IN SITU OBSERVATION OF THE DISTRIBUTION AND LOCATION OF GLIADIN AS A FUNCTION OF MIXING TIME IN WHEAT FLOUR DOUGH USING QUANTUM DOTS

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THESIS
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ABSTRACT

Gliadins are a group of cereal proteins extracted from wheat flour. Gliadins contribute to viscosity and extensibility in dough.

In our study, distribution and location of gliadins as a function of mixing time in model wheat flour dough were investigated for the first time using confocal laser scanning microscopy.

In this research we tagged gliadin proteins with Quantum Dots (QDs) to increase the clarity and specificity of imaging and then investigated the effect of different mixing conditions on distribution of gliadin proteins and their role in building food structure/texture and quality.

Dough samples were prepared in a 300 g Brabender farinograph instrument. A complete farinogram was obtained. The mixing times chosen were the arrival time (AT), peak time (PT), departure time (DT) and breakdown time (5 min after departure time). Small pieces of dough (approx. 4x4x4mm) were taken from the farinograph bowl and were immediately frozen using liquid nitrogen. The samples were cyro-sectioned to a thickness of 6 μm using cryostat. Auto-fluorescence of dough was removed by soaking small pieces of samples in 10 mL aqueous solution of Heparin. Quantum dots were conjugated to anti-gliadin antibody with a covalent crosslinker. Dough sections were conjugated with antibody-QDots mixture. CLSM (Zeiss Lsm 700) was used to investigate the locations of gliadin. Excitation wavelengths of 405 nm and 615 nm were selected for the reflection and fluorescence signal respectively. We also utilized the Image J program to be able to quantify images obtained from CLSM.

We found that antibody-QDots mixture successfully bonded to gliadins located on dough sections. The images obtained from dough sections were very bright and clear that allowed us to distinguish gliadin easily. The QDs were found to be localized not only around the air cells as indicated by higher intensities but also in the bulk dough. We also observed that mixing plays an
important role in distribution of gliadin proteins. Quantum Dots can be used as fluorophore probes to tag and track proteins of interest in food microstructures.
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CHAPTER 1

INTRODUCTION

Dough and bread products predominantly compromise water soluble and insoluble proteins, lipids, carbohydrates, water and entrapped air. Food processing conditions along with food ingredients determine the food microstructure associated with the appearance, texture, taste and stability of the final product (Autio and Laurikainen, 1997).

Classic bread making process consists of kneading or mixing, fermentation, dividing and rounding, molding, proofing and baking, leading to important structural changes that affect quality.

Mixing is very important for dough formation. During mixing, a viscoelastic dough is formed by blending of water and flour in which the mechanical energy formed by mixer blades are transferred to the sample. Mixing leads to formation of two phases: the continuous, three dimensional gluten phase and immiscible aqueous phase consisting of water soluble compounds and starch (Autio and Laurikainen, 1997; Migliori and Correra 2013).

Gluten, which is the main functional protein in the dough, plays a significant role in determining the rheological properties of the dough (Tsiami et al., 1997). Gluten possesses two sub fractions: monomeric gliadins and polymeric glutenins. Gliadins are responsible for plasticity in dough matrix while glutenins possesses robust elastic properties (Southan and MacRitchie, 1999). Gliadins, which are highly surface active and hydrophobic monomeric proteins, play significant roles in gas holding capacity (Ornebro et al, 2000). Gliadins were found
not only in bulk dough but also were present around gluten-gas cell interface by addition of fluorescently labelled gliadins to the dough (Li et al., 2004).

The use of polyclonal antibodies coupled either directly or indirectly to fluorescent probes offers enormous potential for tracing individual proteins of interest at the ultrastructural level. Wheat storage proteins found in wheat endosperm and in baked and unbaked wheat products were identified using immunohistochemistry techniques (fluorescent dyes labelled polyclonal and monoclonal antibodies) by light microscopy (Ariss, 1986).

Currently, organic dyes have been widely known conventional tools used for biolabelling of biological molecules. In food science research, numerous organic dyes such as Rhodamine (Rh), fluoresceinisothiocyanate (FITC) and tetramethylrhodamineisothiocyanate (TRITC), light green, acid fuchsine, are commonly used. However, several drawbacks of using organic dyes as a fluorescent probe are present. A novel fluorescent probe called “Quantum Dots” has been progressively advanced to identify and visualize the biological molecules, which overcomes the problems encountered organic dyes.

Confocal Laser Scanning Microscopy (CLSM) delivers several benefits for examining relationships between composition, processing and the final product quality (Blonk and van Aalst, 1993; Kalab et al, 1995).

Even though a number of publications examined structural changes during mixing process, they focused on the gluten matrix. No study has been documented to investigate the relationships between specific subfractions of gluten such as gliadin microstructure and mixing process. The overall objective of this research was to investigate distribution and location of gliadin and its role in building food structure/texture and quality as a function of mixing time in a model wheat
flour dough using confocal laser scanning microscopy and examine polyclonal antibodies’ ability to recognize gliadins found in wheat flour dough. In our study, we brought a new immunohistochemistry approach to visualize specific wheat proteins of interest using quantum dots. Dough samples were taken from a farinograph at arrival time (AT), peak time (PT), departure time (DT) and 10 min after departure time. Gliadins were tagged with quantum dots labelled with polyclonal antibodies by means of SMCC crosslinker using immunohistochemistry techniques. Location and distribution of gliadins were investigated in AT, PT, DT and 10 min after departure time. This offers a better understanding of gliadin microstructure formation during dough mixing, a very important and critical unit operation for the cereal industry.
CHAPTER 2

LITERATURE REVIEW

Seed storage proteins are classified based on their solubility and extraction in different solvents. These include the water soluble albumins, the dilute saline soluble globulins, dilute acetic acid or alkali soluble glutelins, and aqueous alcohol soluble prolams, gliadins (Osborne, 1908).

Prolamins are recognized as proteins extractable in aqueous alcohol solutions due to their substantial levels of glutamine and proline content. The prolams can be divided into two groups on the basis of their solubility: monomeric gliadins which are soluble in aqueous alcohols 60-70 % (v/v) ethanol or 50% (v/v) aqueous propan-1-ol and polymeric glutenins which are soluble in aqueous alcohols by the addition of a disulfide bond reducing agent (2- mercaptoethanol (2-ME) or dithiothreitol (DTT). Shewry and Tatham (1990) classified prolams into three groups based on amino acid sequencing as S-rich, S-poor, and high molecular weight (HMW) prolams.

The gluten matrix, accounts for 80-90% of the total proteins in flour. Glutens involve the interactions of numerous intermolecular and intramolecular disulfide bounds between gliadin and glutenin. Wheat gliadins are a group of storage proteins that are extracted from the endosperm of the grain with aqueous alcohols (Kasarda et al., 1976). Monomeric gliadins vary in molecular weight from 30kDa to 100kDa and account for about half of the total prolams of gluten (Rogers and Hoseney, 1990) (Table 2.1).

On the basis of their sequence and electrophoretic mobility gliadins at low pH, were initially characterized into four groups (α, β, γ and ω) (Wieser, 2007). α gliadins, have a MWs
range between 40 kDa and 50 kDa, possess high quantities of glutamine, proline and phenylalanine which account for 80% of its total protein content. Most of these amino acids do not have adequate cysteine levels which play a role in the formation of crosslinks through disulfide bonds.

The aspartic acid and asparagine content of all gliadins is fairly low. Gliadins contain roughly 15% proline, a cyclic amino acid, which triggers conformational transitions through the formation of bends in proteins. In the presence of proline side chains, formation of α-helices, an important secondary structure of gliadin polypeptides is inhibited. Gliadins are not rich in essential amino acids especially in lysine (Lasztity, 1996). From a nutritional standpoint, lysine, the limiting essential amino acids, is found in the storage proteins of wheat (Lasztity, 1996) (Table 2.3). Gliadins are among the least charged proteins due to inadequate levels of lysine, arginine and histidine, together with the low levels of free carboxyl groups (Lasztity, 1996). This gives gliadin their hydrophobic character.

