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Antioxidant Activity of Lutein Entrapped in Poly (DL-Lactide Co-Glycolide) Acid and PLGA/Chitosan Nanoparticles

Toni Borel Lousteau

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ANTIOXIDANT ACTIVITY OF LUTEIN ENTRAPPED IN POLY (DL-LACTIDE CO-GLYCOLIDE) ACID AND PLGA/CHITOSAN NANOPARTICLES

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in Biological and Agricultural Engineering

in

The Department of Biological and Agricultural Engineering

by

Toni B. Lousteau
B.S., Louisiana State University, 2012
May 2015
I dedicate this thesis to my parents and my husband.
ACKNOWLEDGEMENTS

I would like to thank the LSU Biological and Agricultural Engineering Department faculty and staff and for providing the necessary resources and guidance allowing me complete my graduate studies. Of the faculty, my major advisor, Dr. Cristina Sabliov, has been incredibly helpful and influential throughout my graduate career. I would also like to thank Dr. Zhimin Xu, associate professor of food science, for his help with antioxidant studies and for being a part of my committee. I would also like to thank my biological engineering committee member, Dr. Daniel Hayes, for his help and guidance during my graduate career. Lastly of my committee, I would like to thank Dr. Dorel Moldovan, professor of mechanical engineering, for his help and for his support throughout my project. Many thanks to Dr. Carlos Astete for sharing all of his nanotechnology knowledge and for all of his continued help throughout my studies. Thanks to Yixiao Shen, Daniel Bourgeois, Meocha Whaley, Lacey Simon, Thanida Chuacharoen, and Sara Navarro for their assistance with various conducted studies. Thank you to Ying Xiao, Dr. Rafael Cueto, and Dr. Holli Hale-Donze for their assistance with transmission electron microscopy, dynamic light scattering, and confocal microscopy. Lastly, I would like to thank my friends and family who have supported me throughout my college career.
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ABSTRACT

Polymeric nanocarriers improve cellular uptake, stability, solubility, and functionality of entrapped drugs and nutraceuticals. The hypothesis of this study was that entrapping lutein, a hydrophobic antioxidant, in different polymeric nanoparticles (NPs) will improve its stability and antioxidant activity. The following objectives were proposed: 1. Synthesize and characterize polymeric nanoparticles of poly lactic co-glycolic acid (PLGA) and PLGA NPs covered with a layer of chitosan (PLGA/Chi) from a physicochemical perspective, and 2. Assess functionality of the entrapped lutein as a function of type of polymer in which entrapped. Nanoparticles were synthesized by emulsion evaporation method. Characterization included size, zeta potential, and morphology measurement, followed by testing the physical and chemical stability, and antioxidant activity of entrapped lutein. PLGA NPs loaded with lutein were in the size range of 119.2 nm ± 0.98 nm with a PDI of 0.17 ± 0.02 and a zeta potential of −29 mV ± 1.1 mV in nanopure water with 0.05 mg PVA/ mg PLGA ± 0.002 mg PVA/ mg PLGA remaining on the surface of the nanoparticles. PLGA/Chi NPs loaded with lutein were in the size range of 145.9 ± 0.3 nm with a PDI of 0.25 ± 0.01 and a zeta potential of 21.2 mV ± 2.3 mV with 0.06 mg ± 0.002 mg PVA/ mg PLGA/Chi remaining in nanopure water. PLGA NPs and PLGA/Chitosan NPs loaded with lutein were stable in emulsions made with Tween 20 for 72 hours at 37 °C as indicated by a constant size over time. PLGA and PLGA/Chi NPs protected the chemical stability of lutein in Tween 20 emulsions for 24 hours at 37 °C. PLGA and PLGA/Chitosan NPs showed significant reductions in the oxidation of cholesterol by 45 ± 1% and 60 ± 1 % at 48 hours, and 36 ±
1 % and 42 ± 3 % at 72 hours when compared to the free lutein. The addition of chitosan to the PLGA NPs further enhanced the efficacy of lutein as an antioxidant when compared to PLGA NPs. These results support the hypothesis that polymeric NPs are enhancing stability and antioxidant activity of entrapped lutein and hence may find beneficial applications in the biomedical and food science fields for delivery of lutein.
CHAPTER 1: INTRODUCTION

Antioxidants protect carbohydrates, proteins, nucleic acids and lipids from oxidation, for improved overall health of the consumer. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause oxidation of carbohydrates, proteins, and other molecules which is associated with diseases such as cardiovascular and inflammatory diseases, cancer, and premature aging. Mechanisms of oxidation occur through altering the chemical structure of lipids and other molecules inside the body. Natural defenses in the human body are designed to combat this oxidation; these defenses include natural antioxidants such as glutathione, bilirubin, α-tocopherol, and many others (Laguerre, Lecomte et al. 2007). However, the human body does not contain enough of these natural antioxidants to maintain a balance sufficient to diminish all oxidative stress (Laguerre, Lecomte et al. 2007). Therefore dietary antioxidants are necessary for protection against diseases and inflammation. Common exogenic dietary antioxidants include tocopherols, tocotrienols, flavonoids, and carotenoids (Laguerre, Lecomte et al. 2007).

Many of these dietary antioxidants such as carotenoids and tocopherols are lipid soluble, which results in low bioavailability in vivo (Anand, Nair et al. 2010). These molecules are often photosensitive and easily oxidized leading to a further decrease in bioavailability and in vivo activity of these molecules (Canene-Adams and Erdman 2009). A well-investigated solution for enhanced stability and bioavailability is the use of antioxidant nanoparticles as delivery systems (Choi, Zuckerman et al. 2011), (Faraji and Wipf 2009).

In particular, polymeric nanoparticles made of polymers such as poly (lactic-co-glycolic) acid (PLGA), poly-lactic acid (PLA), polyethylene glycol (PEG), zein, and low
molecular weight chitosan (LMWC) have shown to improve the bioavailability of various hydrophobic drugs and nutraceuticals (Arunkumar, Harish Prashanth et al. 2013), (Kumari, Yadav et al. 2010), (Anand, Nair et al. 2010), (Hu, Lin et al. 2012), (Hu, Liu et al. 2011) and provide protection from degradation of multiple photosensitive and unstable antioxidants (Trombino, Cassano et al. 2009), (Konecsni, Low et al. 2012). Nanodelivery systems may also enhance activity of bioactives in vivo through their controlled release properties (Kumari, Yadav et al. 2010), (Hu, Liu et al. 2011), (Hu, Lin et al. 2012).

Nanoparticle behavior in vivo is affected by the properties of the particle, including size and surface charge (Mohamed and van der Walle 2008). Surfactants such as poly vinyl alcohol (PVA, Figure 1.1) are commonly used to enhance these properties (Mohamed and van der Walle 2008), (Sahoo, Panyam et al. 2002), (Astete, Kumar et al. 2007). Concentration of surfactant will affect the properties of size, zeta potential, and hydrophobicity of NPs and can be adapted for specific applications (Sahoo, Panyam et al. 2002).

![Figure 1.1 Structure of poly (vinyl alcohol)](image)

Polymers selected for nanoparticle synthesis largely contribute to nanoparticle properties and their activity in vivo. Poly (lactide co glycolide), PLGA, (Figure 1.2), for
example, is a FDA approved, biodegradable synthetic co-polymer of lactic acid and glycolic acid shown to improve stability and bioavailability of entrapped drugs (Anand, Nair et al. 2010),(Astete, Kumar et al. 2007). It is a highly biocompatible and biodegradable polyester (des Rieux, Fievez et al. 2006). PLGA is soluble in many different solvents, and is available in many different formulations so hydrophobicity of the NP can be optimized through ratio of lactic acid to glycolic acid of the PLGA selected for NP synthesis (Makadia and Siegel 2011).

Chitosan (chi), (Figure 1.3), a natural heteropolymer derived from chitin, is commonly used to supply a positive surface charge to a nanomaterial. Chitosan has been found to provide a stable means of encapsulation and to the positive zeta potential of the Chitosan NP was shown to improve cellular uptake of nanoparticles (Hu, Ting et al. 2012),(He, Hu et al. 2010).
In the present study lutein was entrapped in PLGA and PLGA/Chitosan nanoparticles using PVA as a surfactant in an attempt to improve its efficacy as an antioxidant against peroxyl radicals. Chapter 2- Nanodelivery of Bioactive Components for Food Applications: Types of Delivery Systems, Properties, and their Effect on ADME Profiles and Toxicity of Nanoparticles and will further describe how different nanoparticle properties affect nanodelivery and functionality of the entrapped bioactive. Chapter 3 covers the synthesis and characterization of PLGA (lutein) and PLGA/Chitosan (lutein) NPs and compares the antioxidant functionality of nanoentrapped lutein to antioxidant lutein. Chapter 4 summarizes the findings of this study.

