally designed food products with improved nutritional functionality. The knowledge on the stability and colloidal behavior of such systems during acidification will also help to better predict structural changes occurring to these emulsions during digestion.
CHAPTER 5
IN VITRO DIGESTION OF EMULSIONS LOADED WITH EPIGALLOPECTECHIN GALLATE

5.1. Abstract
The digestion behaviour of a structured emulsion interface loaded with epigallocatechin-gallate (EGCG) was investigated. Oil in water emulsions were prepared with 0.35% sodium caseinate, 0, 2 and 6 mg/ml epigallocatechin-gallate (EGCG) and 0.45% high methoxyl pectin (HMP). Emulsions were subjected to a static in vitro digestion, simulating the three stages of human upper gastrointestinal digestion, to investigate EGCG-salivary protein associations, and the effect of incorporated EGCG on gastric proteolysis and duodenal lipolysis. Confocal microscopy observations demonstrated that the addition of HMP reduced saliva’s mucin-induced aggregation of the emulsions. Gel electrophoresis results indicated that sodium caseinate was fully hydrolysed, regardless of the presence of EGCG or HMP. The presence of EGCG reduced the extent of free fatty acid release during the duodenal phase. The findings from this study suggest that sodium caseinate-stabilized emulsions can be employed as a platform for delivery of EGCG.
5.2 Introduction

Tea polyphenols have been associated with several health-promoting effects such as improving neurologic functions, preventing the development of cardiovascular disease, or having antioxidant, anticarcinogenic, anti-inflammatory, antibacterial, and lipid-lowering properties (Khan and Mukhtar 2007). Amongst the tea catechins, the main compounds are (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate (EGCG). EGCG is the major and most abundant polyphenol in green tea and it has been reported to be the main contributor to these potential health benefits (Khan and Mukhtar 2007; Velayutham et al. 2008). Catechins from tea are poorly bioavailable when orally administered (Lambert and Yang 2003), mainly due to their instability under digestive conditions leading to poor bioaccessibility (Record and Lane 2001; Green et al. 2007), poor permeability across the intestine leading to poor absorption (Vaidyanathan and Walle 2001), high rates of efflux back into the intestinal lumen after absorption (Vaidyanathan and Walle 2001; Cai et al. 2002), extensive and rapid metabolism after absorption and high systemic clearance (Cai et al. 2002; Fang et al. 2006).

Addressing the bioavailability challenges of tea polyphenols as bioactives has drawn a great deal of scientific and industrial attention. The ability of polyphenols to associate with proteins has been well documented. In fact, polyphenols tend to bind to non-globular proteins with extended conformation containing high amounts of proline such as salivary proteins and caseins (Siebert et al. 1996; Richard et al. 2006).

Complex formation of phenolics with milk proteins has been used to develop carriers for targeted delivery (Sahu et al. 2008; Shpigelman et al. 2012). The affinity of tea catechins to milk proteins was reported to boost catechin’s stability against thermal treatment (Song et al. 2015).
Furthermore, in a recent study, complexation of green tea polyphenols with milk proteins was shown to improve catechin’s bioaccessibility (Lamothe et al. 2014).

Sodium caseinate-stabilized emulsions could be an efficient platform for the delivery of tea polyphenols. It has been demonstrated that sodium caseinate, adsorbed at the oil water interface, can associate to high concentrations of EGCG (Sabouri et al. 2015); furthermore, the addition of high methoxyl pectin (HMP) to these emulsions improves their stability at low pH (Sabouri and Corredig 2016). Objective of this work was to study the fate of EGCG-loaded sodium caseinate emulsions during simulated in vitro digestion.

Although human or animal feeding studies offer evidence of the highest quality (Singh and Sarkar 2011), in vitro digestion models can be utilized for rapid screening of a large number of samples to identify differences between treatments. Such in vitro models are also suitable for mechanistic studies and hypothesis building owing to their reproducibility, ability to select controlled conditions and easy sampling at sites of interest (Singh and Sarkar 2011; Minekus et al. 2014). In this study, a standardised digestion model based on the current state of knowledge of in vivo digestion conditions was employed to investigate the digestion behavior of the EGCG-loaded emulsion systems.

In the oral cavity, polyphenols interact with proteins. Tea polyphenols and salivary proteins form insoluble complexes causing precipitation of proteins and thereby decreasing lubrication in the oral cavity. These complexes seem to be linked to the puckering mouthfeel called astringency (Breslin et al. 1993; Baxter et al. 1997; Jöbstl et al. 2004). The inclusion of tea catechins in milk-tea complexes reduces their sensation of astringency and bitterness (Lesschaeve and Noble 2005; Bohin et al. 2013). In the current study, salivary interactions were evaluated, as they play an important role in the physical and chemical changes occurring to EGCG loaded emulsions.
It has been suggested that green tea catechins inhibit diet-induced obesity and reduce adipose tissue mass via lipase inhibition (Nakai et al. 2005; Wolfram et al. 2005; Wolfram et al. 2006). Beside lipase, tea polyphenols have been reported to inhibit other digestive enzymes, including proteases (Rohn et al. 2002). Protease inhibition has been stated as an anti-nutritional factor for polyphenols (McDougall et al. 2008). Therefore, the digestibility of the oil and protein from the emulsions' matrices was assessed.

In this work, the effect of the presence of EGCG and pectin, as well as the original pH of the emulsions on their physical properties in the oral stage and after digestion, also the extent of proteolysis and lipolysis were investigated. Results will contribute to designing novel dairy/emulsion-based vehicles for polyphenols to enable the delivery of additional nutritional functionality in foods.

5.3 Materials and Methods

5.3.1 Materials

Pancreatin from porcine pancreas (4xUSP specifications, P1750- lipase activity: 28.9±0.3 TBU/mg determined by tributyrin assay) (Carriere et al. 1993), porcine bile extract (B8631, 1.34 mmol bile salts per g of extract powder determined by a colorimetric assay) (Minekus et al. 2014), pepsin from porcine gastric mucosa (P7000- 1064 units/mg protein provided by the manufacturer), phospholipase A2 (PLA2) from porcine pancreas (P6534- 6.7 mg protein/mL (Biuret), 903 unit/mg protein provided by the manufacturer), mucin from porcine stomach (M2378), uric acid (U2625), hexane (>95%, spectrophotometric grade), analytical grade hydrochloric acid (HCl), soybean oil (S7381) and Nile Red (72485), were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO, USA).
Urea (U15), sodium Azide (S2271), sodium sulfate (Na₂SO₄-S421), sodium hydroxide (NaOH-S320), sodium bicarbonate (NaHCO₃-S233), sodium chloride (NaCl – S671), calcium chloride dehydrate (CaCl₂·(H₂O)₂-C79), potassium chloride (KCl-P217), monopotassium phosphate (KH₂PO₄-P285), magnesium chloride hexahydrate (MgCl₂·6H₂O-BP214) and ammonium carbonate ((NH₄)₂CO₃-A656) were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Fat free soybean phospholipids (ALCOLEC® PC75) with 75% phosphatidylcholine were provided by American Lecithin Company (Oxford, CT, USA). The non-esterified fatty acid kit (NEFA-HR2) was purchased from Wako Pure Chemical Industries (Richmond, VA, USA). Sodium caseinate (NaCas) (NaCas 180) was purchased from Fonterra Inc. (Rosemont, IL, USA). Ultrapure water (Millipore Corp., Bedford, MA, USA) was used in all reagents and buffers, unless stated differently.

5.3.2 Emulsion Preparation

A sodium caseinate-stabilized oil in water emulsion of 20% w/w soybean oil and 1% w/w protein with an average diameter (d₄₃, as measured using Mastersizer 2000S, Malvern Instruments, Southborough, MA, USA) of 0.24 ± 0.01 μm was prepared as previously described (Sabouri et al. 2015). In brief, a solution of sodium caseinate, containing 0.02% w/v sodium azide was filtered through a 0.8 μm filter (Milllex-HV, Millipore Co., Billerica, MA) and added to soybean oil. The mixture was passed through a microfluidizer (M-110EH, Microfluidics, MA, USA) with four passes and a pressure of 69 MPa.