Dough and bread are made from wheat flour predominantly consisting of proteins (nearly 7-15% of common flour), lipids (about 1% of flour), carbohydrates (63–72% of flour) and water (Table 2.2). The bread making process involves kneading and mixing, fermentation, dividing, rounding, molding, proofing and baking as shown in fig. 2.1. Flour and water are blended, mixed and kneaded to form dough and to integrate air during the kneading or mixing process. Fermentation leads to conversion of basic sugars into alcohol and carbon dioxide by yeast to provide a light and airy texture and improve the flavor of the bread. Numerous smaller gas cells occur by the dispersion of large gas cells in the dividing step generated during mixing. During molding, dough samples are given their final shapes followed by expansion of the volume of
dough samples during proofing. The baking process converts the viscoelastic dough into an elastic foamy cellular bread structure.

The microstructure of bread is associated with its appearance, texture, taste, stability and its digestibility, and is determined by the dough ingredients combined with processing conditions (Autio and Laurikainen, 1997). The rheological behavior of dough is influenced by the mixing. The type of mixing apparatus, energy input, mixing time, and mixing speed all have a lot of influence. (Hoseney, 1985; Janssen et al., 1996). Numerous instruments have been industrialized for assessment of the dough characteristics and further prediction of final dough quality. Examples include farinograph, extensograph, alveograph, amylograph, and mixograph (Lee et al., 2001).

The Farinograph is an instrument that measures the mechanical resistance of dough, which depends on the rheological properties of dough while mixing a mixture of water and flour to form developed dough (Correra and Migliori, 2013). Mechanical stress is applied to dough samples by a couple of rotating sigma shaped blades driven by a motor at constant temperature and the resistance of dough against the blades is measured as torque and recorded in a graph called farinogram in which the horizontal axis gives time in minute and the vertical axis plots the resistance to deformation in arbitrary units called Brabender Units (BU). A hockey-stick shaped farinogram is obtained with the shape and height of the curve depending on the strength of the flour (Fig. 2.2).

Important information can be obtained from a farinogram such as water absorption to reach 500 BU, development time, stability and degree of softening. The points of interest in the farinogram are; arrival time (AT) in which the top of the curve reaches 500 BU, peak time (PT)
where top of the curve reaches a maximum, departure time in which the top of the curve falls back to the 500 BU, stability which is the time interval between AT and DT associated with strength of flour and mixing tolerance index and finally the difference in BU between PT and 5 minutes after peak time which is related to degree of softening.

The curve obtained from a weak flour is narrow whereas strong flour leads to formation of a broad and less acute curve associated with larger water absorption and stability (fig.2.3). The farinograph has been employed to determine stability and other features of doughs to be able to comprehend the effects of several processing conditions or the dough ingredients. (Mohsen et al., 2010; Ji et al., 2013).

Numerous rheological studies have been conducted to understand structural changes that occur in the gluten matrix during mixing (Amend and Belitz, 1990; Martinant et al., 1998; Lee et al., 2001; Keentok et al., 2002; Dobraszczyk and Morgenstern, 2003; Kuktaite et al., 2005). Several studies have been conducted to examine the direct effects of gliadin on rheological characteristics of wheat flour (Fido et al., 1997; Mudgil et al., 2013). Khatkar et al., (2013) studied gliadin addition ranging from 5% (w/w) to 10% (w/w) to the base flour and observed a decrease in the dough stability. Addition of 10% (w/w) gliadin to the base flour resulted in a decline in peak time from 3.20 min. to 1.40 min and the maximum value of the curve at peak time decreased from 510 BU to 491 BU, thus lowering the strength of the dough. Fido et al (1997) demonstrated that the largest weakening effect on the flour was observed with ω gliadin addition followed by α-, β-, γ gliadin addition respectively and peak resistance of these flours declined by the addition of each type of gliadins (α-, β-, γ, and ω).

Since rheological properties of dough samples are related to microstructure of dough samples, rheological measurements are often coupled with microscopy and imaging techniques.
Several microscopic studies have been carried out to assess the effect of mixing on microstructural characteristics of gluten network using light microscopy (LM) (Autio and Salmenkallio-Marttila, 2001; Kuktaite et al., 2005), atomic force microscopy (AFM) (Tatham et al., 1999), transmission electron microscopy (TEM) (Amend and Belitz, 1990) epifluorescence light microscopy (EFLM) (Peighambardoust et al., 2010) scanning electron microscopy (SEM) (Amend and Belitz, 1990; Bache and Donald, 1998; Létang et al., 1999; Watanabe et al., 2002; Calderón-Dominguez et al., 2003; Kuktaite et al., 2005; Khatkar et al., 2013), and laser scanning confocal microscopy (Lee et al., 2001; Li et al., 2004; Upadhyay et al., 2012). However, typical light microscopy requires special sample preparation composed of exhaustive fixation and embedding leading to artifacts. Sections must be thin to be able to obtain good quality images of microstructure with high resolution (Blonk and Aalst, 1993). SEM also requires extended sample preparation time and some essential techniques such as mounting and coating with gold nanoparticles to make samples ready for SEM imaging. Modifications in the microstructure of the sample may occur due to several chemical and physicochemical events before examination during sample preparation time including mounting and coating (Peighambardoust et al., 2010; Lee et al., 2001).

Confocal laser scanning microscopy (CLSM) offers numerous benefits to overcome problems encountered by light microscopy and TEM. It does not require long sample preparation time (Autio and Laurikainen, 1997) and allows to scan thicker sections (5-10 µm and even more) (Lee et al., 2001). CLSM has the capability of producing blur-free images of thick specimens at various depths (three-dimensional specimen). Numerous procedures have been developed to prepare the dough and bread samples for imaging (Fardet et al., 1998; Hug-Iten et al., 1999; Li et al., 2004). In one approach described by Hug-Iten et al. (1999), small pieces of dough samples
were soaked in heparin solution to remove auto fluorescence of dough. The samples were then frozen with carbon dioxide followed by sectioning to the desired thickness at -20ºC using a cryostat. Then, sections were placed onto microscopy slides and covered with glycerol/gelatin to improve the adhesiveness of the cryosections during staining. As proteins were stained with light green solution, starches were stained with lugol solution.

Lee et al (2001) studied the microstructural characteristics of non-developed, partially developed and developed dough using CLSM. They found that a good protein network was observed in developed dough while the protein network was not well formed in non-developed dough. The distribution of gluten proteins and lipids in bread dough was examined using CLSM (Li et al., 2004). Purified gliadin and glutenin were stained with Rhodamine B via covalent bonding to form fluorescence labelled proteins. Small amount of fluorescence labelled proteins were added to the base flour and observed under CLSM. Results illustrated that gliadin was not only found in the bulk dough but were also located in the gas cell walls representing higher fluorescence intensities. The use of fluorescently labelled immunoglobulins (antibodies) enables the detection and helps locate specific components of interest by using immunohistochemistry techniques (Harlow and Lane, 1999).

Antibodies are a large group of globular, Y shaped proteins formed by the body’s immune system. They possess special patterns which recognize the target molecules similar to a lock-key interaction (fig. 2.4). A typical antibody comprises of two identical heavy polypeptide chains (≈55 kDa) and two identical light polypeptide chains (≈25 kDa), which are linked by disulfide and noncovalent bonds to form a Y shaped molecule of ≈150 kDa composed of antigen binding domains (Fab) and tail (Fc) (Lipman et al., 2005).
Antibodies are divided into two groups—polyclonal antibodies and monoclonal antibody. Polyclonal antibodies involve complex mixtures of different antibodies produced by multiple B cell clones. A single B lymphocyte clone functions to produce a monoclonal antibody targeting just one specific epitope. Specificity of an antibody is defined by its ability to recognize a specific epitope in the presence of other epitopes. Affinity of an antibody refers to its binding strength to a specific epitope. It is important to have an antibody with high affinity which enhances stability of interaction among an antibody and an antigen.