Literature Cited


CHAPTER 2: NANODELIVERY OF BIOACTIVE COMPONENTS FOR FOOD APPLICATIONS: TYPES OF DELIVERY SYSTEMS, PROPERTIES, AND THEIR EFFECT ON ADME PROFILES AND TOXICITY OF NANOPARTICLES

Introduction

Antioxidants, probiotics, polyunsaturated fatty acids, and proteins are common bioactives that can be added to food to improve nutritional value. Food bioactives can be used to prevent diseases such as cancer and heart disease and to improve overall health (Jiménez-Colmenero 2013). Nanodelivery of these components in food may improve their stability (Trombino et al. 2009), solubility (Fathi et al. 2011), functionality (Swarnakar et al. 2011), cellular uptake (Gaumet et al. 2010, Hamdy et al. 2009, Harush-Frenkel et al. 2007, Hu et al. 2012, Li et al. 2010, Sahoo et al. 2002, Win & Feng 2005, Wang et al. 2012, Yoo & Mitragotri 2010, Zhang et al. 2008), and bioavailability (Anand et al. 2010) and may also provide controlled release (Kumari et al. 2010) for better efficacy of the bioactive.

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It is therefore widely accepted that nanoparticles may offer distinct advantages for delivery of bioactives, ranging from improving stability to controlling the release and
targeting of the bioactive for enhanced functionality. Due to the increasing interest in the use of nanoparticles for food and oral drug delivery, consumer safety has become a serious concern (Vega-Villa et al. 2008). Safety concerns stem from the potential of the nanoparticles to translocate to tissues due to their small size and the higher-than-physiologically normal concentrations of the delivered bioactive in tissues. The type of nanoparticle and related chemical, physical, and morphological properties affect their interaction with living cells and also determine the route of clearance from the gastrointestinal (GI) system and possible toxic effects.

Types Of Delivery Systems

Nanodelivery systems (Figure 1) are classified into two main groups, liquid and solid. The three types of liquid nanodelivery systems are nanoemulsions, nanoliposomes, and nanopolyemulsomes. Nanoemulsions can be separated into either emulsions or stabilized emulsions. The three types of solid nanodelivery systems are lipid nanoparticles, polymeric nanoparticles, and nanocrystals. The group of lipid particles is composed of solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs). Polymeric nanoparticles can take the form of nanospheres and nanoencapsulates.

Emulsions and Stabilized Emulsions

In its most simple form, an emulsion is the mixture of two immiscible liquids, generally oil and water. Nanoemulsion droplets measure between 10 and 100 nm in diameter and are typically transparent due to these sizes being on a scale smaller than the ultraviolet-visible light range. Emulsions stabilized by surfactants or other types of stabilizing
agents, including phospho-lipids, amphiphilic proteins, or polysaccharides (Guzey & McClements 2006), namely stabilized.

Figure 1: Classification of nanodelivery systems. Abbreviations: MLVs, multilamellar vesicles; SLVs, single lamellar vesicles

emulsions, have been developed to provide controlled release, improved entrapment efficiency, and protection from degradation (Carrillo-Navas et al. 2012, McClements & Li 2010). In the instance of nanodelivery, the hydrophobic food bioactive is to be dissolved in the internal organic phase of an oil-in-water emulsion, whereas double emulsions are employed for delivery of hydrophilic molecules (Sapei et al. 2012).

Two types of methods are commonly used in nanoemulsion synthesis, mechanical or chemical processes. Mechanical processes employing sonicators and microfluidizers are considered high-energy processes as mechanical forces are employed to break larger emulsion droplets into smaller ones. Low-energy or chemical methods result in spontaneous formation of emulsion droplets due to the hydrophobic effect of lipophilic molecules, in the presence of emulsifiers (McClements & Li 2010). Emulsions and stabilized emulsions are commonly used in the food industry. Emulsions of olive oil stabilized with sodium caseinate, whey protein concentrate, and polyglycerol ester were used to replace the animal fat in meat products (Cofrades et al. 2013); locust bean gum was used for reduced-fat salad dressings and desserts (Chung et al. 2013); additionally,
lemon oil emulsions are being developed for food and beverage flavorings (Rao & McClements 2012).

Liposomes
Liposomes are self-assembled nanoparticles formed from phospholipids in the form of a bilayer. Liposomes are ideal for carrying hydrophilic molecules due to the polar environment of the vesicle (Tamjidi et al. 2013). However, hydrophobic molecules can be delivered using liposomes as well, when these components are entrapped in the bilayer. When delivered with liposomes, the encapsulated materials are protected from external conditions. Another advantage is that liposomes can be made without the use of organic solvents (Madrigal-Carballo et al. 2010). Liposomes can be either unilamellar or multilamellar, referred to as small unilamellar vesicles (SUVs) and multilamellar large vesicles (MLVs), respectively. SUVs are in the range of 100 nm, whereas MLVs are commonly between 500 nm and 5 µm.

Methods for synthesizing liposomes include the gentle hydration method (Wang et al. 2010) and the layer-by-layer electrostatic deposition (Madrigal-Carballo et al. 2010). Liposomes have been developed for food applications as a method for creating iron-enriched milk (Xia & Xu 2005), antioxidant delivery (Madrigal-Carballo et al. 2010), and codelivery of vitamins E and C with orange juice (Marsanasco et al. 2011).

Polymersomes
Polymersomes are vesicles formed from a bilayer of amphiphilic copolymers with bioactives entrapped in the formed cavity (Bouwmeester et al. 2009), similar to liposomes. The difference is that a copolymer of amphiphilic nature is used instead of
phospholipids. Similarly to liposomes, Polymersomes can be used to entrap both hydrophilic and hydrophobic drugs. Advantages of polymer carriers, as opposed to lipid carriers, include controlled release and increased stability and versatility (Rastogi et al. 2009). Polymersomes can be made with tri-block copolymers such as poly(caprolactone)-poly(ethylene glycol)-poly(caprolactone) and can be synthesized using methods similar to those used for polymeric nanoparticles (Rastogi et al. 2009).

Nanocrystals
Nanocrystals consist of a bioactive surrounded by a surfactant (Florence 2005).
Nanocrystals improve the solubility of poorly water-soluble drugs by increasing the surface-area-to-volume ratio, which increases the dissolution rate of the bioactive in vivo (Sun & Yeo 2012). The distinct advantage of nanocrystals is that they have 100% bioactive loading. Another advantage is that they can be prepared without organic solvents. Nanocrystals have shown not only improved solubility but also improved cellular interaction of the bioactive in nanocrystal form (Hunter et al. 2012).
Nanocrystals can be made using mechanical or chemical methods (Sun & Yeo 2012). Starch nanocrystals are commonly prepared using acid hydrolysis, ultrasonication, and stirring to disperse and prevent the nanocrystals from aggregating (Kristo & Biliaderis 2007, Xu et al. 2010). Wet milling is applied to reduce the size and increase the uniformity of the crystals (Sun & Yeo 2012). Another technique of forming nanocrystals is nanoprecipitation (Sun & Yeo 2012). Food grade starch and protein nanocrystals are being developed for various applications in the food and biomedical realms (de Mesquita et al. 2012, Flauzino Neto et al. 2013, Tzoumaki et al. 2011).
Lutein nanocrystals for oral delivery are in the process of development, but lutein crystals in the 100-nm range have not yet been achieved (Mitri et al. 2011).

**Lipid Nanoparticles**

SLNs are similar to emulsions, with the exception that the lipids are in a solid phase. The lipids in these SLNs are digested at a slower rate than lipids in a liquid phase (McClements & Li 2010). An advantage of SLNs is that they provide a means of entrapping lipophilic molecules in stable particles without the use of organic solvents. SLNs have the potential to provide controlled release of various lipophilic components due to decreased mobility of the bioactive in the solid matrix (Mehnert & Ma¨der 2012). However, these particles have a low loading efficiency and drug may be expelled during crystal structure transformation during storage (Tamjidi et al. 2013). NLCs are lipid nanoparticles with both crystallized and liquid phases of lipid used for nanodelivery (Tamjidi et al. 2013). The inner liquid phase dissolves and entraps the bioactive to provide the advantages of chemical stability, controlled release, and a higher loading efficiency (Tamjidi et al. 2013).

High-energy methods are often used to synthesize SLNs and NLCs for food applications (McClements & Li 2010, Tamjidi et al. 2013), including microfluidization and ultrasonication (Mehnert & Ma¨der 2012). Hot and cold homogenization techniques are able to form both SLNs and NLCs (Tamjidi et al. 2013). SLNs are often varied in size depending on the surfactant type and concentration; if the surfactant concentration is too low or too high, the SLNs will aggregate. The use of ionic surfactants often results in smaller particle sizes, and stability is increased by using more than one surfactant to stabilize the emulsion (Mehnert & Ma¨der 2012). SLNs were developed for food
applications with the goal of improving guava shelf life (Zambrano-Zaragoza et al. 2013), the stability of quercetin (Li et al. 2009), beta carotene, and alpha tocopherol (Trombino et al. 2009).