EGCG solution was prepared by dissolving a green tea polyphenol extract containing 94% EGCG (DSM Nutritional Products, Ayr, Ontario, Canada) in ultrapure water. A high methoxyl pectin (HMP) solution was prepared by dispersing the HMP (DE 71.4, unstandardized, CpKelco, San
Diego, CA, USA) in 70 °C ultrapure water, stirring for 3 h and storing overnight at 4 °C. The pH of the solution was adjusted to 6.8.

Freshly prepared EGCG solutions were mixed with the primary emulsion and then the mixture was incubated at room temperature (22°C) for 15 min before mixing with the pectin solution. The concentration of EGCG and pectin solutions and their ratio to the primary emulsion were set such that final emulsion formulations of 7% oil, 0.35% sodium caseinate containing 0, 2 and 6 mg EGCG/ml emulsion with or without 0.45% HMP were obtained. For the emulsions containing no EGCG or no pectin, ultrapure water was used instead of their portion of EGCG or pectin solutions. The concentrations of EGCG and HMP were chosen based on previous results (Sabouri et al. 2015; Sabouri and Corredig 2016). For emulsions containing pectin, aliquots were acidified with glucono delta lactone to pH 5 (Sabouri and Corredig 2016).

5.3.3 In Vitro Digestion

To mimic the fed state in the human GIT, a three-step in vitro static digestion model was adapted from the literature (Versantvoort et al. 2005; Minekus et al. 2014) with some modifications suited to the food system being investigated. Emulsion samples were submitted to simulated oral, gastric, and duodenal environments and after each stage aliquots were taken for further analysis.

5.3.4 Oral Phase

To investigate the interaction of salivary proteins such as mucin with the interface-bound EGCG, the emulsions were exposed to a simulation of the human oral phase of digestion. Simulated salivary fluid (SSF) was composed of 12 mM KCl, 2 mM KSCN, 7.4 mM NaH$_2$PO$_4$, 4 mM Na$_2$SO$_4$, 5 mM NaCl, 0.2 mM NaHCO$_3$, 3 mM urea, 0.09 µM uric acid, and 3% w/v porcine mucin, and the
The final pH was adjusted to 6.8 ± 0.2. Each emulsion was placed in an amber glass jar with SSF at a 1:1 ratio (Minekus et al. 2014). The jars were incubated at 37°C for 2 min in a horizontal shaking water bath (model 89032-226, VWR International, USA) with a 4 Hz (220 rpm) shaking speed. The mucin concentration was higher than that found in human saliva, as the viscosity of human saliva can be reproduced with porcine or bovine mucin at concentrations of about 3% w/w (Sarkar et al. 2009b; Chung et al. 2012). The simulated saliva also did not contain α-amylase, as no starch was present in the emulsion system.

5.3.5 Gastric Phase

Immediately after the oral phase, simulated gastric fluid (SGF) containing salt solutions and enzyme was added to the jars in a 1:1 ratio of SGF to bolus (Minekus et al. 2014), and the incubation under the same condition as oral phase was continued for 30 min. SGF was composed of 6.9 mM KCl, 0.9 mM KH₂PO₄, 25 mM NaHCO₃, 47.2 mM NaCl, 0.1 mM MgCl₂(H₂O)₆, 0.5 mM (NH₄)₂CO₃, 0.15 mM CaCl₂, and pH was adjusted to 3 using 6M HCl. Porcine pepsin was freshly dissolved in SGF to obtain a final concentration of 2000 U/ml in the gastric digestion mixture of SGF and bolus. Aliquots (chyme) were withdrawn from the jars at the end of 30 min of gastric processing for protein analysis (see below). Proteolysis was stopped by raising the pH of aliquots to 6.5 using 1 M NaHCO₃.

5.3.6 Duodenal Phase

Simulated duodenal fluid (SDF) containing salt solutions, pancreatin, bile salts, phospholipids, and phospholipase A₂ (PLA₂) with a 1:1 SDF:chyme ratio (Minekus et al. 2014) was added to the jars immediately after the gastric phase and incubation continued for 2 h. SDF contained 6.8 mM KCl,
0.8 mM KH$_2$PO$_4$, 85 mM NaHCO$_3$, 38.4 mM NaCl, 0.33 mM MgCl$_2$(H$_2$O)$_6$, 0.6 mM CaCl$_2$(H$_2$O)$_2$, and pH adjusted to 7 with 1M NaOH. A lower pancreatin concentration was chosen compared to previous reports (Minekus et al. 2014), to decrease lipolysis kinetics during the *in vitro* digestion (Malaki Nik et al. 2010). Pancreatin was freshly dissolved in SDF providing a final concentration of 5 mg pancreatin/mL of SDF-chyme mixture, equivalent to 144.5 TBU/mL SDF-chyme mixture. The bile salt concentration in the final digestion mixture of SDF-chyme was 10 mM. Phospholipid particles were dissolved in bile solution by means of sonication providing 1 mM final concentration in the SDF-chyme mixture. Phospholipase A$_2$ was also used in the current study to hydrolyze phospholipids and mimic the physiological enzymatic profile of pancreatic lipases. The concentration of PLA$_2$ in the SDF-chyme mixture was 0.84 µg/mL; this concentration was sufficient to hydrolyse all the phospholipid in SDF-chyme mixture.

### 5.3.7 Confocal Laser Scanning Microscopy (CLSM)

Confocal microscopy was employed to characterize the microstructure of the emulsions after being exposed to simulated saliva. Nile red (0.1% w/w) was added to the soybean oil and stirred at room temperature for 1 h to selectively stain the oil droplets. The oil was filtered through 0.45 µm nylon filters (Fisher Scientific) and used to prepare the emulsions. Emulsions (100 µl) before and after mixing with SSF were transferred onto a concave microscopy slide. Images were immediately taken using a 63× magnification oil immersion objective at 543 nm. An upright Leica DM 6000B microscope connected to a Leica TCS SP5 system (Heidelberg, Germany) coupled with the Leica LAS AF Imaging software was used.
5.3.8 Dynamic Light Scattering (DLS)

The overall surface potential of the emulsions before and after in vitro oral processing was measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd., Malvern, UK). To minimize multiple scattering effects, emulsions before and after the oral phase were diluted with Milli-Q water or simulated salivary fluid at a ratio of 1:300. The measurements were conducted at 25 °C in disposable capillary cells (Malvern Instruments, UK).

5.3.9 Particle Size Measurements

A laser diffraction instrument (Mastersizer 2000S, Malvern Instruments Inc., Southborough, MA) was employed to determine the particle size distribution of the emulsions before and after incubation with SSF and after completion of the three stages of in vitro digestion. Refractive indices of 1.46 and 1.33 were used for the emulsion droplets and dispersant (i.e. water), respectively.

5.3.10 Proteolysis Profiles

To investigate the effect of the EGCG-sodium caseinate complexes at the emulsion interfaces on the sodium caseinate proteolysis, aliquots of gastric chyme were submitted to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) under reducing conditions.