Numerous factors such as interaction with other proteins, temperature lead to conformational changes of an antibody. Structural changes resulting from numerous factors such as interactions with other proteins, posttranslational modification, temperature, pH, salt concentration, and fixation lead to changes in the binding affinity of an antibody for an antigen. Polyclonal antibodies often possess better specificity than monoclonal antibody and are less vulnerable to the loss of epitopes because conformational changes may not influence all epitopes similarly (Lipman et al., 2005).

Anti-gliadin polyclonal antibodies have been created in B cells of mice or rabbits by injecting whole purified gliadins or subfractions of gliadin, or proteolytic fragments of gliadin, or synthetic peptides that characterize epitopes of (Li et al., 2008; Leszczynska et al., 2008). Li et al 2008 indicated that wheat quality parameters during the mixing process can be predicted and screened using polyclonal antibodies.

There are numerous straightforward biolabelling procedures to identify and describe structures and are of interest in biological samples. Various key features described by Genger et al (2010) must be considered when a biological label or marker is chosen:
1. It must excite by a light source without interfering with the fluorescence of the biological matrix and be measureable with conventional equipment. 2. It must be remarkably bright; 3. It must be soluble in an aqueous medium such as buffers or bodily fluids. 4. Be thermally and photo chemically durable when exposed to related environments. 5. It should possess functional groups for site-specific labelling of molecules or process of interest. 6. It needs to preferably have long fluorescent life time to be able to make time gated imaging if desired. 7. It should be reproducible and enable surface modification and functionalization. 8. It should be biocompatible and non-toxic and 9. it should be ideal for multiplexed detection of molecules or processes.

Isotopic tracers, organic dyes, quantum dots, and other tools have been employed in biolabelling for a long time. However, isotopic labelling possesses various drawbacks including high cost, alterations in protein structure and restrictions to the number of proteins and variables that can be studied at a time (Lederman, 2007). Therefore, fluorescent labelling techniques using quantum dots with superior sensitivity and selectivity have been developed for analyzing biomolecules.

A fluorophore is a fluorescent molecule which is capable of being excited by absorbing the light energy when exposed to a light source. As shown in figure 2.5, fluorophores consist of two energy levels, ground level (the lowest energy level) and excited level. The energy difference between excited level and ground level is defined by the band gap energy (E_v). The minimum energy required to jump electrons from the ground state to excited state must exceed E_v. In the ground state, the electrons have relatively low energy compared to the excited level. The electrons that become mobile are shifted from ground level to excited level (a higher energy state) when aroused from an optical or electrical energy source. This process is defined as “
excitation”. The electrons stay for a finite time in excited state. The length of this time is called “the excited state lifetime”. The excited electrons revert back to the ground level by reordering themselves, which is accompanied by emission of the light. This is the origin of “fluorescence”. Some of the energy is captured by a neighboring molecule and lost in the form of heat resulting from microscopic vibrations of electrons in the excited state. Hence, the emitted light has lower energy, thereby longer wavelength than absorbed light. The color of emitted light is significantly different than the absorbed light due to differences in the amount of energy carried by lights at different wavelengths. When the fluorophore is exposed to a light source, the light is absorbed within the range of wavelengths they emit.

There is a wavelength located in the range of wavelengths in which the light is absorbed more effectively. It is defined as the wavelength for “excitation maximum”. Each fluorophore possesses its specific excitation range. The excited fluorophore emits light within the same range of wavelengths. “An emission maximum” is the wavelength inside the range of wavelengths in which the light is emitted more effectively than other wavelengths within the range.

The difference between the lowest energy peak of absorbance and the maximum energy of emission is called “Stokes Shift” (Gispert, 2008). Stokes Shift, expresses the ease with which to differentiate excitation from emission and is one of the distinctive properties of each fluorophore.

Organic dyes have been widely used for biolabelling of biological molecules. Numerous organic dyes such as Rhodamine (Rh), fluoresceinisothiocyanate (FITC) and tetramethylrhodamineisothiocyanate (TRITC), light green, acid fuchsine, are frequently employed in food science research. However, numerous disadvantages of using organic dyes as a fluorescent probe exist.
When organic dyes are stimulated by intense laser illumination, they tend to lose their intensity due to photochemical damage to the chemical structure of the dye over time. This is called “photobleaching”. Therefore, the amount of light emitted from the organic dyes gradually decreases and lose their brightness.

A novel technique able to detect and visualize biological molecules and clarify the biological function and activity of biological molecules such as proteins, nucleic acids, and others or processes occurring in living cell tissues has been developed. This technique uses inorganic materials called “Quantum Dots” which overcome the problems encountered by organic dyes. The first applications of quantum dots including synthesis and characterization of luminescent quantum dots, colloidal semiconductor nanocrystals, emerged in the early 1990s (Bawendi et al., 1990; Mattoussi et al., 1996; Hines and Guyot-Sionnest, 1996). Utilization of quantum dots as fluorescent probes for biolabelling were demonstrated by several laboratories (Bruchez et al., 1998; Chan and Nie, 1998). Quantum dots are highly luminescent, semiconductor nanocrystals which vary in size from 2 nm to 10 nm and possess roughly 200-10000 atoms (fig. 2.6).

The sizes and shapes of quantum dots can be precisely controlled by the duration, temperature, and the ligand molecules used in synthesis. The emission wavelength can be tuned from blue to red wavelengths by changing the particle size (quantum confinement effect) as shown fig.2.7.

Quantum dots absorb light at all wavelengths shorter than their emission wavelength. This allows multiple colors of quantum dots to be effectively excited by a single light source. Their stokes shifts can be hundreds of nanometers simplifying the design of instrumentation used for
collection and detection of the emitted light. In addition, this property facilitates multiplexing in which two or more quantum dots can be simultaneously excited using a single laser source. Typically a quantum dot emission spectrum is nearly Gaussian shaped, which is in contrast with most fluorescent dyes that display asymmetric emission spectra.

Quantum dots are brighter than organic dyes and fluorescent proteins because they have larger extinction coefficients (~10-100 times greater than most dyes). Chan and Nie (1998) indicated that brightness of quantum dots are approximately 20 times higher than organic dyes. This can also be explained by “quantum yield”. Quantum yield, an indicator of the brightness of a fluorescent molecule, is expressed as the proportion of the light emitted to light absorbed by a fluorophore. Quantum yields of numerous organic dyes can reach up to 100% initially prior to binding. When they bind to affinity molecules and form fluorophore-affinity molecule conjugates, their quantum yields significantly decrease. However, even when quantum dots create a quantum dots–affinity molecule conjugate, no reduction in quantum yield is observed (Hotz, 2005).

Stability is one of the most important characteristics of a fluorophore. Organic dyes are easily degraded when exposed to intense laser illumination. Therefore, the intensity of light emitted by them fade away over time. As indicated fig. 2.8, quantum dots are much more stable than organic dyes in terms of photo bleaching because of their inorganic composition (Wu et al., 2003). For example, a superior long-term stability for CdSe/ZnS and rhodamine labelled tubulin (Reigler et al., 2003) as well as CdSe and Texas red (Gao et al, 2006) compared to organic dyes has been observed.
The fluorescence time of quantum dots is nearly 10 to 40 ns, and most of the conventional dyes have shorter fluorescence time (approximately < 5 ns) (Dahan et al., 2001) (fig. 2.9). This is not only an indicator of how stable quantum dots are compared to organic dyes, but also allows the use of time-gated detection by removing autofluorescence of various organic compounds which possess shorter fluorescent life time than quantum dots (Dahan et al., 2001).