Polymeric Nanoparticles
Polymeric nanoparticles are matrices of polymers and entrapped molecules surrounded by an emulsifier or surfactant (des Rieux et al. 2006, Hunter et al. 2012, Plapied et al. 2011). Polymeric nanocapsules are nanoparticles in which the polymer forms a wall surrounding the entrapped core containing the bioactive; the polymer encapsulates the bioactive. These particles, nanospheres, and nanocapsules are designed to protect the entrapped bioactive from degradation (Mishra et al. 2010); polymeric particles can be engineered to have mucoadhesive or enhanced intestinal permeability (Chen et al. 2011). Methods of synthesizing polymeric nanoparticles include solvent displacement or nanoprecipitation or desolvation, salting out, and emulsion evaporation methods (Sabliov & Astete 2008). Other methods are pH cycling, thermal treatment, atomization spraying, and the use of super-critical fluids; these are mostly applied to nanoparticle synthesis for proteins and hydrocolloids. Natural polymer nanoparticles such as gum arabic maltodextrin were developed to improve the stability and bioavailability of epigallocatechin gallate (Peres et al. 2011). Protein polymer (zein) nanoparticles successfully entrapped essential oils with antioxidant properties (Wu et al. 2012). Gelatin nanoparticles encapsulated polyphenol antioxidants to protect their antioxidant activity and provide controlled release (Shutava et al. 2009).
Properties of Nanodelivery Systems of Importance to Nano-Bio Interactions
Physicochemical properties of the delivery systems will affect their absorption, distribution, metabolism, and excretion (ADME), which is essential in modulating the in vivo activity of the delivered bioactive. Properties of importance in nanoparticle in vivo interactions are size, charge, hydrophobicity, and targeting properties, among others (Figure 2).

Size is important for entry into cells, immune cell stimulation, and particle clearance (Naahidi et al. 2013). Cell uptake mechanisms are partially dependent on size. Endocytosis is the process through which nanoparticles or small molecules enter cells; the specific type of endocytosis through which the nanoparticle enters the cell determines the translocation of the entrapped molecule inside the endosome (Sahay et al. 2010). The common sizes of vesicles formed through endocytosis are between 60 nm and 1,000 nm. Particles larger than 500 nm are not endocytosed by enterocytes; therefore, they are commonly excreted before reaching the bloodstream (Table 1) (Yoo & Mitragotri 2010). The size of particles may affect the immune response; the larger the particle, the more likely it is to be eliminated by cells in the mononuclear phagocyte system (MPS), which will reduce the nanoparticle blood circulation time (Choi et al. 2011). Nanoparticles between 1 and 20 nm remain in the vasculature longer than larger particles; particles between 30 and 100 nm are able to evade the MPS and avoid clearance by macrophage cells, allowing for long circulation times (Table 1) (Faraji & Wipf 2009).

Hydrophobicity affects cellular uptake, distribution, interaction with immune cells and plasma proteins, and clearance from the body (Naahidi et al. 2013). Hydrophobic nanoparticles diffuse
into epithelial cells more easily than hydrophilic particles because of a large activity
coefficient (Table 1) (Acosta 2009, Powell et al. 2010). Hydrophobic nanoparticles are
recognized as foreign substances by macrophages of the MPS present in the blood and
are often cleared through biliary excretion (Table 1) (Bertrand & Leroux 2012, Naahidi et
al. 2013).

Hydrophilic nanoparticles may be quickly cleared from the kidney if they are smaller
than 10 nm (Table 1) (Choi et al. 2011). Hydrophilic nanoparticles are less likely to be
recognized as foreign objects in the blood (Naahidi et al. 2013). Hydrophilic
nanoparticles traverse through mucus at a rate faster than that of hydrophobic particles
due to hydrophobic interactions in the mucus layer (Lai et al. 2009).
### Table 1: Ranking of characteristics for absorption, distribution, and excretion

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Rank of properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSORPTION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucus Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 nm</td>
<td>High absorption</td>
<td>(Lai et al. 2007)</td>
</tr>
<tr>
<td>200 nm</td>
<td>Moderate absorption</td>
<td>(Acosta 2009)</td>
</tr>
<tr>
<td>300–500 nm</td>
<td>Low absorption</td>
<td>(Norris et al. 1998)</td>
</tr>
<tr>
<td>&gt;500 nm</td>
<td>No absorption</td>
<td>(Norris et al. 1998)</td>
</tr>
<tr>
<td>Charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No charge</td>
<td>High absorption</td>
<td>(Lai et al. 2009)</td>
</tr>
<tr>
<td>Positive</td>
<td>Adheres to mucus</td>
<td>(Lai et al. 2009)</td>
</tr>
<tr>
<td>Negative</td>
<td>Low absorption</td>
<td>(Lai et al. 2009)</td>
</tr>
<tr>
<td>Water affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enterocytes/M cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 nm</td>
<td>High absorption</td>
<td>(Faraj &amp; Wipf 2009, Florence 2005)</td>
</tr>
<tr>
<td>100–400 nm</td>
<td>Moderate absorption</td>
<td>(des Rieux et al. 2006)</td>
</tr>
<tr>
<td>500–5,000 nm</td>
<td>Low absorption</td>
<td>(Sahay et al. 2010)</td>
</tr>
<tr>
<td>Charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No charge</td>
<td>Moderate absorption</td>
<td>(Sahay et al. 2010)</td>
</tr>
<tr>
<td>Positive</td>
<td>High absorption</td>
<td>(Sahay et al. 2010)</td>
</tr>
<tr>
<td>Negative</td>
<td>Moderate absorption</td>
<td>(Sahay et al. 2010)</td>
</tr>
<tr>
<td>Water affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>High absorption</td>
<td>(Acosta 2009)</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>Low absorption</td>
<td>(Acosta 2009)</td>
</tr>
<tr>
<td><strong>DISTRIBUTION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 nm</td>
<td>Longest circulation</td>
<td>(Faraj &amp; Wipf 2009)</td>
</tr>
<tr>
<td>&gt;200 nm</td>
<td>Rapid clearance</td>
<td>(Faraj &amp; Wipf 2009)</td>
</tr>
<tr>
<td>Charge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No charge</td>
<td>Longest circulation</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td>Positive</td>
<td>Fastest clearance</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td>Negative</td>
<td>Rapid clearance</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td>Water affinity</td>
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<td></td>
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<tr>
<td>Hydrophobic</td>
<td>Faster clearance</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>Longest circulation</td>
<td>(Naahidi et al. 2013)</td>
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<tr>
<td><strong>EXCRETION</strong></td>
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<tr>
<td>Kidneys Size</td>
<td></td>
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<tr>
<td>&lt;5 nm</td>
<td>Excreted in urine</td>
<td>(Choi et al. 2011, Hunter et al. 2012)</td>
</tr>
<tr>
<td>&gt;5 nm</td>
<td>Not cleared from kidney</td>
<td>(Choi et al. 2011, Hunter et al. 2012)</td>
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*(Continued)*
Charge is important for entry into cells, immune cell stimulation, plasma proteins, and toxicity (Naahidi et al. 2013). Positive charges are more prone to promote an immune response than negative and neutral charges (Table 1) (Bertrand & Leroux 2012, Naahidi et al. 2013). Nanoparticles without charge are able to remain in the bloodstream for a longer time than positively or negatively charged particles, and negative charges are associated with longer circulation times in mice than positive charges (Bertrand & Leroux 2012).

Targeting affects nanoparticle distribution and immune response (Naahidi et al. 2013). Targeting molecules may exploit receptors to gain entry into specific types of cells (Markovsky et al. 2012).

<table>
<thead>
<tr>
<th></th>
<th>Rank of properties</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Charge</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No charge</td>
<td>Cleared</td>
<td>(Choi et al. 2011)</td>
</tr>
<tr>
<td>Positive</td>
<td>Accumulated in kidney</td>
<td>(Hunter et al. 2012)</td>
</tr>
<tr>
<td>Negative</td>
<td>Cleared</td>
<td>(Choi et al. 2011)</td>
</tr>
<tr>
<td><strong>Water affinity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>Not cleared from kidney</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>Excreted in urine</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td><strong>Liver/Intestines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–200 nm</td>
<td>Cleared in bile</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td>&gt;500 nm</td>
<td>Cleared in feces</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td><strong>Charge</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No charge</td>
<td>Cleared</td>
<td>(Choi et al. 2011)</td>
</tr>
<tr>
<td>Positive</td>
<td>Adhered to mucus</td>
<td>(Ensign et al. 2012)</td>
</tr>
<tr>
<td>Negative</td>
<td>Hindered biliary excretion</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td><strong>Water affinity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic</td>
<td>Cleared</td>
<td>(Bertrand &amp; Leroux 2012)</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>Cleared</td>
<td>(Ensign et al. 2012)</td>
</tr>
</tbody>
</table>
The physical and chemical properties, specifically size and surface charge, of the targeting molecule affect its immune response (Markovsky et al. 2012).

Adsorption, Distribution, Metabolism, And Excretion (ADME) of Nanoparticles
The ADME profile of a nanoparticle is greatly determined by properties such as size, charge, hydrophobicity, and targeting molecules, and these properties are dependent on the type of delivery system (Card et al. 2011). Nanoparticle ADME (Figure 3) will determine the potential toxicity.

Figure 3: Flow chart describing a nanoparticle’s path through the body from ingestion to excretion of the nanoparticle (Bouwmeester et al. 2009). Absorption refers to the nanoparticle interaction with the gut mucosa and epithelial cells, which ends in the nanoparticle entering into systemic circulation. Distribution or biodistribution occurs through systemic circulation, during which the particles are distributed to organs such as the liver, kidneys, spleen, heart, lungs, and brain. Metabolism describes the biotransformation of the nanoparticle that occurs through interactions with proteins and lipids in tissues such as the liver and intestinal tissue.
The liver, kidneys, and colon are primarily responsible for excretion of nanoparticles and their metabolites (Bertrand & Leroux 2012, Bouwmeester et al. 2009).