The resolving gel (15%) was composed of 4.8 mL of water, 5 mL of resolving buffer pH 8.9 (1.5M Tris, 0.2% SDS, pH 8.9), 10 mL of acrylamide bis (30%), 200 μL of ammonium persulfate (10%), and 14 μL of TEMED. The stacking gel (4%) was composed of 3.56 mL of water, 5 mL of stacking gel buffer pH 6.7 (0.1M Tris, 0.2% SDS, pH 6.7), 1.34 mL of acrylamide bis (30%), 100 μL of ammonium persulfate, and 7 μL of TEMED. Aliquots (100 μL) of gastric digestates were mixed
with 200 μL of sample buffer composed of 3.9 mL of 1 M Tris-HCL (pH 6.8), 2.0 mL of 75% glycerol, 1.6 mL of 10% w/v SDS, 0.4 mL of 2-mercaptoethanol, and 0.1 mL of 1% bromophenol blue in water. The mixtures were heated at 95 °C for 5 min while shaking using the Eppendorf Thermomixer 5436 (Eppendorf-Netheler-Hinz GmbH, 22331 Hamburg, Germany). After cooling for 15 min, samples were centrifuged at 10,600 rpm for 10 min using an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY) and aliquots (10 μL) of the subnatant were loaded onto the gels and run at a constant voltage of 200 V in a Bio-Rad mini-protein electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A sample of non-digested emulsion diluted to the same dilution as the gastric chyme was used as control for sodium caseinate. After each run, the gels were immediately stained for 30 min using Coomassie blue R-250 and destained for 2 h with a destaining solution of 45% ultrapure water, 45% methanol and 10% acetic acid, with two changes, then destained overnight with a destaining solution of 72.5% ultrapure water, 22.5% methanol and 5% acetic acid. A SHARP JX-330 scanner (Amersham Biosciences, Quebec) was employed to scan the gels.

5.3.11 Lipolysis Determination

An enzymatic colorimetric assay was employed to measure the extent of lipid hydrolysis (NEFA kit, Wako Diagnostics, VA, USA) (Shimizu et al. 1980). Immediately after the duodenal phase, 100 μL of digestate was mixed with 100 μL HCl (0.1M), to stop lipolysis, and then 900 μL hexane was added to the mixture (Reis et al. 2008; Malaki Nik et al. 2010). The mixture was mixed and centrifuged in an eppendorf centrifuge (Brinkmann Instruments, Westbury, NY) at 13000 g for 30 min, and tested according to manufacturer’s instructions. Water and water solutions of 2 and 6 mg EGCG /ml were also submitted to the same in vitro digestion system and the percent FFA of their
final digestate was measured via NEFA kit and was used as controls for counteracting the possible optical density contribution of digestive juices and EGCG. Standards were prepared using oleic acid with concentrations ranging from 0.1 to 1 mM.

5.3.12 Statistical Analysis

All experiments were conducted in three independent replicates and their average value with a standard error was reported. In the case of images, representative images of three independent replicates are shown. To evaluate treatments for statistically significant differences, Tukey’s multiple comparisons testing in XLSTAT software was used. Significance was considered at p<0.05.

5.4 Results and Discussion

5.4.1 Interactions of EGCG and Salivary Proteins

The microstructure of emulsions containing 0, 2 and 6 mg/mL of EGCG before and after simulated oral digestion is shown in Figures 5.1, 5.2 and 5.3, respectively. The particle size distribution of all emulsions, as measured using integrated light scattering, is summarized in Figure 5.4.

Before the in vitro oral stage, the emulsions at neutral pH, regardless of the presence of EGCG, and in the absence of HMP, showed no aggregation (Figures 5.1A, 5.2A and 5.3A). At this pH, the emulsion droplets had a monomodal distribution with a diameter below 1 µm (Figure 5.4A-C). In the presence of HMP, the emulsions before oral digestion showed flocculation by depletion, regardless of the presence of EGCG (Figures 5.1B, 5.2B and 5.3B).
Figure 5.1 Representative images of emulsions containing 0 mg/ml EGCG, before (A, B, C) and after (D, E, F) in vitro oral processing taken by Confocal Laser Scanning Microscopy. Scale bar corresponds to 50 µm. No HMP added, pH 6.8 (A, D); pH 6.8, 0.45% HMP (B, E); pH 5.0, 0.45% HMP (C, F).
Figure 5.2 Representative images of emulsions containing 2 mg/ml EGCG, before (A, B, C) and after (D, E, F) *in vitro* oral processing taken by Confocal Laser Scanning Microscopy. Scale bar corresponds to 50 µm. No HMP added, pH 6.8 (A, D); pH 6.8, 0.45% HMP (B, E); pH 5.0, 0.45% HMP (C, F).
**Figure 5.3** Representative images of emulsions containing 6 mg/ml EGCG, before (A, B, C) and after (D, E, F) *in vitro* oral processing taken by Confocal Laser Scanning Microscopy. Scale bar corresponds to 50 µm. No HMP added, pH 6.8 (A, D); pH 6.8, 0.45% HMP (B, E); pH 5.0, 0.45% HMP (C, F).
Figure 5.4 Particle size distribution of emulsions containing 0 (A,D,G), 2 (B,E,H) and 6 mg/ml (C,F,I) EGCG. Emulsions at pH 6.8 (A-F) or at pH 5.0 (G-I) and with no pectin HMP (A-C) or 0.45% HMP (D-I). Particle size distribution was measured after extensive dilution either before (●) or after (□) *in vitro* oral processing. Results are representative data for three separate experiments.
Depletion flocculation caused the formation of large dark areas depleted of oil droplets with a network of structured oil droplets. With dilution, the emulsions showed a particle size distribution similar to that of the original control emulsion (Figure 5.4 D-F), confirming the presence of depletion flocculation (Dickinson et al. 1991; Surh et al. 2006).

Microstructural observations of acidified emulsions suggested that there was a decreased extent of aggregation compared to the same emulsions at neutral pH (i.e. comparing Figure 5.1C to Figure 5.1B). The increase in stability was well aligned with previous reports: at acidic pH, HMP form complexes with sodium caseinate at the interface, contributing to increased stability (Dickinson et al. 1998b; Bonnet et al. 2005). Compared to the same emulsions at neutral pH, the emulsions containing HMP at pH 5 showed a decreased extent of flocculation also in the presence of EGCG (Figure 5.2C and 5.3C). These results were also confirmed by light scattering, where at pH 5, the emulsions containing HMP had a similar average droplet size distribution (Figure 5.4G-I) to the emulsions at neutral pH, with the majority of droplets <1 µm of diameter.

After exposure to the simulated oral fluid, extensive aggregation was observed in all emulsions. At neutral pH and in the absence of EGCG, aggregation was noted both with microscopy (Figure 5.1D and 5.1E) as well as with light scattering (Figure 5.4A and 5.4D). The aggregation was caused by the ions and mucin molecules present in the simulated oral fluid. The screening of the charges caused a decrease in the charge repulsion between droplets, as confirmed by the decrease in the electrophoretic mobility of the emulsion droplets after the addition of simulated oral fluid (ζ-potential data not shown). In the presence of simulated saliva, depletion flocculation may also occur because of the presence of unadsorbed negatively charged mucin. Mucin-induced depletion has been reported for sodium caseinate and β-lactoglobulin-stabilized emulsions at neutral pH (Sarkar et al. 2009b; Ritzoulis et al. 2012). In the case of sodium caseinate-stabilized emulsions, a
critical concentration of about 0.5 % (w/w) of porcine gastric mucin has been shown to cause depletion flocculation (Vingerhoeds et al. 2005; Ritzoulis et al. 2012). Close proximity of the flocculated droplets will also lead to coalescence, as shown by the large oil droplets visible in the confocal images (Figure 5.4).

With the addition of EGCG (Figures 5.2 and 5.3), regardless of the presence of HMP and/or pH, emulsions showed a larger extent of aggregation after oral in vitro digestion (Figures 5.2D-F and 5.3D-F compared to Figures 5.1D-F). The same trend was also observed in the particle size distribution of emulsions, with the droplet size shifting towards larger sizes from emulsions with no EGCG (Figures 5.4A,D, and G) to emulsions with 2 mg/ml of EGCG (Figures 5.4B, E, and H) and 6 mg/ml of EGCG (Figures 5.4C, F, and I). This could suggest that, in addition to the screening effect of the ions present in the simulated oral fluid and mucin-induced flocculation, the formation of complexes between EGCG and mucin also caused extensive aggregation during in vitro oral processing.