Among II–VI, III–V and IV–VI compounds and their alloys, II–VI ((zinc sulfide (ZnS), Cadmium selenide CdSe, Zinc selenide (ZnSe) cadmium sulfide (CdS)) compounds and their alloys emit visible light, III–V (Indium antimonide (InSb), Indium phosphide (InP)) compounds and their alloys fluorescence from red to near-infrared, and the IV–VI (PbS) compound emits near-infrared and mid–infrared (Murray et al. 2000).

A typical quantum dot’s structure shown in fig. 2.10 can consist of the following layers: (Hotz, 2005)

1. The quantum dot core is typically a semiconductor such CdSe and is responsible for its optical properties.
2. core/shell quantum dot possesses a crystalline inorganic shell to render them brighter and more stable
3. Water-Soluble Quantum dots~ typically have a core-shell which is hydrophobic. They are manufactured by capping a hydrophilic ligand on quantum dot surface
4. Quantum dot-bioconjugate~affinity ligand is attached to a water soluble quantum dots.

Murray et al. (2000) demonstrated that noble metals which are resistant to corrosion and oxidation such as, Ag, Au., groups of II–VI (zinc sulfide (ZnS) and cadmium sulfide (CdS)), III–V (Indium antimonide (InSb)) and IV–VI (PbS) compounds and their alloys can be used to
produce colloidal semiconductor nanocrystals. Principles of preparation of monodisperse colloidal growth of nanocrystals follow the La Mer theory (La Mer et al., 1950) and the Oswald ripening process (Ostwald, 1896). The concentration of precursor is increased until it reaches the nucleation threshold when maximum supersaturation is reached. At maximum supersaturation, nuclei formation occurs and the growth of nuclei proceeds until the precursor concentration falls below the nucleation threshold. The particles unceasingly grow until the precursor concentration reaches the saturation concentration. Then, Oswald ripening or competitive growth, involves dissolution of small crystals and redeposition of small crystals as larger crystals. Numerous less energetically favored small crystals previously formed are depleted by more energetically favored larger particles following Ostwald ripening (Ostwald, 1896).

The first approach to produce quantum dots is organic synthesis shown in fig. 2.11. Murray et al (1993) established the first synthetic approach to produce well-defined semiconductor nanocrystals on the basis of thermal decomposition of organometallic components by injection into a hot coordinating solvent.

To obtain nearly monodisperse nanocrystals, Dimethyl cadmium (Me₂Cd) in tri-n-octylphosphine (TOP) solution and Tri-n-octylphosphine selenide (TOPSe) stock solution were collected in a syringe and rapidly injected in one shot in a vessel tank kept at 300°C containing tri-n-octylphosphine oxide TOPO solution used as a reaction medium. Reaction between Me2Cd and TOPSe occurred in a vessel tank followed by separation and purification of nanocrystals by precipitating in a solvent. TOPO and TOP solutions used as a capping agent provide protection of core from environment. The high yield of quantum dots in the range of 50% to 80% and nearly monodisperse quantum dots (less than 5%) was obtained without surface defects.
Nevertheless, Cd (CH$_3$)$_2$ has undesirable properties such as, being poisonous which leads to irritation on skin and mucous membranes. It is also unstable and can spontaneously ignite in air and is expensive.

Alternative green methods have been developed to produce other types of high quality nanocrystals. Peng and Peng (2001) showed that CdO can be employed as the Cd precursor in lieu of Cd (CH$_3$)$_2$. Brennen et al (1989), demonstrated that nanoclusters of CdSe were synthesized with a yield of 60-70% using a single-source precursor Cd(SePh)$_2$, which is less toxic than Cd(CH$_3$)$_2$.

The second approach to synthesize quantum dots is traditional aqueous synthesis or precipitate synthesis. Thiol-capped CdSe nanocrystals were synthesized based on the reaction between cadmium perchlorate hydrate (Cd(ClO$_4$).6H$_2$O) as a source of metal salt and NaHTe or NaHSe in the presence of thiol compounds (thioalcohol or thioacid) as capping reagents in aqueous solution (Rogach et al., 1999).

The thiol stabilized nanocrystals possessed less than 0.1% quantum yield and a wide emission band. Higher quantum yields and less synthesis time were achieved by using higher temperatures in an autoclave (Zeng et al. 2003), microwave irradiation and illumination (Bao et al., 2004).

Zhang et al (2003) found that CdTe nanocrystals grown at 180º C had a narrower particle size distribution and higher quantum yields (up to 38%). The fluorescence quantum yield of CdTe nanocrystals improved up to 85% when they were exposed to illumination under room light for 20 days based on photodegradation of thioglycolic acid due to illumination and redeposition of sulfide ions released from thioglycolic acid on CdTe surface and forming a shell.
layer (Bao et al., 2004). High quality CdSe-CdS alloyed quantum dots with enhanced quantum yields were manufactured by means of microwave irradiation (Qian et al., 2005) (fig. 2.12).

CdSe nanocrystals were formed by reaction of CdCl₂ with NaHSe in the presence of 3-mercaptopropionic acid as a stabilizer. When CdSe nanocrystals were heated by microwave irradiation, and Cadmium sulfide formed due to decomposition of 3-mercaptopropionic acid which reaccumulated on CdSe surface by forming CdSe-CdS alloyed quantum dots. The synthesis of CdSe-CdS alloyed quantum dots with a florescence quantum yield up to 25% and the full width at half maximum of the emission spectrum peak nearly 28 nm was achieved.

Various continuous flow systems have been developed in which the growth of nanocrystals can be controlled by altering temperature, flow rate, precursor concentration. Chan et al., (2002) utilized controlled, continuous flow synthesis of high-quality CdSe nanocrystals using a microfabricated chip-based micro reactor in which CdSe nanocrystals were formed by the reaction of precursors and octadecene in a heated micro reactor channel made of borosilicate glass wafers. The size of nanocrystals varied in size from 2.23 to 2.64 nm depending on manufacturing parameters used. In varying flow rate ratios in the range of 1/1 to 5/1 the effective diameter of nanocrystals could be made smaller with a decrease in precursor/1-octadecene ratio. The temperature of 180 °C gave the smallest size in the range of 180 °C to 210 °C. When the flow rate of precursor was decreased, and consequently the residence time was increased, larger nanocrystals were obtained. Mirhosseini Moghaddam et al (2013) conducted a similar study. They developed a continuous flow processing reactor made of a stainless steel coil having a 1 mm inner diameter in which precursor solution was pumped into the reactor by means of an HPLC pump to synthesize CdSe nanocrystals. When the temperature in the reactor was increased from 240 °C to 260 °C and residence time of precursor solution was increased from 2
to 20 min, larger quantum dots in the range of 3 to 6 nm were formed. Increased residence time and higher temperatures led to the formation of CdSe nanocrystals possessing low quantum yields in the range from 11% to 28%. Significant size distribution differences between different treatments were not observed.

“Blinking” occurs in quantum dots because of either charging or discharging of the nanocrystals core driven by light or charging and discharging of surface electron traps resulting from surface defects due to oxidation and other chemical reactions. Galland et al (2011) pointed out that blinking decreases quantum yields of quantum dot nanocrystals. To prevent and overcome blinking and enhance photoluminescence efficiency, atoms located on the surface of quantum dots nanocrystals can be protected from undesirable environmental conditions causing degradation and oxidation by depositing a shell layer on the core of quantum dots with a wider band gap. The coating of quantum dots nanocrystals with a shell makes them more robust and leads to higher quantum yields. This is achieved by passivation non-radiative sites on quantum dot nanocrystals surface.