**Ingestion**

Knowledge of the fate of nanoparticles in the GI tract is needed to optimize delivery systems for improvement of bioavailability of food nutraceuticals. Ingested nanoparticles begin their GI journey when they enter the mouth and are partially digested through saliva and mastication at a pH between 5 and 7 (McClements & Li 2010). Then the nanoparticles enter the stomach through the esophagus, where they will remain for 30 min to 4 h, depending on whether the stomach is in a fasting or fed state (Kompella & Lee 2001, McClements & Li 2010). The acidic pH of the stomach (between 1 and 2), along with enzymes, is designed to break down proteins and carbohydrates (Ensign et al. 2012). If the nanoparticle is made out of hydrocolloids and proteins, degradation of the nanoparticles can be significant under the extreme pH of the stomach. Assuming that the particles are able to withstand the acidic pH and remain suspended in the media as an integral nanoparticle, they travel to the small intestine. Nanoparticles enter the intestines through the duodenum, where they are introduced to a pH between 6 and 7.5 and various bile salts from the gall bladder that are designed to emulsify fats (Kompella & Lee 2001, McClements & Li 2010). Nanoparticles remain in the small intestines for 3–6 h before traveling to the large intestine or colon (Kompella & Lee 2001, McClements & Li 2010). It is mainly in the small intestine that the nanoparticle and/or the released bioactive are absorbed.
Absorption
For nanoparticles to be absorbed, they have to overcome enzymes, the low pH of the stomach, the mucosal lining of intestines, and the cellular membranes with selective permeability (Chen et al. 2011, Ensign et al. 2012). The inner surface of the small intestine contains epithelial cells for the uptake of necessary nutrients or nanoparticles. These cells are covered in microvilli and mucus to help them absorb various nutrients and avoid absorption of harmful chemicals. Enterocytes in the small intestine are covered with a thin layer of firmly adherent mucus and a thick layer of loosely adherent mucus making it the easier for nutrients to be absorbed (Ensign et al. 2012). The surface properties and concentration of the particles determines the type of transport and extent of nanoparticle absorption. The optimal size for mucous transfer is

![Figure 4: Mechanisms of entry into cells (Sahay et al. 2010). Abbreviations: CCVs, clathrin-coated vesicles; CLICs, clathrin-independent carriers; GEEC, glycosylphosphatidylinositol-anchored protein-enriched compartment. Reprinted from J. Control. Release, Vol 145(3), Guarav Sahay, Daria Y. Alakhova, Alexander V. Kabanov, Endocytosis of Nanomedicines, Page 14, Copyright (2010), with permission from Elsevier.](image)
less than 100 nm, which is the average pore size of human mucus (Lai et al. 2007, 2009). However, a nanoparticle size of 200 nm is preferred over one of 100 nm because 200-nm nanoparticles have three times the diffusion coefficient of 100-nm nanoparticles (Acosta 2009) (Figure 3). The size cutoff for mucosal transfer is approximately 500 nm (Norris et al. 1998). M cells in Peyer’s patches make up approximately 1% of intestinal cells, and they are able to absorb large particles of approximately 1 µm as well as small particles of approximately 100 nm. Particles may enter M cells through endocytosis such as clathrin-mediated endocytosis or fluid-phase endocytosis (Florence 2005). Enterocytes are responsible for the rest of the cellular uptake in the intestine through clathrin-mediated endocytosis, caveolea-mediated endocytosis, macropinocytosis, caveolea, and clathrin-independent endocytosis (Figure 4) (Sahay et al. 2010). Nanoparticles endocytosed via clathrin-mediated endocytosis enter early endosomes with a low pH and eventually enter lysosomes in which they will be metabolized. Nanoparticles that enter cells via caveolea-mediated endocytosis are enveloped in caveosomes, which enter the nucleus.

Nanoparticle transport through cellular membranes and organelles is dependent on cell type, surface charge, nanoparticle composition, and hydrophobicity. Nanoparticle path and localization within the cellular environment will be responsible for functionality of the entrapped bioactive.

Distribution and Metabolism

Following absorption through the gut, very hydrophobic nanoparticles are transported to the lymph, if they are taken up in the gut-associated lymphoid tissue and reach lymphoid circulation (Bouwmeester et al. 2009); alternatively, particles can be sent to the liver via the hepatic portal vein, if they are not taken up by the gut-associated lymphoid tissue.
The liver is where they are processed and sent to systemic circulation (Chen et al. 2011) (Figure 3).

If the nanoparticles enter the lymph, they will reach circulation before being metabolized in the liver (Hunter et al. 2012). This is one way that nanoparticles are able to bypass first-pass metabolism and increase concentration of bioactives in the bloodstream. The lymphatic system delivers the nanoparticles to systemic circulation through the caudal vena cava (Lu et al. 2010). Upon reaching systemic circulation, nanoparticles are transported to various organs such as the heart, lungs, spleen, kidney, liver, and potentially the brain (Aillon et al. 2009). Organs with higher blood flow such as those listed above are exposed to higher concentrations of nanoparticles (Faraji & Wipf 2009).

The metabolism of nanoparticles greatly depends on the type of nanoparticle. Polymeric and lipid nanoparticles are often designed to be biodegradable. Natural polymers such as proteins and carbohydrates are degraded in the digestive tract via enzymes. Little information is known on the metabolic processes of nanoparticles and their components.

Excretion
Nanoparticles can also pass by the epithelial cells and mucus without being absorbed; if this happens, nanoparticles enter the colon directly, and they are prepared for excretion. From the liver, the nanoparticles may also reenter the intestines for excretion (Lu et al. 2010). Hydrophilic particles may be excreted through the kidneys into the urinary tract if they are smaller than 10 nm (Choi et al. 2011) (Figure 3).

Nanoparticles can be designed to improve absorption, enhance distribution, and reduce excretion (Table 2). Neutrally charged, hydrophobic nanoparticles that are smaller than 100 nm are
Table 2: Optimal characteristics of nanodelivery systems for improved absorption, distribution, and excretion

<table>
<thead>
<tr>
<th></th>
<th>Optimal property</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSORPTION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>&lt;100 nm</td>
<td>(Lai et al. 2007, Florence 2005, Faraji &amp; Wipf 2009)</td>
</tr>
<tr>
<td>Charge</td>
<td>Neutral</td>
<td>(Lai et al. 2009)</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Hydrophobic</td>
<td>(Acosta 2009)</td>
</tr>
<tr>
<td><strong>DISTRIBUTION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>&lt;100 nm</td>
<td>(Faraji &amp; Wipf 2009)</td>
</tr>
<tr>
<td>Charge</td>
<td>Neutral</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Hydrophilic</td>
<td>(Naahidi et al. 2013)</td>
</tr>
<tr>
<td><strong>EXCRETION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>&lt;5 nm for urine; &gt;500 nm for fecal matter</td>
<td>(Hunter et al. 2012)</td>
</tr>
<tr>
<td>Charge</td>
<td>Not Available</td>
<td>Not Available</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>Both cleared</td>
<td>(Bertrand &amp; Leroux 2012, Ensign et al. 2012)</td>
</tr>
</tbody>
</table>

Ideal for absorption. Neutrally charged, hydrophilic nanoparticles that are smaller than 100 nm remain in circulation longer, making them ideal for distribution. Hydrophilic nanoparticles smaller than 5 nm are excreted via the kidneys. Nanoparticles larger than 500 nm are not likely absorbed in the intestines and are excreted in the feces.

Fate of Bioactives

The entrapped bioactive is eventually released into the body either in the intestines, during systemic circulation, or in the cells of organ tissues (Bouwmeester et al. 2009, Chen et al. 2011). The surface chemistry of the nanodelivery system is responsible for the time and location of the release. Release can be stabilized through the use of polymers such as alginate or chitosan, which are applied in a layer-by-layer technique to prevent a burst release (Quintanilla-Carvajal et al. 2009).
The location of release can be controlled through the use of polymers that are sensitive to a specific pH range. For example, PEG has been shown to increase pH-stimulated lysozyme release (Quintanilla-Carvajal et al. 2009).

If released into the intestines, the bioactive will undertake intracellular trafficking into enterocytes or M cells. The chemical, morphological, and physical properties of the bioactive will determine the rate, method, and gravity of uptake (Sahay et al. 2010).

Non-ionic hydrophobic bioactives will be taken up in greater quantities than charged or hydrophilic molecules (Florence 2005). The bioactive may remain in the intestinal cells; it may be released into the lymphatic system, or it may be released into a portal vein routing it to the liver (Hunter et al. 2012, Markovsky et al. 2012).

If released into systemic circulation, the properties of the bioactive will determine how it interacts with blood components. A hydrophilic non-ionic bioactive will remain in circulation longer than a hydrophobic bioactive due to the hydrophobic and electrostatic interactions required for plasma proteins to bind to molecules (Sheng et al. 2009).