In the presence of EGCG and HMP and at neutral pH (Figures 5.4E and 5.4F), the particle size distribution was quite similar to that of the same emulsions without HMP (Figures 5.4B and 5.4C), but the microstructure showed much larger areas depleted of oil droplets, indicating phase separation (Figures 5.2E and 5.3E compared to Figures 5.2D and 5.3D). Furthermore, the particle size distribution was similar between the acidic and neutral emulsions containing HMP and EGCG (Figure 5.4), albeit their microstructure was quite different. Although the acid emulsions also showed aggregation under simulated salivary conditions, the extent of aggregation appeared to be less when compared to emulsions with no HMP or the ones at neutral pH, containing un-adsorbed HMP (Figures 5.2F and 5.3F compared to Figures 5.2E, 3E, 5.2D and 5.3D).
The results indicated that changes in the amount of EGCG or the presence of HMP and pH are important factors that affect not only the aggregation state of the emulsions, but also the microstructure during oral processing. It was clear that HMP and mucin could cause phase separation of the oil droplets, but, more importantly, that EGCG interacted with the mucin molecules forming large aggregates. These findings demonstrate that fine tuning of the composition of the emulsion is critical to the development of beverages containing high loads of EGCG. Indeed, different physical properties can be achieved before or after oral consumption and it may be possible to reduce astringency and obtain products with different sensory attributes.

5.4.2. Proteolysis

The presence of residual polypeptides after incubation with the oral, followed by gastric simulated fluids, was tested by performing SDS-PAGE electrophoresis on the residual digestate after the gastric stage. After in vitro gastric digestion, no residual bands were noted in the gel, regardless of HMP, EGCG or pH treatment (data not shown).

It has been previously suggested that polyphenols may have inhibitory effects on digestive enzymes during digestion due to their interactions with proteins (He et al. 2007). These results indicated that complex formation between EGCG and caseinate at the interface did not cause the casein molecules to be less accessible to gastric pepsin. The presence of HMP in the aqueous phase or at the interface of the emulsions did not affect caseinate proteolysis, as both emulsions originally at pH 6.8 or 5.0 showed hydrolysis after digestion. These results were in full agreement with recent investigations on in vitro proteolysis of milk-EGCG or black tea and milk indicating no inhibitory effect induced by EGCG on casein hydrolysis (van der Burg-Koorevaar et al. 2011; Haratifar et al. 2014). It is important to note that in this work the distribution of peptides after digestion was
not tested. Possible changes in the peptide distribution due to the presence of complexes between EGCG, HMP and proteins, should be further evaluated, as it could be of biofunctional and nutritional importance.

5.4.3 Particle Size Distribution of Final Digestates

To investigate the stability and structural changes of emulsions containing EGCG and pectin at the end of the \textit{in vitro} digestion, their particle size distribution was measured (Figure 5.5). All emulsions after digestion, regardless of presence of EGCG and HMP, or the initial pH, showed complex size distributions with average diameters much larger than those of the original emulsions before digestion ($d_{4,3}$ of 0.24 ± 0.01 µm). Although after \textit{in vitro} oral processing (Figure 5.4) the majority of the droplets where still below 1 µm, at the end of three consecutive stages of our \textit{in vitro} digestion model, the emulsions were highly aggregated, with two main droplet sizes, between 1-10 µm and >100 µm of diameter.

5.4.4 Lipolysis

To study the effect of interface structure on the emulsion’s lipid digestion, the free fatty acid (FFA) content in the final \textit{in vitro} digestates was measured as shown in Figure 5.6. Independently of pH or presence of HMP, less fatty acids were released from emulsions containing EGCG compared to emulsions without EGCG, with a statistically significant decrease between control and emulsions containing 6 mg/ml EGCG. The same trend was noted for emulsions containing HMP, regardless of the initial pH. The presence of HMP in the emulsions did not significantly change the lipolysis level, regardless of the emulsion’s pH.
Figure 5.5 Particle size distribution of final digestates containing 0 (A), 2 (B) and 6 (C) mg/ml EGCG with 0 pectin (O), 0.45% pectin at neutral pH (▲) and 0.45% pectin at pH = 5 (■). Results are representative data for three separate experiments.
**Figure 5.6** Free fatty acid released after *in vitro* duodenal digestion (preceded by oral and gastric) for emulsions containing 0, 2 and 6 mg/ml EGCG, and without HMP (A), or with 0.45% HMP added (B,C) and adjusted to pH 6.8 (A,B) or pH 5.0 (C). Values are the means of three independent experiments and error bars represent standard errors. Letters indicate statistical differences between all treatments at p < 0.05.
In a recent study, inclusion of tea polyphenols into dairy products (milk, yogurt and cheese) was reported to lower the release of free fatty acids during in vitro digestion. The authors suggested that tea polyphenol-milk protein complexation enhances the stability of polyphenols against intestinal conditions, protecting their biofunctional properties (Lamothe et al. 2014). This study clearly indicated that the presence of EGCG hindered lipid hydrolysis. The mechanism underlying the lipid-lowering effect of tea polyphenols is still under debate. Several studies reported an inhibitory activity against lipase for tea polyphenols (Nakai et al. 2005; Gondoin et al. 2010; Cha et al. 2012). Binding of tea polyphenols close to the active site of lipase may be responsible for the reduced enzyme activity (Raghavendra et al. 2007). In addition, in the small intestine, EGCG interacts with phosphatidylcholine (Shishikura et al. 2006), causing profound changes to the composition of the interface and of the mixed micelles. Green tea catechins also were shown to inhibit pancreatic phospholipase A_2 (PLA_2) a key enzyme in the hydrolysis of phosphatidylcholine (Wang et al. 2006).

It was concluded that presence of ECGG hindered lipid hydrolysis. Further analysis is warranted to confirm and clarify the underlying mechanisms.

**5.5 Conclusions**

The formation of complexes between sodium caseinate, EGCG, and high methoxyl pectin (HMP) at the oil-water interface can be modulated to obtain complexes with different physical properties, which can be fine tuned to cause differences in sensory perception, and may reduce the catechin’s perceived astringency. It is important to note that oral processing is often excluded from in vitro digestion studies, justified by the short transit time of liquid food in the oral cavity. The results from this study show that extensive physical changes are induced by saliva components. This
stresses the importance of including the oral processing stage in *in vitro* digestion models for emulsions, given that emulsion stability, colloidal and rheological behaviour are determinant factors in their sensory, digestive and absorptive properties.

The presence of EGCG in the emulsions did not inhibit proteolytic activity, but reduced the fatty acid release in the *in vitro* duodenal stage. Sodium caseinate-stabilized emulsions can be ideal carriers for tea polyphenols. The structures obtained could be employed to develop new emulsion-based nutritional beverages, with important biological functionality. These emulsions may also reduce lipid digestion, although *in vivo* evidence is still needed.
CHAPTER 6
SODIUM CASEINATE STABILIZED EMULSIONS AS A DELIVERY SYSTEM FOR EPIGALLOCATECHIN-GALLATE: BIOACCESSIBILITY, ANTI-PROLIFERATIVE ACTIVITY AND INTESTINAL ABSORPTION

6.1 Abstract

The objective of this study was to investigate the effect of complexation of epigallocatechin-gallate (EGCG) to a caseinate-stabilized oil-water interface on the bioefficacy of EGCG. Emulsions with final concentration of 7% soy oil, 0.35% sodium caseinate, 0, 2 and 6 mg/mL EGCG and 0.45% high methoxyl pectin were prepared and digested using a static three stage in vitro model. The post digestion recovery (bioaccessibility), anti-proliferative activity, and intestinal uptake of EGCG are reported. Higher amount of intact EGGC was present in the digestate of emulsions containing EGCG compared to their equivalent solutions after digestion. It was concluded that incorporation of EGCG in sodium caseinate emulsions improved its bioaccessibility. This was confirmed by cytotoxicity assays on Caco-2 cells, whereby proliferation was decreased after exposure to EGCG-loaded emulsions. Intestinal absorption of EGCG was also studied with in vitro transport experiment using a Caco-2 monolayer model, and collecting the extracts transmitted through the cells in the basolateral fraction. Albeit it was difficult to obtain a quantitative measurement of the EGCG in the basolateral fraction, these fractions showed a clear anti-proliferative activity on Caco-2. These results would suggest the use of a cytotoxicity assay on cell cultures to estimate the extent of intestinal absorption of the bioactive. The findings demonstrated that sodium caseinate-stabilized emulsions can be used as a platform for delivery of EGCG.
6.2 Introduction