Dabbousi et al (1997) reported production of (CdSe)ZnS core-shell quantum dot nanocrystals using two step synthetic routes. In the first route, CdSe core quantum dots were formed by pyrolysis of organometallic components by injection into a hot coordinating solvent. In the second route, Diethylzinc (ZnEt₂) and hexamethyldisilathiane ((TMS₂)S) as sources of Zn and S dissolved in TOP were poured into CdSe core quantum dot nanocrystals solution in TOPO under vigorously stirring at an intermediate temperature. This temperature and shell precursor concentration are important. The temperature should be in the range of 160 °C – 220 °C which only supports the growth of ZnS on CdSe surface. This temperature range inhibits Oswald ripening of CdSe cores which causes wider spectral line widths and inadequate of ZnS
nanocrystals. Shell precursor concentration should be high enough and addition rate should be fast enough to favor homogenous growth of ZnS on CdSe surface. The study demonstrated that the quantum yield of CdSe over coated with ZnS (50 %) was enhanced three times compared to bare TOPO capped CdSe core nanocrystals (15%).

Quantum dots are typically formed in nonpolar organic solvents which makes them insoluble in water. Numerous solubilization approaches have been developed in order to render them hydrophilic, thereby biocompatible and capable of being used in vivo (Fig.2.13). One procedure used to make quantum dots water soluble is ligand displacement with molecules comprising of thiols. Reagents used for this purpose include , Mercaptoacetic acid (Mitchell et al., 1999), Mercaptopropionic acid (Willard et al., 2001), Mercaptoundecanoic acid (Aldana et al., 2001), 16-Mercaptohexadecanoic acid (Aldana et al., 2001), Dihyrolipoic acid (Mattoussi et al., 2000), Ditiotheitol (Pathak et al., 2001), cystamine (Nann et al., 2008), cysteine (Liu et al., 2007), dendimers (Guo et al., 2003), thiol-derivatized sugar (Babu et al., 2007), carboxyl acids-containing oligomeric phosphines (Kim and Bawendi, 2003) or phytochelatin-related peptides (Pinaud et al., 2004) in which TOPO (triotylphosphine oxide). Hydrophobic ligand, located on quantum dot surface are displaced by one or more of the long list of hydrophilic ligands mentioned above which possess greater affinity than TOPO ligand for quantum dots surface.

Compact versatile ligands have been used to synthesize quantum dots with improved stability and biological functionalities as shown in Fig. 2.14 (Susumu et al., 2007; Susumu et al., 2009). A ligand molecule consists of three units: polyethylene glycol (PEG) to promote hydrophilicity, a functional unit to provide further modification on quantum dot surface linked to the one end of PEG (-COOH, -NH2, -OH and Biotin) and dihydrolipoic acid (DHLA) to offer anchoring on quantum dot surface as illustrated in fig. 2.15. Thiotic acid (TA) is attached to one
end of PEG and then, DHLA is formed by reduction of 1, 2-dithiolanein groups of TA in the presence of NaBH4 via ring opening reaction (figure 2.16).

The ligand exchange reactions are performed to replace the native hydrophobic ligands (TOP/TOPO) on quantum dot surface with hydrophilic ligands to be able to form water soluble, biocompatible quantum dots having free functional units for further modification.

The second approach is to use of amphiphilic molecules (phospholipids micelles (Dubertret et al., 2002), poly (maleic anhydride-alt-1-octadecene) (PMAO)-PEG (Yu et al., 2007), amphyphilic polymer shell (Pellegrino et al., 2003), triblock copolymer (Gao et al., 2004) to obtain biocompatible quantum dots. Amphiphilic molecules possess both hydrophobic and hydrophilic moieties. As hydrophobic TOPO molecules binds to hydrophobic ends of amphiphilic molecules via hydrophobic-hydrophobic interactions, hydrophilic end extends from quantum dots surface towards aqueous environment.

There are several approaches to bioconjugate quantum dots to biomolecules (e.g., antibodies, proteins, peptides, oligonucleotides). One approach uses electrostatic interactions between the negatively charged lipoic acid capped CdSe-ZnS quantum dots and positively charged engineered adapter having not only binding affinity to Fc region of antibodies, but also are able to bond through electrostatic interactions with charged quantum dots (Jaiswal et al. 2003; Goldman et al. 2001).

The second approach is streptavidin-biotin interaction used to link quantum dots to antibodies. This approach benefits from the high binding affinity that streptavidin and biotin have for each other (K_d~10^{-14}) in which streptavidin coated antibodies are linked to biotinylated F_c region of antibodies.
The third approach is covalent crosslinking of quantum dots with antibodies. This gives a more stable and stronger bond than electrostatic interactions. Amine or carboxyl derivatized quantum dots have been covalently linked to compounds containing thiol groups (Dubertret et al., 2002; Mitchell, 1999); or N-hydroxysuccinimyl ester moiety (Bruchez et al., 1998) using standard bioconjugation techniques. The first reaction is the reduction of antibody to make its sulfhydryl–SH groups available for conjugation reaction using DL-Dithiothreitol (DTT) as shown in fig. 2.17.

Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) is a non-cleavable and membrane permeable crosslinker. It possesses two groups including an amine-reactive N-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. NHS esters react with primary amines at pH 7-9 to form stable amide bonds whereas Maleimides bind to sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds.

(fig. 2.18). Quantum dots are excellent probes for confocal laser scanning microscopy which offer timed-gated studies because of their high photostability and multiplexing (2 or more quantum dots can be observed in the same time). The basic confocal laser scanning microscopy involves a laser power source, objective lens, dichroic mirror, oscillating mirrors screen composed of a pinhole and photomultiplier tubes as detector (fig. 2.19). The excitation light in a range of different distinct wavelengths is provided a laser source. Assume that the light emanating from the laser source is red and the light emitted from sample is green. The light passes through a dichroic mirror in which light shorter than a certain wavelength is reflected whereas the light longer than the wavelength is passed. Since the violet light possesses a lower wavelength and higher energy, it is reflected through the dichroic mirror. The reflected violet light hits the one or two special oscillating mirrors to be able to scan the laser across a sample
pixel by pixel in the horizontal plane and is then focused on a sample plane by an objective lens. The emitted green light having a longer wavelength and lower energy is rescanned by the oscillating mirrors and passes through the dichroic mirror due to its longer wavelength and a pinhole conjugate to the focal point of the screen. Out of focus light is blocked by the screen with a pinhole. The green light passing through the pinhole is collected by a detector such as a photomultiplier tube. The different horizontal planes (Z stacks) are created by moving the sample holder up or down direction. 3D images of samples can be obtained when a series of such Z stacks at various depths are combined.

Even though there are many applications of QDs in biomedical field, only one application exists emerging from our laboratory (Sozer and Kokini, 2013) in imaging the distribution of gluten proteins in dough and bread. Carboxyl-functionalized water soluble CdSe/ ZnS core/shell quantum was used to tag and identify cereal proteins in bread. Quantum dots were attached to protein surface in the presence of EDC (1-Ethyl-3-[3- dimethylaminopropyl] carbodiimide hydrochloride), a zero length crosslinker. EDC reacted with carboxyl group on quantum dot surface and formed an amine-reactive O-acylisourea intermediate. This intermediate covalently bonds to free amine groups on protein surface.