If released into the cells of organ tissues, cellular interactions will determine the intracellular trafficking of the bioactive (Sahay et al. 2010). The method of entry the nanoparticle employed to enter the cell will determine the location of bioactive release. If lysosomal release occurs, small hydrophobic molecules are able to escape the endosome or lysosome membranes, and large or hydrophilic molecules will remain inside the lysosomes (Romberg et al. 2008). Bioactives may follow the pathways of typical water-soluble or fat-soluble biomacromolecules depending on their chemical and physical properties (Sahay et al. 2010).
Safety Of Nanoparticles For Bioactive Delivery

Nanotechnology application in the food and agriculture sectors has been hindered to some extent by concerns about the safety of the engineered nanoparticle (ENP), as well as ethical, policy, and regulatory issues (Food Agric. Org. & World Health Org. 2012), all rooted in the lack of knowledge about the potential effects of nanomaterials on human health and the environment. Large differences in the toxicokinetic properties for ENPs of varying types are expected (European Commission 2009). Even for the same type of ENP, when nanoparticles do not show any acute toxicity, questions of long-term effects and bioaccumulation remain unanswered (Tiede et al. 2008). For poly(lactic-co-glycolic acid (PLGA) ENPs, making up a well-studied biodegradable and biocompatible polymeric delivery system, little to no histopathological changes were found in tissues derived from animals treated orally with PLGA nanoparticles. For example, high doses of PLGA ENPs (4 mg/0.2 ml) did not lead to any tissue lesions as compared to the same high dose of ZnO nanoparticles that led to toxicity in Balb/C mice (Semete et al. 2010). Similarly, in Sprague Dawley rats, histopathological examination of tissue indicated absence of any inflammatory response in the liver and spleen (Mittal et al. 2007).

Cationic bovine serum albumin–PLGA ENPs showed mild and transient inflammatory reactions in the liver and kidney; mild reactions in the spleen; and no reaction in the cerebral cortex, hippocampus region, cerebellum, lung, or heart (Lu et al. 2007). To date, little work has been done in vivo to evaluate the fate of polymeric nanoparticles purposely designed for food and agricultural uses (European Commission 2009) and to correlate with the fate of nanoparticles after ingestion with their possible toxic effects. The challenge for characterizing nanoparticles with regard to human toxicity is due to the diversity of the size, shape, and surface chemistry.
(Elder et al. 2009) of ENPs. In addition, agglomeration, degradation, and mucus interaction of particles as a function of ENP material composition and surface characteristics will dictate the ADME profile of the ENPs and inherently ENP safety. Nanoparticles made of food grade materials, intended specifically for food and agriculture uses, are expected to be nontoxic to the animal host on the premise that the materials used in the ENP synthesis are carefully selected to hold generally-recognized-as-safe status. Yet, ENP safety to humans and farm animals exposed to nanoparticles is a major concern, and understanding the effect of the particle type and properties on the fate of the nanoparticle in the GI tract, of ENP uptake, and of biodistribution as well as the potential toxic effects is critically important for the responsible development and use of ENPs in food and agriculture.

Toxicological studies provide information on carcinogenicity, teratogenicity, reproductive toxicity, developmental toxicity, and neurotoxicity of the nanoparticle prior to their use (Cockburn et al. 2012). Cockburn et al. proposes a two-tiered system for engineered nanomaterial (ENM) safety characterization, where in silico, in vitro, and subacute in vivo studies comprise tier 1 and acute in vivo studies make up tier 2. In tier 1, the beginning criteria to evaluate engineered nanodelivery systems are biodegradability, biocompatibility, and solubility compared to their microscale counterparts.

The metabolism and elimination profiles of the nanomaterial are important factors to consider in preventing accumulation of the nanodelivery systems in tissues and clearance organs (Markovsky et al. 2012). In silico studies provide computed data describing the potential toxicity and ADME profile. In vitro studies use real or simulated biological
material to determine possible cytotoxicity, genotoxicity, reactive oxygen species
generation, inflammatory response, and chemical degradation of nanomaterials. In vivo
studies offer data on toxicity, genotoxicity, and the ADME profile; for tier 1, the
suggested timeline for in vivo repeat dosing is 14 or 28 days (Cockburn et al. 2012). In
tier 2, the dosing time for in vivo studies increases to an acute dose time of 90 days to
determine carcinogenicity, reproductive toxicity, neurotoxicity, and teratogenicity
(Cockburn et al. 2012).
Safe application of nanoparticles to food and agriculture requires the nanoparticle
colorization, functionality, ADME profile, and toxicity to be determined. A tiered
approach to ENM testing provides a means to establish the type and quantity of
toxicological studies necessary to determine safety.

Conclusion
Nanodelivery systems offer a multitude of physical and chemical advantages for
improved bioavailability and stability of bioactives. Each type of nanodelivery system
offers distinct benefits, and the properties of the bioactive along with the purpose of
delivery should determine which nanoparticle type is most appropriate for the
application. The system’s chemical, physical, and morphological properties will
determine the nano-bio interaction and ADME profile. Properties of importance for
cellular and immunological interaction are size, charge, hydrophobicity, and targeting
molecules. Degradation in the stomach (low pH, presence of enzymes), the presence of
the firmly adherent mucosal lining, selectively permeable epithelial and M-cell
membranes, as well as enzymatic degradation in the intestines hinder uptake of the
nanodelivery system and bioactive absorption. Upon absorption, the nanodelivery system must evade the MPS, which will remove nanoparticles from systemic circulation. Distribution of the nanodelivery system begins once nanoparticles enter systemic circulation through the lymph or caudal vena cava. The fate of the bioactive greatly depends on where it is released, but it also depends on its chemical, physical, and morphological properties. The location of bioactive release can be tailored using nanomaterials with specific surface chemistry, enabling release at the target. A layer-by-layer approach for engineering the nanodelivery system prevents the burst release of the bioactive and ensures a gradual release profile of the bioactive. The safety of nanodelivery systems is determined by the materials’ biocompatibility, biodegradability, and nanoparticle ADME profile. A combination of in silico, in vitro, and in vivo studies reveals the safety and toxicological profile of the nanodelivery system and is required for the safe application of nanodelivery systems in food and agriculture.

Summary Points

1. Nanodelivery of food bioactives enhances their bioavailability and biodistribution through the improvement of their uptake, stability, and solubility.
2. Nanodelivery systems are classified into two main groups, liquid and solid. Liquid particles can be nanoemulsions, nanoliposomes, or nanopolymersomes. Solid particles consist of nanocrystals, lipid particles, or polymeric particles.
3. Physicochemical properties of importance for nano-bio interaction include nanoparticle size, charge, hydrophobicity, and targeting molecules, among others. Size is important for nanoparticle entry into cells, immune system interactions, and nanoparticle clearance.
Hydrophobicity affects absorption, distribution, interaction with immune cells and plasma proteins, and route of excretion. Charge is important for mucoadhesion or diffusion, cellular uptake, and toxicity. Targeting affects biodistribution and immune responses.

4. Orally delivered nanoparticles must overcome the chemical and physical barriers to absorption, which include the acidic pH of the stomach and enzymes, the loosely and firmly adherent mucosal linings of the intestines, and selectively permeable enterocyte membranes.

5. The entrapped bioactive is eventually released in the intestines, in the circulatory system, or in the cells of various organs. The location of release and the chemical and physical properties of the bioactive determine its biological fate.

6. The safe application of nanoparticles in food and agriculture require knowledge of their ADME and toxicological profiles.

Disclosure Statement
The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Literature Cited


European Commission. 2009. Scientific opinion of the Scientific Committee on a request from the European Commission on the potential risks arising from nanoscience and nanotechnologies on food and feed safety. EFSA J. 958:1–39


Yoo J-W, Mitragotri S. 2010. Polymer particles that switch shape in response to a


CHAPTER 3: ANTIOXIDANT ACTIVITY OF ENTRAPPED LUTEIN

Introduction

Unsaturated fatty acids and lipids are a prime target of oxidation in foods and biological systems (Laguerre, Lecomte et al. 2007). Macular degeneration and cataracts, UV induced sun damage, and risk of atherosclerosis can be decreased by enhanced consumption of foods or supplements containing lutein (Kritchevsky, Bush et al. 2000; Dwyer, Navab et al. 2001; Krinsky, Landrum et al. 2003; Alves-Rodrigues and Shao 2004; Lee, Faulhaber et al. 2004).

Lutein, a dietary antioxidant (Figure 3.1), is essential for diminishing photo-induced oxidative stress and is useful for filtering high energy blue light in the lens and macula lutea in the retina (Alves-Rodrigues and Shao 2004; Ahmed, Lott et al. 2005).

Figure 3.1 Lutein Structure

Lutein is reactive at quenching both singlet oxygen radicals, formed through UV photonic exposure (Laguerre, Lecomte et al. 2007), and peroxyl radicals, which result from the oxidation of unsaturated fatty acids (Kiokias and Oreopoulou 2006). Singlet oxygen radicals are electrophilic and form hydroperoxides, which break down into radicals causing autoxidation of lipids. Hydroxyl, hydroperoxyl, peroxyl, and lipid
Alkoxy radicals are mainly responsible for non-enzymatic lipid oxidation, which results in the formation of toxic hydroxy and ketocholesterols (Laguerre, Lecomte et al. 2007; Lordan, Mackrill et al. 2009). In the macula lutea of the eye, cholesterol is photooxidized to 7-ketocholesterol and 7α and β-hydroxycholesterols, process linked to macular degeneration (Breuer, Dzeletovic et al. 1996; Rodriguez and Larrayoz 2010).