The recognition of certain food compounds with health benefits beyond their nutritional value has triggered the scientific community and food industry to design and formulate food matrices with the purpose of optimizing physical and mental well-being and decreasing the incidence of chronic illnesses. Polyphenols are one of the food components linked to the health improving properties of plant food (Manach et al. 2005). Tea, one of the most significant and concentrated dietary sources of polyphenols, is extraordinarily rich in flavon 3-ols (catechins) (Song and Chun 2008). Traditionally, green tea is consumed to eliminate toxins, relieve joint pain and improve disease resistance (Balentine et al. 1997). In recent years, an expanding body of scientific research noted many beneficial health effects linked to the consumption of tea catechins, such as antioxidant, anti-inflammatory, anti-arthritic and antibacterial effects, also preventive effects on cancer, obesity, diabetes, cardiovascular and neurodegenerative diseases (Balentine et al. 1997; Scalbert et al. 2005; Khan and Mukhtar 2007). Main tea catechins are (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechingallate, and (-)-epigallocatechin gallate (EGCG); EGCG is the most abundant and the main contributor to the reported health benefits (Braicu et al. 2013).

The addition of bioactive compounds such as EGCG in mainstream foods is becoming of interest, as a possible way of delivering additional health benefits through the diet. Bioactive compounds will be effective only if they maintain their activity in the gut and reach the sites of intended action. Catechins from tea have been generally regarded as poorly bioavailable. While a concentration of at least 10 μM seems to be required for therapeutic effects of EGCG (Scalbert and Williamson 2000; Yang et al. 2008), plasma concentrations in the nM range were obtained following oral administration of EGCG to rodents (Chen et al. 1997; Dube et al. 2011b). Tea catechin’s instability to digestive conditions (Record and Lane 2001; Green et al. 2007), and their poor permeability
across the intestine (Vaidyanathan and Walle 2001; Cai et al. 2002; Dube et al. 2011a) are important contributing factors to their low bioavailability.

Among various attempts to improve tea catechin’s bioavailability, complex formation between polyphenols and proteins is of particular relevance to this study. The ability of polyphenols to associate with proteins has been well documented. Polyphenols tend to bind to nonglobular proteins containing high amounts of proline such as saliva protein or caseins (Siebert et al. 1996; Baxter et al. 1997; Richard et al. 2006). The association of phenolics with milk proteins has been used to develop carriers for their targeted delivery (Sahu et al. 2008; Shpigelman et al. 2012; Haratifar et al. 2013).

Sodium caseinate-stabilized emulsion can bind EGCG by forming complexes with proteins at the oil/water interface (Sabouri et al. 2015). The addition of high methoxyl pectin (HMP) to the emulsions can improve the emulsion stability at low pH (Sabouri and Corredig 2016). In the present work it was hypothesized that these structured emulsions could be an efficient carrier of tea polyphenols. To test the hypothesis, the efficiency of the emulsions in improving the bioaccessibility and intestinal uptake of EGCG was measured compared to that of fresh EGCG solutions. *In vitro* models are useful tools providing the opportunity to estimate mentioned parameters and screen several systems before embarking on expensive, resource intensive animal or clinical trials. In this study, the designed emulsions were subjected to a standardised *in vitro* digestion model (Minekus et al. 2014).

Since the anti-proliferative activity against cancer cells is one of the bio-functional effects of EGCG, this activity can be tested on emulsions and it can be related to the amount of EGCG still present after digestion. From these results it is then be possible to estimate the bioaccessible portion of EGCG. *In vivo* digestion is a heterogeneous process and presumably a wide range of emulsion
droplets from non-digested to fully digested will reach the gastrointestinal cells. In this study the anti-proliferative activity of EGCG from both digested and non-digested emulsions and their controls (water solutions containing EGCG) was determined using a cytotoxicity test on Caco-2 cells as a model for colorectal cancer.

To assess the absorptive behavior of EGCG from sodium caseinate-stabilized emulsions, the in vitro digestion of emulsions was followed by an in vitro absorption model using intestinal absorptive Caco-2 cells. This combination has been reported as one of the most effective and peer-accepted in vitro models to simulate in vivo absorption processes (Glahn et al. 1996; Yun et al. 2004). A Caco-2 cell line was employed for in vitro prediction of intestinal absorption of various compounds (Hubatsch et al. 2007). When Caco-2 cells are grown under standard cell culture conditions and reach confluency, they differentiate expressing typical morphological and functional features of mature enterocytes (Hilgendorf et al. 2000).

The results from this research will bring further evidence of the ability of sodium caseinate emulsions to be a vehicle for the delivery of polyphenols.

6.3 Materials and Methods

6.3.1 Materials

Sodium caseinate was obtained from New Zealand Milk Products (Mississauga, Ontario, Canada). Soybean oil was purchased from Sigma Chemical Co. (St. Louis, MO, USA). High metoxyl pectin (DE 71.4, unstandardized) was obtained from CPKelco (San Diego, CA, USA). Tea polyphenol (Teavigo®, DSM Nutritional Products, Ayr, Ontario, Canada) extract contained mostly (−)-epigallocatechin-3-gallate (EGCG) (min. 94 %). HPLC-grade water, acetonitrile (A998), and glacial acetic acid (UN2789) were obtained from Fisher Scientific (Mississauga,
Ontario, Canada). Dulbecco’s Modified Eagle Medium (DMEM) (containing 25 mM glucose) and HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were obtained from Sigma-Aldrich Co. Heat-inactivated fetal bovine serum (FBS), nonessential amino acids (NEAA), 0.25 % trypsin 1 mM EDTA 4Na (1×), L-glutamine, penicillin-streptomycin (10,000 U of penicillin and 10,000 μg of streptomycin per mL), phosphate-buffered saline (PBS), and Hank’s balanced salt solutions (HBSS) were purchased from Invitrogen (Canada Inc., Burlington, Ontario, Canada).

6.3.2 Preparation of EGCG-Loaded Emulsions and Solutions

Oil in water emulsions were prepared at a final concentration of 7% oil, 0.35% sodium caseinate, 0, 2, and 6 mg EGCG/mL, containing 0 or 0.45% high metoxyl pectin, as previously described (Sabouri and Corredig 2016). In brief, primary oil in water emulsion was prepared through microfluidization at 69 MPa with four passes. Solutions of EGCG and pectin in ultrapure water were then mixed with the primary emulsion respectively. The concentrations of EGCG and HMP were chosen based on previous results (Sabouri et al. 2015; Sabouri and Corredig 2016). For emulsions containing pectin, aliquots were acidified with glucono delta lactone to pH 5 (Sabouri and Corredig 2016).

Solutions of 2 and 6 mg/mL EGCG in ultrapure water were also prepared as controls. All the emulsions and respective solutions were prepared freshly before being subjected to the in vitro digestion and other experiments to avoid EGCG degradation.

6.3.3 In Vitro Digestion

To mimic the fed state in the human gastrointestinal tract, a three-step in vitro static digestion model was adapted from literature (Versantvoort et al. 2005; Sarkar et al. 2009a; Minekus et al.
2014) with some modifications (Sabouri et al. 2016). For bioaccessibility experiment, after the duodenal phase, aliquots of the final digestates of emulsions and solutions were immediately cooled in ice water bath and stored at -18°C to stop enzymatic reactions.

For cytotoxicity and uptake experiments, the prepared solutions and emulsions were divided into 2 aliquots. One aliquot was subjected to the in vitro digestion process, then the digestate was cooled in ice water bath immediately after the duodenal stage, diluted in a DMEM medium containing 10 % FBS (1:6, v/v digest to medium) to stop digestive reactions, and stored at -18 °C until use. The other aliquot of each treatment was not subjected to in vitro digestion and was directly diluted with ultrapure water and DMEM medium to the same dilution factor, and then stored at -18 °C.