In our study, we focused on gliadin fractions of cereal proteins and studied their distribution and location as a function of mixing time during dough development and their role in building dough structure/texture and quality as a function of mixing time using confocal laser scanning microscopy.
2.1 Figures and Tables

Table 2.1. Proportions of the Main Protein Fractions of Flour and Their Amino Acid Compositions

<table>
<thead>
<tr>
<th>Extracting solvent</th>
<th>Wheat</th>
<th>Flour</th>
<th>Soluble Proteins</th>
<th>Gluten Proteins</th>
<th>Residue Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Albumin</td>
<td>Globulin</td>
<td>Gliadin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Water 0.5 M NaCl</td>
<td>70% ethanol 0.5 M acetic acid</td>
<td></td>
</tr>
<tr>
<td>Proportion, b %</td>
<td>...</td>
<td>100</td>
<td>15 3</td>
<td>33 16 33</td>
<td></td>
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<tr>
<td>Tryptophan</td>
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<td>1.5</td>
<td>1.1</td>
<td>1.1 0.7 2.2 2.3</td>
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</tr>
<tr>
<td>Lysine</td>
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<td>1.9</td>
<td>3.2</td>
<td>5.9 0.5 1.5 2.4</td>
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<td>2.0</td>
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<tr>
<td>NH₃</td>
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<td>3.9</td>
<td>2.5</td>
<td>1.9 4.7 3.8 3.5</td>
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</tr>
<tr>
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<td>3.1</td>
<td>5.1</td>
<td>8.3 1.9 3.0 3.2</td>
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<tr>
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<td>5.8</td>
<td>7.0 1.9 2.7 4.2</td>
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<tr>
<td>Threonine</td>
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<td>2.4</td>
<td>3.1</td>
<td>3.3 1.5 2.4 2.7</td>
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<td>4.4</td>
<td>4.5</td>
<td>4.8 3.8 4.7 4.8</td>
<td></td>
</tr>
<tr>
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<td>34.7</td>
<td>22.6</td>
<td>15.5 41.1 34.2 31.4</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
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<td>11.8</td>
<td>8.9</td>
<td>5.0 14.3 10.7 9.3</td>
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</tr>
<tr>
<td>Glycine</td>
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<td>3.4</td>
<td>3.6</td>
<td>4.9 1.5 4.2 5.0</td>
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<tr>
<td>Alanine</td>
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<td>4.3</td>
<td>4.9 1.5 2.3 3.0</td>
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</tr>
<tr>
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<td>2.8</td>
<td>6.2</td>
<td>5.4 2.7 2.2 2.1</td>
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<tr>
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<td>3.4</td>
<td>4.7</td>
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<tr>
<td>Methionine</td>
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<td>1.3</td>
<td>1.8</td>
<td>1.7 1.0 1.3 1.3</td>
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<tr>
<td>Isoleucine</td>
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<td>3.0</td>
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</tr>
<tr>
<td>Leucine</td>
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<td>6.8</td>
<td>6.8 6.1 6.2 6.8</td>
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</tr>
<tr>
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<td>2.8</td>
<td>3.4</td>
<td>2.9 2.2 3.4 2.8</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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<td>4.8</td>
<td>4.0</td>
<td>3.5 6.0 4.1 3.8</td>
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</tr>
</tbody>
</table>

(Atwel, 2001)
Table 2.2. Analytical Composition and Primary Component of Flour.

<table>
<thead>
<tr>
<th>Property</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
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</tr>
<tr>
<td>Protein</td>
<td>7–15 (of flour)</td>
</tr>
<tr>
<td>Osborne classification</td>
<td></td>
</tr>
<tr>
<td>Albumins</td>
<td>15 (of protein)</td>
</tr>
<tr>
<td>Globulins</td>
<td>3 (of protein)</td>
</tr>
<tr>
<td>Prolamin (gliadin)</td>
<td>33 (of protein)</td>
</tr>
<tr>
<td>Glutelin (glutenin)</td>
<td>16 (of protein)</td>
</tr>
<tr>
<td>Residue</td>
<td>33 (of protein)</td>
</tr>
<tr>
<td>Gluten</td>
<td>6–13 (of flour)</td>
</tr>
<tr>
<td>Gliadin</td>
<td>30–45 (of gluten)</td>
</tr>
<tr>
<td>Glutenin</td>
<td>55–70 (of gluten)</td>
</tr>
<tr>
<td>Starch</td>
<td>63–72 (of flour)</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>75 (of starch)</td>
</tr>
<tr>
<td>Amylose</td>
<td>25 (of starch)</td>
</tr>
<tr>
<td>Nonstarchy polysaccharides</td>
<td>4.5–5.0% (of flour)</td>
</tr>
<tr>
<td>Pentosans/hemicellulose</td>
<td>67 (of NSP)</td>
</tr>
<tr>
<td>Insoluble</td>
<td>67 (of pentosans/hemicellulose)</td>
</tr>
<tr>
<td>Soluble</td>
<td>33 (of pentosans/hemicellulose)</td>
</tr>
<tr>
<td>Beta glucans</td>
<td>33 (of NSP)</td>
</tr>
<tr>
<td>Lipids</td>
<td>1 (of flour)</td>
</tr>
</tbody>
</table>

(Atwel, 2001)
Table 2.3. Classification of the Amino Acids based on Charge and Hydrophobicity

<table>
<thead>
<tr>
<th>Acidic</th>
<th>Basic</th>
<th>Neutral (Hydrophilic)</th>
<th>Neutral (Hydrophobic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>Lysine</td>
<td>Glutamine</td>
<td>Valine</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Histidine</td>
<td>Asparagine</td>
<td>Leucine</td>
</tr>
<tr>
<td>Arginine</td>
<td>Serine</td>
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<tr>
<td>Tryptophan</td>
<td>Threonine</td>
<td></td>
<td>Alanine</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tyrosine</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Cysteine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cystine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proline</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methionine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycine</td>
</tr>
</tbody>
</table>

(Hoseney, 1994)
Figure 2.1. Schematic illustration of bread making process
Figure 2.2. Schematic representation of a farinogram indicating Arrival time (1), Peak time (2), mixing tolerance index (3) and departure time (4).
Figure 2.3. Farinograms of two flours representing weak (A) and strong (B) mixing characteristics
Figure 2.4. Schematic representation of an antibody consisting of Fab and Fc fractions
Figure 2.5. Schematic representation of fluorescence process.

\[ E_2 - E_1 = \Delta E = h\nu \]
Figure 2.6. Size comparison of quantum dots.

Adopted from Invitrogen website
Figure 2.7. The Emission Spectra of Quantum Dot Nanocrystals. Size tunable florescence properties of quantum dots; From left to right, the emission maxima are located at 480, 510, 530, 560, 590, 610, and 640 nm.

(Sigmatech, 2013)
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(Wu et al. 2003).
**Figure 2.9.** Comparison of Fluorescent Life Time between Organic Fluorophore and Quantum Dots

(Weiss et al., 2000)
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Adapted from Invitrogen website.
Figure 2.11. (A) Monodisperse Colloid Nucleation and Growth Theory, (B) synthesis of core quantum dots via organic synthesis. (Murray et al., 2000)
Figure 2.12. TEM image of CdSe-CdS alloyed quantum dots produced by microwave irradiation (Qian et al 2005)
Figure 2.13. Schematic representation of various approaches to solubilize and functionalize quantum dots. Arrows illustrates the locations where quantum dots are modified. (Michalet et al., 2005)
Figure 2.14. Schematic illustration of chemical composition and synthetic pathways required to form versatile ligands. (a) structure of hydroxyl-derivatized DHLA-PEG. (b) amine-terminated DHLA-PEG formation pathway. (c) conversion of TA-PEG-NH₂ to TA-PEG-COOH or TA-PEG-biotin. (d) transformation of 1,2 dithiolane groups to dihyrolocipic acid groups via ring-opening reaction. (Susumu et al., 2007; Susumu et al., 2009)
Figure 2.15. Schematic representation of modular design of ligand. Adopted from Mattoussi et al (2007).
Figure 2.16. Schematic representation of ligand exchange strategy to functionalize the surface of quantum dots (DHLA-PEG) (Pons and Mattoussi, 2009)
Figure 2.17. The chemical reduction of DTT with disulfide containing molecule (Singh et al., 2005).
Figure 2.18. SMCC crosslinking mechanism. Adapted from Piercenet.
Figure 2.19. Schematic representation of a Confocal Laser Scanning Microscopy (Prasad et al., 2007)
3.1. Materials