Lutein is easily oxidized by photooxidation or free radicals; therefore providing a means of improving its chemical stability until use would preserve the efficacy of the antioxidant (Lakshminarayana, Aruna et al. 2008). Release of preserved antioxidant over time, providing radical scavenging benefits for extended periods of time, would further aid in enhancing its activity for long-term applications.

Nanodelivery systems have been proposed as alternatives for improving stability, release and functionality of bioactives. It has been shown for example that chemical stability of quercetin, alpha-tocopherol, and beta-carotene was protected using chitosan and solid lipid nanoparticles (Trombino, Cassano et al. 2009; Luo, Zhang et al. 2010), while PLA, PLGA, and zein nanoparticles were previously developed to provide controlled release of other hydrophobic drugs and antioxidants (Kumari, Yadav et al. 2010; Hu, Liu et al. 2011; Hu, Lin et al. 2012). Of the studies focused on entrapping antioxidants in nanoparticles many are centered on nanoparticle characteristics, antioxidant stability and release, and very few on the effect of the delivery system on the efficacy of the antioxidant (Bala, Bhardwaj et al. 2006; Pool, Quintanar et al. 2012).

The goal of this study was to determine if stability and antioxidant activity of lutein could be improved by entrapping the bioactive into polymeric nanoparticle of
different surface properties. The hypothesis of this study was that by providing controlled release and protection from degradation of entrapped lutein into polymeric nanoparticles made of poly(lactic-co-glycolic) acid (PLGA) will improve its antioxidant activity. Furthermore, it was hypothesized that the addition of chitosan on the nanoparticle surface will enhance the antioxidant efficacy by acting as a co-antioxidant; chitosan like lutein is a singlet oxygen scavenger (Xie 2001; Xing, Liu et al. 2005; Yen, Yang et al. 2008).

Two nanoparticle types of a similar size, PLGA (lutein) and PLGA/Chitosan (lutein), were used in this study. The particles differed in surface charge due to the positively charged chitosan on the surface of PLGA/Chitosan (lutein) NPs. Release and degradation of lutein were assessed in Tween 20 emulsions in the presence and absence of the chemical initiator, 2,2′-azobisamidinopropane dihydrochloride (AAPH) using spectrophotometry. Antioxidant activity was evaluated using a cholesterol assay to determine the effect of NP entrapment on the antioxidant efficacy of lutein.

Materials and Methods

Materials: PLGA (50:50; Mol. Wt. 30,000-60,000) was purchased from Sigma Aldrich (St. Louis, Missouri). Koumin Foods, LLC (Des Moines, IA) provided lutein. Poly (vinyl alcohol) (PVA; Mol. Wt. 9000), ethyl acetate, acetonitrile, (2,2′-azobisamidinopropane dihydrochloride) AAPH, Tween 20, and all other chemicals were purchased from Sigma Aldrich (St. Louis, Missouri).

Synthesis: Poly (D, L-Lactide-co-glycolide) nanoparticles (NPs) with entrapped lutein and blank polystyrene NPs (as a control) were prepared using emulsion evaporation. The
organic phase was made up of 200 mg of PLGA and 20 mg of lutein (10% loading), which were dissolved in 8mL of ethyl acetate. The organic phase was added via a syringe to 40mL of an aqueous phase containing 2% w/v PVA in deionized water and 4 mL of ethyl acetate at 300 rpm. The emulsion was run three times through the microfluidizer at 30000psi for uniform size reduction. Solvent evaporation occurred under vacuum (70 mm Hg) for 60 minutes by rotovap (Buchi R-124, Buchi Analytical Inc., New Castle, DE, USA). Residual lutein and PVA were removed through dialysis membranes with MWCO: 100,000 Daltons. PLGA/Chitosan NPs were made by adding low molecular weight chitosan to PLGA NPs after dialysis under stirring at a pH of 3 in a ratio of 1:10, Chitosan to PLGA. Polystyrene NPs were made exactly as PLGA NPs without the addition of lutein. After dialysis, trehalose was added in a (1:1) w/v ratio, and particles were lyophilized using Freezone 4.5 (Labconoco Corp., Kansas City, MO, USA) for 72 hours. Freeze dried particles were stored at 0°C until analysis.

Size Analysis and Morphology: Hydrodynamic radius, size distribution, and polydispersity index was determined by dynamic light scattering with a Zetasizer (Malvern Instruments, Malvern, UK). Transmission electron microscopy (TEM) was used for morphology studies of the particles. Zeta potential was also measured with the Zetasizer using the Helmholtz-Smoluchowski equation.

Entrapment: Extraction was performed to remove entrapped lutein from nanoparticle powder. An amount of 6 mg of powder was suspended in 600 µL of water using sonication and next 5.4 mL of acetonitrile was added. The mixture was allowed to set for 4 hours, and the 6 mL sample was centrifuged at 30,000 rpm for 15 minutes at 8 °C. The absorbance of the supernatant was measured on a UV-Vis spectrophotometer (Genesys 6,
Thermo Fisher Scientific, Waltham, MA) at 450 nm for detection of lutein. The absorbance value was used to calculate the concentration of lutein entrapped using a standard curve. The limit of detection on the spectrophotometer for lutein was calculated to be 3.8 µg/mL.

Cholesterol Oxidation: An emulsion was made with 1000 mg of cholesterol with 10 mL of tween 20 in one liter of nanopure water. AAPH was used as an initiator at a concentration of 100 ppm. Each lutein sample was mixed with 27 mL of the cholesterol solution and 3 mL of AAPH for a final lutein concentration of 94 µM. The solution was continuously stirred for 72 hours at 37 °C as a model for human body temperature.

Samples were taken for HPLC analysis at 0, 24, 48, and 72 hours to determine the level of 7-ketocholesterol, the oxidation product of cholesterol. The HPLC analysis method used for 7-ketocholesterol determination was obtained from Xu et al. (Xu, Hua et al. 2001). A 1 mL sample was taken and mixed with hexane. Centrifugation at 5,000g for 10 minutes was performed to remove the aqueous portion of the sample. Sodium sulfate was used to further remove any moisture before injecting the sample. The sample was analyzed using a Waters 2690 separation module with a silica normal phase column and a mobile phase of hexane: isopropanol (99%: 1%) was used at a 1.5 mL/min flow rate. Detection of 7-ketocholesterol (UV wavelength of 234 nm) was done using a 996-photodiode array detector and a Millennium chromatography station (Milford, MA). The concentration was calculated using a standard curve of 7-ketocholesterol (Tian, Wang et al. 2011).

Physical Stability of lutein-loaded nanoparticles in emulsion environments: The physical stability of NP, with entrapped lutein in the cholesterol emulsions was assessed using the
Zetasizer (Malvern Instruments, Malvern, UK). Change in size, PDI, and zeta potential of the NPs were monitored for 72 hours in the cholesterol emulsion using the methods described above. Chemical stability of lutein emulsified and entrapped in PLGA (lutein), and PLGA/Chitosan (lutein) nanoparticles was measured for up to 24 hours in emulsions with and without AAPH.

Localization in the emulsion environment: Confocal imaging was used to determine the localization of NP lutein in the linolenic acid emulsion. Linolenic acid emulsion droplets were stained using oil red (excitation: 589 nm, emission: 615 nm). Fluorescently labeled PLGA with fluorescein isothiocyanate, FITC, (excitation: 495 nm, emission: 519 nm) were used in this study. All images were taken using a Leica TCS SP2 Spectral Confocal Microscope (Buffalo Grove, IL).

Statistical Analysis: All experiments were performed in triplicate, and all data was analyzed for statistical difference (Adj P < 0.05) using the PROC MIXED with the Tukey-Kramer adjustment Procedure in SAS 9.3 (SAS Institute Inc. Cary, NC).

Results

Size, Morphology, Zeta Potential and Remaining PVA analysis:

PLGA (lutein) NPs measured 119 nm ± 0.98 nm in size, and PLGA/Chitosan (lutein) NPs were 146 ± 3 nm in diameter when measured in nanopure water (Table 3.1) with PDIs of 0.17±0.02 and 0.25 ± 0.01, respectively. Zeta potentials in nanopure water of PLGA (lutein) NPs and PLGA/Chitosan (lutein) NPs were -29 ± 1 and 21 ± 2 mV, respectively (pH 6.8). The remaining PVA surfactant after dialysis was corresponding 11 ± 1 and 10 ± 1% of PLGA (lutein) and PLGA/Chitosan (lutein) NP structure, respectively. Based on
TEM pictures, PLGA(lutein) NPs appeared to be spherical and uniform in size, while PLGA/Chitosan (lutein) NPs appeared to be nearly spherical with a rough outer coating (Figure 3.2). Entrapment efficiency measurements revealed that 21.8 ± 0.63 µg and 20.2 ± 0.62 µg lutein per mg of nanoparticles was entrapped in the PLGA (lutein) and PLGA/Chitosan (lutein) nanoparticles, respectively.