### 6.3.4 Bioaccessibility

The amount of EGCG recovered after digestion was measured using a UHPLC (Dionex, Thermo Scientific with Chromeleon®-Chromatography Data System). The concentration of non-degraded EGCG in the final digestate was used as an estimate of the amount of bioaccessible EGCG. Immediately before HPLC analysis, aliquots were defrosted at room temperature, and centrifuged in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY) at 13362 g for 15 min. Then the supernatant was filtered through a 0.45 µm filter and submitted to a reverse phase HPLC Nova-Pak C18 column (4 µm, 3.9×150 mm, Waters Corporation, Milford, MA, USA) using a linear gradient consisting of 2% acetic acid (in HPLC water) and 100% acetonitrile, for 20 min (Ferruzzi and Green 2006). The elution was carried out at 1 mL/min at 35 °C for 30 min. Peaks were detected at 280 nm. Solutions with known concentrations of EGCG dissolved in HPLC grade water were also measured by HPLC to obtain a standard curve.
6.3.5. Cell Culture
Polyphenols’ low systemic absorption puts them in a direct contact with the digestive tract; specifically the intestine is exposed to high amounts of polyphenols after ingestion. Therefore, for many polyphenol’s anti-cancer studies, adenocarcinoma cell lines from human digestive tract such as Caco-2 are cultured and used (Yang et al. 1998; Hong et al. 2002; Chen et al. 2003; de Mejía et al. 2010).

The Caco-2 cell line was obtained from the Canadian Research Institute for Food Safety, Culture Collection (Food Science, University of Guelph, ON, Canada). Cells were subsequently maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, and 1% antibiotic solution of penicillin–streptomycin and 25 mM HEPES buffer (Invitrogen, Canada Inc., Burlington, ON, Canada). Cells were cultured in 75 cm² tissue culture flasks (Fisher Scientific, Mississauga, Ontario, Canada) and maintained in an incubator (Forma Series II Water-jacketed CO₂ Incubator, Model No: 3110, Forma Scientific, California, USA) at 37°C, 5% CO₂ atmosphere at constant humidity and harvested with trypsin-EDTA prior to seeding. Cells between passages 25 to 45 were used throughout this study.

6.3.6 Cell Viability Assay
The anti-proliferative activity of EGCG in emulsions vs. solutions, and digested samples vs. non-digested ones was assessed using cultured colorectal cancer Caco-2 cells followed by a colorimetric cytotoxicity test, called Sulforhodamine B (SRB) assay.

Harvested Caco-2 cells were seeded in 96-well plates (Fisher Scientific, Mississauga, Ontario, Canada) at a concentration of 8×10⁴ per well and allowed to adhere for 24 h at 37 °C and 5% CO₂. After reaching 70% confluency and a change of medium with fresh one, the treatments (provided
in section 6.3.3) were added to the wells at a final v/v dilution ratio of 1:27 (sample: medium). Preliminary work at a range of dilutions showed that 1:27 dilution allowed a distinctive cell viability between different treatments. The plates were then incubated 24 h at 37 °C and 5% CO₂. The cells were then fixed with trichloroacetic acid and stained with SRB dye for 30 min (Skehan et al. 1990). The excess unbound dye was removed by rinsing the cultures repeatedly with 1% v/v acetic acid. After drying, the protein-bound dye was dissolved in 10 mM Tris base solution and the optical density of the plates was measured using an automated microplate reader (Multidetector Microplate Reader, BioTek Synergy HT Model, Vermont, USA), at a wavelength of 570 nm. The results were expressed as % cell viability, calculated as the absorbance of each treatment relative to the absorbance of control (cell with medium only).

6.3.7 Transport Study with Caco-2 Confluent Monolayer

In vitro transport experiment was conducted to mimic human intestinal epithelium to study the epithelial absorption of EGCG from the digestates. Caco-2 cells were successfully grown on transpermeable polyester inserts of 12 well plates (0.4 μm pore size and 1.2 cm diameter, VWR, Mississauga, ON, Canada) at a density of 6 × 10⁶ cells per insert for 21 days and until they reached complete morphological and functional differentiation. The culture medium was replaced every second day. The development of the monolayers was monitored daily by means of Trans-Epithelial Electrical Resistance (TEER) using an EVOM2 voltmeter (WORLD Precision Instruments, Sarasota, FL, USA).

After 3 weeks of complete differentiation, Caco-2 cells were washed with HBSS buffer to remove any cell debris from the cell monolayer and then incubated with 375 μL of DMEM medium (without FBS) on the apical compartment and 1500 μL of HBSS containing Ca²⁺ and Mg²⁺ on the
basolateral compartment for 30 min at 37°C to achieve equilibration prior to the transport experiment. After that, 125 µL of each treatment (emulsion and solution digestates from section 6.3.3) was added to the apical and incubated for 2 h at 37°C in a 5% CO₂ environment. The dilution of digestates in the apical (1 sample: 27 medium (v/v)) was based on the results obtained from a cytocompatibility assessment, since the integrity of the Caco-2 cell monolayers needs to be maintained during the experiment. For this purpose, the dilution factor used was determined using the same viability test described in the previous section but for 2 h incubation, to mimic the same condition of the transport study, maintaining at least 80% of Caco-2 cells’ viability.

TEER values were recorded before (t = 0 h) and after (t = 2 h) the transport study to monitor the integrity of the monolayer. After 2 h of incubation, the mixture present in the basolateral compartment was collected and its pH was decreased to 2.5 by a 1% ascorbic acid solution containing 0.28% H₃PO₄ as previously described (Zhang et al. 2004; Xie et al. 2013; Li et al. 2014). The solutions were then immediately freeze dried overnight and kept frozen at -20°C until further processing. Ascorbic acid has been reported to protect tea catechins from oxidative degradation (Chen et al. 1998; Zhu et al. 2003).

### 6.3.8 Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) Analysis

To determine the uptake and transport of EGCG from Caco-2 cells, the basolateral fractions after transport experiments were analyzed by LC-MS/MS. Freeze dried basolateral contents for each treatment was reconstituted in 500 µL methanol and vortexed for 60s to solubilize EGCG. Afterwards the solutions were incubated for 15 min on ice for a better extraction followed by centrifugation at 17,000 g using a microcentrifuge (accuspinMicro 17R, Fisher Scientific, Mississauga, Ontario) to eliminate the precipitated salts which interfere with the performance of
the mass spectrometer. An aliquot of the supernatant was injected into an Agilent 1290 infinity UHPLC system, interfaced to a LTQ Orbitrap XL™ Hybrid Ion Trap-Orbitrap Mass Spectrometer instrument (Thermo Scientific, Mississauga, Ontario). The column used for chromatographic separation was Eclipse XDB-C18 column (3.5 µm, 2.1 mm ×100 mm, Agilent, Mississauga, Ontario). The mobile phase consisted of 0.1% formic acid in ultrapure water (buffer A) and 0.1% formic acid in acetonitrile (buffer B). The initial mobile phase conditions were 90% buffer A and 10% buffer B, hold for 2 min, then a linear gradient to 100% buffer B over 7 min, hold for 2 min, back to the initial condition in 1 min and hold there for 3 min. The flow rate throughout the 14 min elution was kept at 0.4 mL/min and the injection volume was 10 µL. Electrospray ionization was carried out in negative mode in full scan for masses between m/z 100 to 800 at 60,000 resolutions. Sheath gas was at 40 (arbitrary units), auxiliary gas at 10 (arbitrary units) and the capillary temperature was at 300°C. The capillary voltage and ESI voltage were -8 V and 4.00 V respectively. Mass calibration was done using standard ESI calibration solution prior to the injection of samples. The parent ion, m/z 457.2, and the MS² fragment ion, m/z 169, were monitored for quantitation. Xcalibur software (Thermo Scientific) was used for data analysis.