Polyclonal Anti-gliadin antibody, Hi Trap Protein G High Performance affinity column and prolong media were purchased from Sigma Aldrich (St Louis, MO). Qdot 625 was purchased from a local market in Champaign, IL. (Gold Medal brand, manufactured by General Mills, Minneapolis, MN, approximate protein content 10.5% and approximate carbohydrate content of 79%) Tissue-Tek™ CRYO-OCT Compound, Tissue-Tek cyromolds and TRUBOND 380 microscope slides were acquired from Fisher Scientific (Pittsburgh, PA) through the Institute of Genomic Biology of the U of I. PBS buffer concentrate was purchased from Sigma Aldrich and was diluted in the ratio of 1 to 10. All other chemicals used in experiments were of analytical grade. Acetone and paraformaldehyde were purchased from Sigma Aldrich (St Louis, MO). They were used for fixation of dough sections.
3.2. Methods

3.2.1 Preparation of Dough Samples

To prepare the dough samples, constant flour weight procedure (AACC Method 54-21.02) was followed. Prior to starting the experiment, moisture content of flour was measured. 2 grams of flour was weighed and placed into an aluminum sample pan. Moisture content of flour was determined by using a HR83 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH). A Farinograph (C.W. Brabender® Instruments, Inc, South Hackensack, NJ) with a 300 g bowl was utilized to prepare the dough samples. The required amount of flour based on a 14 percent moisture basis was placed into the farinograph bowl. The Farinograph bowl was kept at 30 ºC ±1 by a thermostatically controlled circulating water bath. The samples were mixed for 1 min. and DI water from a burette was carefully poured into the flour to form dough. When the dough is being developed as a result of the addition of water and mixing the resistance to mixing is plotted as a curve (farinogram) on a graph paper by the built in Farinograph plotter. Water was added until the center of curve reached 500 Brabender unit (B.U.) and water absorption of flour to reach 500 BU was recorded. Then, the essential quantity of flour based on a 14 percent moisture basis was weighed and placed into the farinograph bowl and mixed for 1 min. respectively. The predetermined amount water was added to the farinograph bowl using the burette to create a dough and water-flour mixture was mixed for 20 min. to obtain an entire farinograph curve. Arrival time (AT), peak time (PT), departure time (DT), and comminute time (10 min after departure time) were recorded from the farinogram curve. Dough samples were obtained from
the farinograph bowl at arrival time (AT), peak time (PT), departure time (DT), and comminute time (10 min after departure time) for further studies.

3.2.2 Sectioning of Samples for Microscopy Imaging

Small pieces of dough samples (0.5 cm³) were taken from the farinograph bowl at four different mixing times which included arrival time, peak time, departure time and 10 min. after departure time and placed into a beaker containing 20 ml of heparin solution (500 IU/ml) for 10 min. to remove auto fluorescence of dough. The samples were then rinsed with DI water and placed into disposable plastic Tissue-Tek cryomolds after their surfaces were covered by a thin layer of O.C.T embedding compound. Tissue freezing medium (O.C.T) was also smeared on samples to entirely cover the dough samples to avoid dehydration of samples and to offer a suitable specimen medium for cryostat sectioning at temperatures of −10°C (14°F) and below. Each disposable plastic cryomold containing dough sample was covered with labelled, precut pieces of aluminum foil. Each aluminum pack was immersed into liquid nitrogen in a beaker and incubated for 10 min. Aluminum packs were taken out by means of forceps and stored at -80 °C. Dough samples were sectioned using a LEICA CM 3050 S Cryostat (Leica Biosystems Nussloch GmbH, Germany). They were transferred to a cryostat in liquid nitrogen and aluminum foil packs were removed inside the cryostat. A thin layer of O.C.T compound was poured into the cryostat chuck and the samples in disposable plastic cryomolds were laid on the O.C.T coated chuck. These samples were frozen as an ensemble containing sample/O.C.T/chuck at -20 °C for 10 min. The inlet temperature of the cryostat was set at -20 °C and kept between -18 °C and -20 °C. The samples were longitudinally carefully cut to a thickness of 10 µm. The sections
were then placed onto special hydrophilic adhesive microscope slides and air-dried overnight at room temperature.

The slides were placed in a slide rack and immersed in a staining dish containing reagent-grade acetone or 4 % paraformaldehyde/PBS for ten minutes. The samples were divided into two groups. One group was fixed in a reagent-grade acetone which provides greatest staining by preserving antigenic determinants but morphology and the other one was fixed in 4 % paraformaldehyde/PBS which provides superior morphology. The slides were air-dried for 10 minutes at room temperature. Then, a hydrophobic barrier on the each slide was created by encircling each section using a special wax pan to avoid the antibody-quantum dots mixture from spreading over the entire surface of the slide.

### 3.2.3 Antibody Purification

In order to obtain antibody with maximum selectivity and sensitivity it needs to be purified. A procedure by GE Healthcare Life Sciences was followed to purify polyclonal Anti-gliadin antibody. In this procedure, Hi Trap Protein G High Performance affinity column was used to purify and isolate polyclonal Anti-gliadin antibody from blood, serum, and ascites. The column was composed of Protein G coupled with highly cross linked agarose gel by using N-hydroxysuccinimide. Protein G, with molecular weight of 17000, is an Fc Type III receptor produced by streptococcus. Protein G has affinity for Fc regions of polyclonal antibodies. The 1 ml column was placed into a fast liquid protein chromatography system (AKTAFLPC) (General Electric Company, USA). One vial of anti-gliadin polyclonal antibody solution (1 ml) in 0.01 M phosphate buffered saline (PBS), pH 7.4, was thawed at room temperature. Then, 1 ml of
antibody solution was diluted in 10 ml PBS solution. Absorbance of the antibody solution was measured in a spectrophotometer at 280 nm.

There were two different buffers used in purification of antibodies. A binding buffer contained 20 mM sodium phosphate at pH 7.0, and the elution buffer was composed of 0.1 M glycine-HCl at pH 2.7. The affinity column was washed with 10 ml of binding buffer at a constant flow rate of 1 ml/min by means of FLPC where solvent velocity is controlled by a peristaltic pumps. The antibody solution was diluted to 700 µg/ml and was loaded to the column, recirculated several times with the FLPC system until most of the antibodies were bound to the column. The column was then washed 10 times with binding buffer. Antibody fractions bound to the column were collected by eluting with 5 ml of elution buffer in 1 ml centrifuge tubes containing 60 µl 1 M Tris-HCl, pH 9.0 to neutralize to the final pH. The final concentration of antibodies were determined and adjusted to 1 mg/ml. Purified and isolated antibodies were stored at -20 °C in a freezer.

3.2.4 Preparation of Antibody-Quantum Dot Conjugate

To prepare antibody-quantum dot conjugate, a method offered by Invitrogen was followed (fig 3.1). In this method, 1 L of 1X PBS solution was prepared from 10 X PBS solution. 1 X PBS solution is composed of 10 mM phosphate, 138 mM NaCL, 2.7 mM KCL. The pH of the solution was adjusted to 7.2 by adding 0.1 M HCL.

50 µl of 10 mM Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) solution in dimethylsulfoxide (DMSO) was thawed in a water bath at 37 °C for 20 min. 1 mg/ml gliadin antibody solution in PBS was prepared.
14 µl of SMCC solution was pipetted into 1.5 ml centrifuge tube. 125 µl of 4 µM QDot solution was added to this centrifuge tube. The tube was briefly mixed using a vortex mixer from Fisher scientific. In order to activate quantum dots crystals, the mixed solution was incubated for one hour at 26 ºC.

In the next step 300 µl of antibody solution at 1 mg/ml was pipetted into 1.5 ml centrifuge tube. 6.1 µl of 1 M Dithiothreitol DTT solution was added to the tube containing antibody. The tube was again mixed using a vortex mixer.

Two desalting columns with exchange buffers were prepared prior to the end of the antibody reduction. One of these columns was used to “reduce the antibody” and the other to activate quantum dots nanocrystals and were accordingly labelled. Top and bottom lids were detached from the both columns. Meanwhile the liquid in each column was approaching the top of the column gel bed. In the next step, 10 mL (3 column volumes) of exchange buffer (50 mM MES, 2 mM EDTA, pH 6.0) was added to equilibrate each column gel. The bottom of each column was capped when sufficient exchange buffer was left above the gel bed on each column.