Table 3.1 Nanoparticle physical/chemical properties, including size, polydispersity, zeta potential and surfactant concentration for PLGA and PLGA/Chitosan nanoparticles with entrapped lutein

<table>
<thead>
<tr>
<th>Nanoparticle Type</th>
<th>Size (nm)</th>
<th>PDI (units)</th>
<th>Zeta Potential (mV)</th>
<th>PVA Surfactant (mg PVA/mg Particle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA (Lutein)</td>
<td>119.2 ± 0.98</td>
<td>0.17 ± 0.02</td>
<td>-29 ± 1.10</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>PLGA/Chitosan (Lutein)</td>
<td>145.9 ± 3</td>
<td>0.25 ± 0.01</td>
<td>+21.2 ± 2.30</td>
<td>0.06 ± 0.00</td>
</tr>
</tbody>
</table>

Figure 3.2: TEM images of A) PLGA (lutein) and B) PLGA/Chitosan (lutein) NPs
Physical stability of NPs at 37 °C in the presence of Tween 20 emulsions was evaluated using DLS, as these conditions were employed for cholesterol oxidation experiments. PLGA and PLGA/Chitosan NPs with entrapped lutein remained stable for 24 hours in the tween emulsion (Figure 3.3). Sizes increased at 48 hours and retained the larger size until 72 hours, but size changes over time were not found statistically different (Figure 3.3).

![Figure 3.3: Size Stability of NPs in Tween Emulsions at 37°C](image)

Lutein chemical stability was determined by measuring the amount of lutein present in the NP over time at 37 °C in the presence and absence of a radical initiator, AAPH. Chemical stability of NP entrapped lutein was compared to a Tween 20 lutein emulsion. In the presence of AAPH, 71% ± 15% of the lutein remained after 12 hours, and 50% ± 30% remained after 24 hours in the emulsified form, whereas in the PLGA (Lutein) NP emulsion, 81% ± 3% of the lutein was still available after 12 hours, and 65%± 10% after 24 hours. In the PLGA/Chitosan (lutein) NP emulsion 84% ± 7% lutein was preserved after 12 hours, and 60% ± 28% after 24 hours (Figure 3.4 A). These changes over time
were not statistically significant. In the absence of AAPH, 0% of lutein in emulsified lutein, PLGA (lutein) NPs and PLGA/Chitosan (lutein) NPs was degraded over 24 hours (Figure 3.4 B).

Figure 3.4: Chemical Stability of lutein in Tween Emulsions in the A) presence and B) absence of AAPH at 37 °C: Results are reported as the percentage of lutein remaining in the NP or in the emulsion at 12 and 24 hours.

Release of lutein from the nanoparticles suspended in the cholesterol emulsions was examined and PLGA NPs showed 50% release of lutein after 24 hours and 89% release after 72 hours. PLGA/Chitosan NPs showed 23 % release of lutein after 24 hours and 69% release after 72 hours from PLGA NPs. The change in percent of lutein release was statistically significant for each time point and was statistically different between
PLGA (lutein) and PLGA/Chitosan (lutein) NPs (Figure 3.5) with more lutein being released from PLGA (lutein) than from PLGA/Chitosan (lutein) NPs.

Figure 3.5: Release of Lutein in Cholesterol Emulsion: PLGA NPs and PLGA/Chitosan NPs over 72 hours

Cholesterol Oxidation
Cholesterol oxidation results in the formation of 7-ketocholesterol; this compound can be extracted and measured using normal-phase high-pressure liquid chromatography. Presence of 7-ketocholesterol is associated with a low effectiveness of entrapped lutein as an antioxidant and can be used to determine lutein antioxidant activity over time. 7-ketocholesterol concentrations are shown for blank control and lutein treatments at 24, 48, and 72 hours (Figure 3.6).

No statistical difference was found between the unloaded treatments and the control at 24 hours (Figure 3.6 A). Polystyrene NPs showed no difference in the amount of 7-ketocholesterol present after 24, 48, and 72 hours when compared to the blank control,
whereas blank PLGA significantly reduced the oxidation of cholesterol at 72 hours and PLGA/Chi unloaded nanoparticles significantly inhibited cholesterol oxidation at 48 and 72 hours (Figure 3.6 A). At 24 hours no statistical significance was found between lutein loaded treatments, free lutein, and control (Figure 3.6 B). Lutein, PLGA (lutein), and PLGA/Chitosan (lutein) showed significantly lower concentrations of 7-ketocholesterol at 48 hours versus the control. PLGA/Chitosan (lutein) was significantly lower than lutein at 48 and 72 hours when compared to the control (Figure 3.6 B).

Figure 3.6 A): 7-Ketocholesterol Concentrations at 37 °C for Blank NP Treatments: 24, 48, and 72 hours * Empty PLGA and PLGA/Chitosan significantly lower than Control B) 7-Ketocholesterol Concentrations at 37 °C for Lutein NP Treatments: 24-Hours, 48 hours, and 72 hours *A Lutein, PLGA(lutein), PLGA/Chitosan (lutein) significantly lower than control *AB PLGA/Chitosan (lutein) significantly lower than lutein
Discussion

PLGA and PLGA/Chitosan nanoparticles with entrapped lutein were synthesized by emulsion evaporation method, using poly (vinyl alcohol) as a surfactant. All particles were determined to have a similar size, but differed in zeta potential due to the addition of positively charged chitosan. Stability, release and antioxidant activity of lutein were assessed in Tween 20 emulsions. Lutein stability in the presence and absence of the chemical initiator, 2,2’-azobisamidinopropane dihydrochloride (AAPH) and lutein release were assessed using spectrophotometry. Antioxidant activity of free and entrapped lutein was evaluated by cholesterol oxidation analysis to assess the effect of entrapment and nanoparticle properties on the activity of the bioactive.

PLGA (119.2 nm) and PLGA/Chitosan (145.9 nm) nanoparticles loaded with 21.8 ± 0.63 and 20.2 ± 0.62 µgs lutein/ mg NP, respectively were used in these studies. In terms of size stability over time, PLGA nanoparticle size increased by 30 nm over the 72 hour period, and the PLGA/chitosan nanoparticle size increased by 50 nm over the 72 hour period at 37 °C, but the difference was not found statistically significant. Overall, nanoparticles maintained their size stability over time under conditions relevant for antioxidant experiments. Chemical stability of lutein was tested in the presence and absence of the oxidizing agent, AAPH. In the absence of AAPH, 0% of entrapped and free lutein was degraded over 24 hours, as expected. The percentage of lutein degraded after 24 hours in the presence of AAPH was not statistically different for emulsified lutein, PLGA (lutein) and PLGA/Chitosan (lutein) due to the amount of lutein released in this environment after 24 hours (Figure 3.5).
The cholesterol assay based on the measurement of 7-ketocholesterol, the final oxidation product of cholesterol oxidation by radicals was used to measure antioxidant activity of entrapped lutein. Cholesterol, an unsaturated lipid, undergoes the same three-stage oxidation process as unsaturated fatty acids (Laguerre, Lecomte et al. 2007). The double bond at carbons 5,6 make cholesterol easier to oxidize than other unsaturated lipids (Iuliano 2011). Oxysterols are seen as end products of radical chain reactions with cholesterol (Iuliano 2011). The final end product of non-enzymatic cholesterol oxidation is 7-ketocholesterol, 3% -hydroxycholest-5-en-7-one, which results from the addition of a ketone group to carbon 7 (Iuliano 2011).

Based on cholesterol oxidation experiments, all PLGA and PLGA/Chitosan nanoparticles acted as antioxidants by significantly reducing the oxidation of cholesterol. In order to determine whether this occurred via the physical presence of the nanoparticles or via chemical reactions of the nanoparticles confocal microscopy was performed. Imaging showed that PLGA nanoparticles moved throughout the emulsion independently from the emulsion droplets (data not shown) therefore proving that protection from oxidation was not simply the result of a NP physical association with the emulsion droplets, but rather the chemical composition of the nanoparticle. To further prove this, polystyrene nanoparticles were included in the study as a control in the cholesterol experiment. The polystyrene nanoparticles did not show any antioxidant activity (Figures 3.6A), clearly indicating that the antioxidant properties of PLGA and PLGA/Chitosan nanoparticles were associated with chemical activity of the materials making the delivery systems or the antioxidant loaded in the nanoparticles. The free carboxylic acid end groups of PLGA or the positively charged amine groups of chitosan may have contributed to the
antioxidative effects of the NPs through hydrogen donation, explaining the lack of antioxidant activity of polystyrene nanoparticles.

The lutein release (Figure 3.5) coincided with the increased antioxidant activity of lutein over time (Figure 3.6B), for antioxidant activity improved as more lutein was released over time. For example, PLGA/Chitosan (lutein) showed 23 % lutein release after 24 hours and 69%, a much higher release after 72 hours (Figure 3.5), significantly different between all time points. It was therefore inferred that the significant reduction of oxidation of cholesterol observed at 48 hours and not at 24 hours was due to the time needed for the lutein to be released from the nanoparticle and to reach the interface of the emulsion. After 48 hours, lutein, PLGA (lutein), PLGA/Chitosan, PLGA/Chitosan (lutein) significantly reduced oxidation of cholesterol compared to the control, and PLGA/Chitosan (lutein) significantly reduced oxidation compared to the unentrapped lutein control (Figure 3.6B).