6.3.9 Cell Viability Assay on the Basolateral Contents

In addition to the analytical determination (LC-MS/MS), the basolateral samples were tested using a Caco-2 model, to determine any residual anti-proliferative activity after transport. The same cell viability assay described in section 6.3.6 was used on the basolateral fractions.
6.3.10 Statistical Analysis

All experiments were conducted in three independent replicates and their average value with a standard deviation was reported. To determine the statistically significant differences between treatments, Tukey’s multiple comparisons testing in SigmaPlot software was used. Significances were considered at p<0.05.

6.4 Results and Discussion

6.4.1 Bioaccessibility of EGCG

To determine the efficiency of the emulsions in protecting EGCG from digestive degradation, the amount of EGCG recovered in the digestates was measured and compared to that of solutions containing the same initial concentration of EGCG. The amount of EGCG remaining as a percentage of initial values is summarized in Figure 6.1. The difference in percent recovery between emulsions and solutions containing 2 mg/mL was not statistically significant; on the other hand, in the emulsions containing 6 mg/mL EGCG, there was a significant increase in EGCG recovery, compared to the same amount of EGCG in solution. It was concluded that the complex formation at the interface of emulsions with caseins protected EGCG during digestion. At both EGCG levels and both pH values of emulsions, the presence of HMP did not affect the recovery of EGCG.
Figure 6.1  Amount of EGCG recovered after *in vitro* digestion of emulsions (filled bars) and solutions (white bars) initially containing 2 (A) and 6 (B) mg/ml EGCG. Emulsions containing EGCG (gray bars); emulsions containing EGCG and 0.45% HMP (black bars) at pH 6.8 and pH 5.0. Values are the means of three independent experiments and error bars represent standard errors. Within a graph, letters indicate statistical difference (*p* < 0.05).
The present results are in agreement with recent studies reporting that green tea polyphenols complexed with milk proteins in milk, yogurt and cheese are protected during digestion (Green et al. 2007; Lamothe et al. 2014).

### 6.4.2 Anti-Proliferative Activity of EGCG

To analyze the bioaccessibility, the anti-proliferative activity of EGCG either in solution or in emulsions was assessed using a Caco-2 model. Table 6.1 illustrates the viability of adenocarcinoma Caco-2 cells incubated with emulsions and solutions initially containing 0, 2 and 6 mg/mL EGCG, before and after digestion (same dilutions applied to all samples for accurate comparison).

In the absence of EGCG, digested emulsions decreased the viability of cancer cells, regardless of the presence of pectin. However, non-digested emulsions both with and without pectin did not show any anti-proliferative activity. This decrease in cell viability could not be the result of digestive juices since control digestates (water with no EGCG, digested solution Table 6.1), which contained all components of digestion did not have any anti-proliferative effect on cells. The decline in cell viability for emulsions digested in vitro with no EGCG was caused by the presence of free fatty acids. Digested soy oil dispersions were also tested and showed a significant decrease in the cell viability in a direct relationship with their oil concentration (data not shown). This finding was in full agreement with another study on viability of Caco-2 cells after digestion of algal and fish oil (van Beelen et al. 2007).

The presence of EGCG significantly decreased the cell viability, and the effect was proportional to the concentration of EGCG.
Table 6.1 Differences in cell viability for Caco-2, after 24 h incubation with solutions of EGCG, and emulsions, with and without high methoxyl pectin; before and after digestion. Initial concentration of EGCG, before digestion was 0, 2 and 6 mg/mL. Values are the means of three experiments ± standard deviation. Different letters indicate significant differences at p<0.05; within a row (a, b), within a column (x, y, z).

<table>
<thead>
<tr>
<th>Concentration of EGCG (mg/mL)</th>
<th>Cell viability (% of control)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emulsion</td>
<td>Emulsion with HMP (0.45%)</td>
</tr>
<tr>
<td></td>
<td>Initial</td>
<td>Digested</td>
</tr>
<tr>
<td>0</td>
<td>108±7$^{a,x}$</td>
<td>60±4$^{b,x}$</td>
</tr>
<tr>
<td>2</td>
<td>89±2$^{a,y}$</td>
<td>45±2$^{b,y}$</td>
</tr>
<tr>
<td>6</td>
<td>65±6$^{a,z}$</td>
<td>31±1$^{b,z}$</td>
</tr>
</tbody>
</table>
These results did not only confirm the ability of EGCG to decrease Caco-2 cell viability, but also demonstrated that the presence of EGCG-casein complexes at the interface did not interfere with the bioefficacy of EGCG. In addition, the presence of HMP had no effect on the anti-proliferative activity of EGCG (Table 6.1).

Comparing the results of digested emulsions containing EGCG with their corresponding EGCG solutions demonstrated that, both at 2 and 6 mg/mL of EGCG, emulsions decreased the cell viability 40% more than solutions, regardless of the presence of HMP. This data indicated that the structured incorporation of EGCG in the emulsion matrix protected EGCG from digestive degradation. There was an improved bioaccessibility of EGCG in the sodium caseinate emulsion system.

These results support previous research, showing that green tea-whey protein or casein complexes retained polyphenols’ anti-proliferative activity also after in vitro digestion (von Staszewski et al. 2012; Guri et al. 2014). The bioavailability of tea catechins after digestion in the presence of milk proteins has been a source of debate. Results from human intervention studies have suggested that the bioavailability of tea catechins is not impaired by the presence of milk in the diet (van het Hof et al. 1998; Kyle et al. 2007). Conversely, in a recent study, the bioaccessibility of green tea flavan-3-ols was significantly lower in a mix with sodium caseinate compared to green tea solutions or green tea-non-fat dry milk solutions (Moser et al. 2014). The differences in the experimental conditions of these studies may be the reason for these discrepancies.

In solutions containing free EGCG (Table 6.1), while at low concentrations (2 mg/mL), there was no significant difference between digested and non-digested samples, at 6 mg/mL EGCG the digested solution showed much less anti-proliferative activity than the non-digested one. Digestion had an adverse effect on the bioactivity of free EGCG, as previously reported (Record and Lane
2001; Zhu et al. 2003; Green et al. 2007). This was not the case for emulsions; on the contrary, digested emulsions decreased the viability of cancer cells to a higher extent than non-digested emulsions (Table 6.1). Possible synergistic effects with other digestion metabolites will need to be evaluated, as this was outside the scope of this work. This effect was also reported in a recent study, where encapsulated EGCG in casein micelles showed less anti-proliferative activity than free EGCG in solution, but after in vitro digestion they had comparable anti-proliferative effects (Haratifar et al. 2014).

6.4.3 Transport Study on Caco-2 Monolayer

To investigate the ability of the emulsion matrices to deliver EGCG to intestinal cells, the absorption behaviour of EGGC from emulsions and solutions was studied with an in vitro transport experiment, using a Caco-2 monolayer model. The digestates of emulsions and solutions were diluted and added to the apical compartment of the cells. Table 6.2 summarizes the amount of EGCG measured after digestion, as well as the concentration applied to the apical compartment of the cell monolayer. The aliquots of EGCG administered to the cells ranged between 5-22 µg/mL. The absorption and transport of the digestates was measured by collecting the basolateral fraction after 2 h of incubation. The basolateral fraction was analyzed using LC/MS/MS. A very low amount of EGCG (estimated at <0.5% of the amount loaded in the apical compartment of the cells) was recovered in the basolateral compartments, with no difference between treatments.
Table 6.2 EGCG concentration measured in the digestates of emulsions and solutions, and estimated concentrations loaded in the apical compartment during Caco-2 monolayer experiments. Values are the means of three independent experiments ± standard deviation.

<table>
<thead>
<tr>
<th>Initial concentration of EGCG (mg/mL)</th>
<th>Emulsion</th>
<th>Emulsion with HMP (0.45%)</th>
<th>EGCG Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After digestion (μg/mL)</td>
<td>Apical (μg/mL)</td>
<td>After digestion (μg/mL)</td>
</tr>
<tr>
<td>2</td>
<td>168±14</td>
<td>6±1</td>
<td>144±12</td>
</tr>
<tr>
<td>6</td>
<td>604±51</td>
<td>22±2</td>
<td>582±36</td>
</tr>
</tbody>
</table>
Quantitation of such low amounts was not possible. Furthermore, the EGCG content in basolateral compartment may have been underestimated as a result of EGCG interaction with proteins and peptides. These complexes would precipitate during extraction and sample preparation (Zimmermann et al. 2009).