After 72 hours, the treatments of lutein, PLGA, PLGA (lutein), and PLGA/Chitosan (lutein) reduced the oxidation of cholesterol compared to the blank, but PLGA/Chitosan (lutein) further reduced the oxidation compared to lutein and PLGA(lutein), which is explained by the addition of chitosan (Figure 3.6B). Low molecular weight chitosan was found to be a scavenger of singlet oxygen and hydroxyl radicals, so the addition of chitosan should make a significant difference in antioxidant activity (Xie 2001; Xing, Liu et al. 2005; Yen, Yang et al. 2008).

PLGA/Chitosan (lutein) NPs showed significantly better antioxidant activity than PLGA/Chitosan at 72 hours, which is explained by the addition of lutein and the increase in lutein release at this time.
PLGA (lutein) and PLGA/Chitosan decreased the oxidation of cholesterol compared to PLGA, which can be explained by the addition of material with antioxidant properties lutein and chitosan.

Conclusions

In an effort to determine suitability of polymeric nanoparticles for delivery of antioxidants, specifically lutein, the stability and antioxidant activity of lutein entrapped in PLGA and PLGA/Chitosan NPs in comparison with unentrapped lutein, were measured. Both PLGA and PLGA/Chitosan NPs with entrapped lutein maintained their size throughout antioxidant experiments. PLGA and PLGA/Chitosan NPs did not improve the chemical stability of lutein when compared to emulsified lutein in the presence of AAPH. However, PLGA/Chitosan NPs improved the antioxidant activity of lutein by 21% ± 1% at 48 hours and 21%± 3% at 72 hours compared to emulsified lutein using the cholesterol assay (Figure 3.6B). Blank PLGA and PLGA/Chitosan NPs also appeared to act as antioxidants in these experiments, whereas polystyrene NPs did not; this suggested that blank PLGA and PLGA/Chitosan NPs improved the antioxidant activity through chemical reactions rather than providing a physical barrier to oxidation. It was concluded that entrapping lutein in PLGA and PLGA/Chitosan NPs did not significantly improve lutein stability over that of free lutein, but due to the release of lutein over time achieved by entrapping it in PLGA and PLGA/Chi nanoparticles, it increased its antioxidant efficacy.
The addition of materials of antioxidant activity such as chitosan to the nanoparticles further improved the antioxidant activity of the nanoparticle system.

Future Experiments
The chemical mechanism by which unloaded PLGA and PLGA/Chitosan exhibited antioxidant properties is not covered in the published literature. Additional studies determining the effect of oxygen concentration and pH of the experimental environments on cholesterol oxidation is important for determining the chemical mechanism of action by which PLGA and PLGA/Chitosan prevented oxidation of cholesterol. Further experiments testing effects of NP properties on antioxidant activity of entrapped lutein could also provide a clearer understanding of the enhanced particle antioxidant activity of nanodelivered lutein.

Literature Cited


metabolites by HPLC and LC-MS (atmospheric pressure chemical ionization)." 


CHAPTER 4. CONCLUSIONS

Nanodelivery systems offer many physical and chemical advantages for improved functionality of entrapped bioactives; i.e. the addition of chitosan significantly improved the antioxidant efficacy of lutein against the oxidation of cholesterol. Each type of nanodelivery system offers distinct benefits, and the properties of the bioactive along with the purpose of delivery should determine which nanoparticle type is most appropriate for the application, i.e. nanodelivery systems for oral delivery should be biodegradable and biocompatible. Lutein is one of the few carotenoids found in the retina and is essential for diminishing photo-induced oxidative stress in the lens and macula lutea in the retina (Alves-Rodrigues and Shao 2004), (Ahmed, Lott et al. 2005). Also in the macula lutea of the eye, cholesterol is photooxidized to 7-ketocholesterol and the process is linked to macular degeneration (Rodriguez and Larrayoz 2010), (Breuer, Dzeletovic et al. 1996). Lutein was proven to significantly reduce the oxidation of cholesterol to 7-ketocholesterol, and PLGA/Chitosan (Lutein) was found to improve this effect when compared to lutein after 48 hours of oxidation. Further studies should be done to determine the nano-bio interaction, bioavailability, and ADME profile of PLGA/Chitosan (lutein) compared to free lutein in order to determine if PLGA/Chitosan (Lutein) as a nanodelivery system could improve the efficacy of lutein as a treatment for macular degeneration.

Literature Cited


APPENDIX

3.1 Lutein Standard Curve: All lutein concentrations were calculated using a lutein standard. The absorbance was measured on a UV-Vis spectrophotometer (Genesys 6, Thermo Fisher Scientific, Waltham, MA) at 450 nm.

\[
y = 0.1725x \\
R^2 = 0.99562
\]
3.2 PVA Standard Curve: All NP PVA Concentrations were calculated using a PVA standard. The absorbance was measured on a UV-Vis spectrophotometer (Genesys 6, Thermo Fisher Scientific, Waltham, MA) at 690 nm.

![PVA Standard Curve](image)

\[
y = 0.029x - 0.0235
\]

\[R^2 = 0.99721\]
3.3 Size Stability in Cholesterol and Linolenic Acid Emulsions: Size in (nm) and Zeta Potential in (mV) and of PLGA and PLGA/Chitosan (lutein) NPs at 0, 24, 48 and 72 hours.

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3.4 Cholesterol Oxidation: 7-ketocholesterol Concentrations 0, 24, 48, and 72 hours and % Inhibition at 24, 48, and 72 hours.

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<td>PLGA</td>
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<td>14%</td>
<td>16%</td>
<td>4%</td>
<td>4%</td>
<td>2%</td>
</tr>
<tr>
<td>PLGA(lutein)</td>
<td>52%</td>
<td>45%</td>
<td>36%</td>
<td>2%</td>
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<td>1%</td>
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<tr>
<td>PLGA/Chitosan(lutein)</td>
<td>54%</td>
<td>60%</td>
<td>42%</td>
<td>4%</td>
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Appendix 3.5: **Cholesterol Oxidation from 0 è72 Hours**: The general trend of 7-ketocholesterol increase between the treatments is depicted in this graph.
3.6 SAS Programs

3.6A: Size Stability
Title 'Size Stability in Emulsion';
Proc Mixed data=work.SIZEE;
Class NP Beaker Time;
Model Y = NP|Time / outp= resids;
Random Beaker(NP) Beaker(NP*Time);
LSMeans NP|Time / adjust=tukey CL;
run;

Proc Univariate data = resids plot=normal;
var resid;
run;
PROC IMPORT OUT= WORK.SIZEE DATAFILE= "C:\Users\snavar4\Documents\SAS1\EmulsionSizeStability.xlsx"
   DBMS=EXCEL REPLACE;
   RANGE="Sheet1$";
   GETNAMES=YES;
   MIXED=NO;
   SCANTEXT=YES;
   USEDATE=YES;
   SCANTIME=YES;
RUN;

3.6 B: Chemical Stability
Title 'Lutein AND Cholesterol Chemical Stability in Emulsion Experiment';
Proc Mixed data=work.RELEASE;
Class NP Beaker Time;
Model Y = NP|Time / outp= resids;
Random Beaker(NP) Beaker(NP*Time);
LSMeans NP|Time / adjust=tukey CL;
run;

Proc Univariate DATA = resids plot = normal;
var resid;
run;
PROC IMPORT OUT= WORK.Release DATAFILE= "C:\Users\tborel1\Documents\RELEASE.xlsx"
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   RANGE="Sheet4$";
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   MIXED=NO;
   SCANTEXT=YES;
   USEDATE=YES;
   SCANTIME=YES;
RUN;

dm 'log; clear; output; clear';
options nodate;
ods html close;
ods html;
ods listing;
3.6 C: Cholesterol Oxidation

    dm 'log; clear; output; clear';
options nodate;

    ods html close;
ods html;
ods listing;

    Proc Mixed data=WORK.SASCHOL3;
    Class REP TREATMENT BEAKER TIME;
    Model POSTY = TREATMENT TIME (TREATMENT*TIME)/ output=resids;
    Random REP(TREATMENT) BEAKER(TREATMENT) REP(TREATMENT*TIME)
    BEAKER(REP*TREATMENT*TIME);
    LSMeans TREATMENT TIME (TREATMENT*TIME)/ adjust=tukey CL;
    run;

    proc univariate data=resids plot normal;
    Var resid;
    run;

    PROC IMPORT OUT= WORK.SASCHOL3
        DATAFILE= "C:\Users\snavar4\Documents\SAS1\SASCD2.xlsx"
        DBMS=EXCEL REPLACE;
        RANGE="Sheet1$";
        GETNAMES=YES;
        MIXED=NO;
        SCANTEXT=YES;
        USEDATE=YES;
        SCANTIME=YES;
    RUN;
VITA

Toni Borel Lousteau, a Louisiana native, received her bachelor’s degree in biological engineering from Louisiana State University in May of 2012. She immediately entered graduate school with hopes to earn her master’s degree in biological engineering, which she will receive in May of 2015. Upon graduation, she plans to begin a career as a process engineer.