It has been reported that EGCG when absorbed by intestinal cells, would undergo extensive catabolism in enterocytes, transforming to a wide range of sulfated, glucuronidated, or methylated metabolites (Lambert et al. 2007; Henning et al. 2008). The EGCG metabolites will also show bioactivity, but were not identified by MS-MS in this study (Del Rio et al. 2010).

### 6.4.4 Cell Viability Induced by Basolateral Contents

To determine if any bioactivity was still present after absorption and transport through Caco-2 cells, the cytotoxicity of the basolateral fraction collected from transport experiments was examined. This allowed further investigating the uptake of EGCG and its bioefficacy after absorption. Caco-2 cells were incubated with aliquots of the basolateral fraction, and the viability of the cells was measured. The initial TEER values were about 1450 Ω cm². There was no change in TEER after incubation, demonstrating that the monolayer integrity was preserved in all cases after addition of the digestates.

After absorption experiments, the basolateral fraction was used for Caco-2 cell viability studies (Table 6.3). The viability was measured relative to a control treatment, containing only media. The basolateral fraction collected after transport experiments of digested emulsion without EGCG did not show a significant change in the viability of the cells, compared to the control.
Table 6.3  Percent cell viability of Caco-2 cells after 24 h incubation with basolateral content of transport experiment for emulsions and solutions containing EGCG. Values are the means of three experiments ± standard deviation. Different letters indicate significant differences to control treatment at p<0.05.

<table>
<thead>
<tr>
<th>Initial concentration of EGCG (mg/mL)</th>
<th>Cell viability (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>Emulsion</td>
<td>Emulsion with HMP (0.45%)</td>
<td>EGCG Solutions</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>91±1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>100±1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>84±7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87±7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79±2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>77±5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84±5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82±2&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>
On the other hand, the basolateral fraction collected from cells subjected to either solutions and emulsions containing EGCG significantly decreased cell viability, clearly indicating the presence of EGCG or bioactive metabolites in the basolateral fraction, after digestion of the sample, and transport through the enterocyte monolayer. There was no statistically significant difference between solutions and emulsions. It is important to point out that the amounts of EGCG measured after digestion were as high as 0.6 mg/mL in emulsions (Table 6.2), and, under the present conditions, the amounts recovered in the basolateral fraction was too low for an accurate detection with LC/MS/MS.

Other studies also demonstrated that the formation of complexes between milk proteins and green tea catechins could induce delayed release of EGCG following digestion (Song and Chun 2008; Xie et al. 2013; Moser et al. 2014). This delayed release, together with a protection imposed by protein-polyphenol complexation against oxidative degradation of small intestine may contribute to improved bioaccessibility.

### 6.5 Conclusions

The bioaccessibility of EGCG was higher for EGCG bound to sodium caseinate at the oil water interface of emulsions, compared to aqueous solutions. The increased anti-proliferative activity of emulsions containing EGGC compared to their equivalent solutions was also an indication of preserved bioactivity for EGCG carried in the emulsion system. These results also highlighted that to have accurate/realistic assessment of bioactives’ functional properties, it is very important to include digestion, as different results were obtained with samples before and after digestion.

While detecting intact EGCG via LC/MS/MS in the basolateral compartment of the transport experiment was a challenge, it was possible to show molecular absorption and transport by
determining the cytotoxicity of the basolateral content on Caco-2 cells. This approach offers the advantage of taking into account not only the intact molecules, but also the metabolites of intestinal transformation, and complexes formed. Longer incubation times for transport experiment may be needed to understand differences in the absorption kinetics from complex food matrices. The results from this study showed the application of EGCG complexation to a sodium caseinate-stabilized oil-water interface to be beneficial towards preserving the bio-functional properties of EGGC during digestion and intestinal uptake. Such matrices could be employed to develop new emulsion-based nutritional beverages as novel delivery vehicles for EGCG.
CHAPTER 7
GENERAL CONCLUSIONS

While consumption of tea polyphenols has been linked to several health benefits, a low oral bioavailability has been reported for them. To overcome the bioavailability challenges, a food matrix can be designed to encapsulate or incorporate the bioactive, in order to protect it from digestive degradation and/or facilitate its dissolution and absorption. This enables the bioactive to reach the targeted site and induce its health effect. The high affinity of tea polyphenols for proline rich proteins such as caseins was suggested to be employed in engineering food carriers for them. However, tea polyphenol-casein interactions in different food matrices and their effect on protein’s functionality, matrix digestibility and even the tea polyphenols’ bio-functional properties, has been a subject of controversy. Hence, more investigation is needed to clarify the structural-functional relation of these complexes.

The results from this thesis demonstrated that EGCG molecules bind to sodium caseinate at the emulsion interface. The binding ratio of EGCG-sodium caseinate at such interfaces was much higher than the binding efficacy of casein micelles for EGCG. The emulsion interface is therefore a novel food matrix and its effectiveness needs to be studied. In this research it was shown that complex formation of EGCG with sodium caseinate at the oil-water interface did not affect the stability, size, and charge of the emulsion. The viscoelastic properties of the interface were also studied to evaluate if complex formation may affect the properites of the bulk emulsion. It was revealed that the association of EGCG to the interface did not influence the interfacial tension but increased the dilational modulus which validated the binding of EGCG to sodium caseinate layer at the interface.
Since a wide range of emulsion/dairy based products are present at an acidic pH, it appeared of significance from a technological point of view to study the colloidal behavior of emulsions containing EGCG at low pH. The presence of EGCG at the emulsion interface delayed acid induced destabilization of emulsions. Also in the presence of EGCG, a different gel microstructure was observed at pH 4.5, with less coalescence compared to the gel microstructure of control emulsion with no EGCG. While addition of pectin to sodium caseinate-stabilized emulsions has been used to prevent emulsion destabilization at low pH (close to protein’s pI), in the presence of EGCG, the adsorption of pectin to sodium caseinate during acidification was delayed and even partly hindered at higher EGCG concentrations.

To determine the digestion behavior of the designed matrix and the bioaccessibility of incorporated EGCG, a three-stage static in vitro digestion model was employed. It was concluded that the formation of complexes between sodium caseinate, EGCG, and pectin at the oil-water interface might reduce the catechin’s perceived astringency through affecting the EGCG-salivary proteins aggregations during mouth processing. Another finding was that the proteolysis of caseinate was not affected by the presence of EGCG in the emulsions, while lipid hydrolysis was lessened in the EGCG-containing emulsions. The latter could be of potential in engineering food structures with reduced lipid absorption, and in turn lowered energy intake.

To evaluate the efficacy of the designed matrices in improving EGCG bioaccessibility, the amount of EGCG retained in the emulsion digestates was determined and compared to that of digested solutions of EGCG. The results indicated that the digestive stability and bioaccessibility of EGCG was improved by incorporating it in the emulsions stabilized with sodium caseinate. There was a higher anti-proliferative activity of digested emulsions compared to digested EGCG solutions, suggesting the presence of EGCG as well as possible synergies with other metabolites.
To simulate and determine the intestinal uptake of EGCG from the designed matrices, transport experiment was conducted. The detection of EGCG in the basolateral fraction via LC/MS/MS was challenging. However, evaluating the induced cytotoxicity by using the basolateral fractions after digestion and transport through Caco-2 monolayers enabled exploring the intestinal uptake of the bioactive from complex matrices. It is important to note that, in this novel approach, not only intact EGCG, but also the bioactive products of intestinal transformation of EGCG can be taken into account.

The results from this study found EGCG complexation to a sodium caseinate-stabilized oil-water interface beneficial towards preserving the bio-functional properties of EGCG during digestion and intestinal uptake, and demonstrate the potential of such matrices for the development of functional dairy beverages.
CHAPTER 8
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