500 µL of water was then pipetted into a 1.5 ml centrifugation tube. The level of water was marked with a pen. A second 500 ml aliquot of water was added to the 1.5 ml centrifugation tube containing 500 µL of water and put a second mark outside the tube at the meniscus by following discarding the water. After the antibody reduction ended, 20 µL of dye labeled marker was added to the reduced antibody. The exchange buffer above the gel bed surface was then introduced into the “Reduced antibody” desalting column. Once the exchange buffer reached the gel surfaces, the reduced antibody mixed with the dye labelled marker was added to the desalting column. As soon as the reduced antibody mixture entirely entered the gel, 1 mL of
exchange buffer was added to the top of the gel bed to elute the antibody. 500 µl of reduced antibody was collected from the column in a previously marked centrifuge tube after the first dyed drop was separated.

“Activated Qdot nanocrystals” desalting column was then opened and the exchange buffer was introduced above the gel bed surface. Once the exchange buffer reached the gel surfaces, the activated Qdot nanocrystals were added to the top of the gel bed. Once the activated Qdot nanocrystals entirely diffused into the gel, 1 mL of exchange buffer was added to the top of the gel bed to elute the Qdot nanocrystals. Activated Qdot nanocrystals were collected from the column in a centrifuge tube containing reduced antibody until the final volume reached 1 ml. The first drop of dyed material was discarded.

Conjugation between reduced antibody and activated Qdot nanocrystals occurred at room temperature for one hour. 10 mM, 10 µl of 2-mercaptoethanol solution was prepared and added to the labelled centrifuge tube in which conjugation reaction took place and incubated for 1 hour at room temperature.

To prepare the separation column, top and bottom lids were uncapped and 8 ml separation media containing 20% ethanol as a preservative were uniformly dispersed in the bottle by using vigorous shaking in a vortex mixer. Then, the separation column was marked with two lines which were indicators of height of the gel bed. One was located at 45 mm above the frit, and second one at 55 mm above the frit. Sufficient amount of uniformly suspended separation media was poured into the separation column with a 1 ml pipette until it reached the 55 mm line. Dripping began to occur at the bottom of the column and a packed gel bed formed at about the 45 mm line. As a level bed surface formed, 0.5 ml of DI water was introduced into the top of the column. The separation columns were connected to a syringe using a tubing which had two edges. When one edge of the
tubing was combined with the tip of the column, second one was attached to the syringe. The solvent from the column was removed by pulling out the syringe plunger. As the level of solvent approached the top of the gel bed, the column was filled out with PBS solution. The level of PBS was lowered to above the top of the gel by drawing the syringe plunger down. This filling and draining step was done in triplicate. In the last one, the column was filled to a level which was 3 mm higher than top of the gel. The syringe was disconnected by removing the tubing and top and bottom lids of the column were replaced.

Once quenching the conjugation reaction ended up, the volume of tube was divided into two equal aliquots in two ultrafiltration devices. The samples containing both conjugated antibody and unconjugated antibody, were centrifuged at 4000x g for 15 min. in a Mini Spin Plus Benchtop Microcentrifuge (Eppendorf, Germany) until the final volume of the sample in each device was reduced to 20 µl. This step enabled concentration of the substrate in each solution.

Next, the top and bottom lids were uncapped and PBS was eluted into the samples by gravity. As soon as PBS level reached the top of the gel surface, concentrated samples (total volume of 40 µl) were poured into the separation column and were allowed to enter the gel following addition of 50 µl of PBS. A dead space formed between the frit and the column tip. The first ten drops of the antibody-quantum dots conjugate was collected in a centrifuge tube when the color was seen in the dead space. The conjugate was stored at 4 ºC in a refrigerator. Optical density of the conjugate was measured at 610 nm by using a UV-VIS spectrophotometer in the visible range. The conjugate concentration was obtained by using this formula;

\[
A = \frac{\text{absorbance}}{
\text{value for Qdot 625 nanocrystals} = 500000}
\]

In which A is the absorbance, \(\epsilon\) is the molar extinction coefficient, \(c\) is the molar concentration, and L is the path length.
3.2.5 Staining the Sections

The staining procedure starts by placing the slides in a slide rack, immersed in a staining dish containing PBS and incubated for 5 min. PBS was removed from slides by tipping the slides and allowing the PBS solution to drip out. Residual PBS around the samples was also removed by gently absorbing the solution with Kim wipes without contaminating and damaging the samples. Diluted antibody solution (1/10 v/v in PBS) were directly inoculated to the regions encircled with wax pen and incubated for one hour without letting the sections dry. The slides were washed in PBS solution for 10 min. This step was triplicated. One drop of prolong media as an antifading agent was applied to each section and covered with a coverslip to preserve the QDs from photobleaching during fluorescence microscopy experiments. The edges of the coverslips were sealed with nail polish to prevent drying. Slides were placed in a dark room and we waited until the nail polish dries at room temperature for 12 hours. They were kept for another 12 hours at 4 ºC in a refrigerator.

3.2.6 Examination of Dough Samples by Confocal Microscopy

A confocal laser scanning microscope (Zeiss LSM 710, Carl Zeiss Micro imaging GmbH, Germany) was used to visualize dough sample microstructure. Starch granules were identified by simple polarized light. The excitation wavelengths of the QDs were 405 nm for the reflection and 615 nm for the fluorescence signal for QDs. Carl Zeiss Plan-Apochromat 20x/0.8 M27 objective lens was employed to view each dough samples. Digital image files in 512X512 pixel resolution were recorded with the Zen LSM software (Carl Zeiss Micro imaging GmbH, Germany).
3.2.7 Digital Image Processing and Analysis

Imaje J, an open source java program, was used to be able to quantify CLSM images (National Institutes of health, Bethesda, MD, USA). Prior to analyze sections of dough samples in different mixing times 5 digital images for each dough sample, the images were converted to grayscale. A fuzzy thresholding algorithm developed by Huang et al. (1995) was used to apply the segmentation of protein to background by forming a binary images. Particle count ($\Sigma P$), average size $\Phi A$ ($\mu m^2$), % area, average intensity and perimeter ($\mu m$) values were calculated from binary images using Imaje J program.
3.3 Figures and Tables

Table 3.1 Extinction coefficients of quantum dots and their measurement wavelengths

<table>
<thead>
<tr>
<th>Product</th>
<th>Extinction Coefficient</th>
<th>Measurement Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qdot® 525 nanocrystals</td>
<td>200,000 M$^{-1}$ cm$^{-1}$</td>
<td>At the highest absorbance value between 504 and 512</td>
</tr>
<tr>
<td>Qdot® 565 nanocrystals</td>
<td>300,000 M$^{-1}$ cm$^{-1}$</td>
<td>At the highest absorbance value between 540 and 556</td>
</tr>
<tr>
<td>Qdot® 585 nanocrystals</td>
<td>400,000 M$^{-1}$ cm$^{-1}$</td>
<td>At the highest absorbance value between 567 &amp; 575 nm</td>
</tr>
<tr>
<td>Qdot® 605 nanocrystals</td>
<td>650,000 M$^{-1}$ cm$^{-1}$</td>
<td>At the highest absorbance value between 596 &amp; 604 nm</td>
</tr>
<tr>
<td>Qdot® 625 nanocrystals</td>
<td>500,000 M$^{-1}$ cm$^{-1}$</td>
<td>At the highest absorbance value between 605 &amp; 612 nm</td>
</tr>
<tr>
<td>Qdot® 655 nanocrystals</td>
<td>800,000 M$^{-1}$ cm$^{-1}$</td>
<td>638 nm exactly</td>
</tr>
<tr>
<td>Qdot® 705 nanocrystals</td>
<td>1,700,000 M$^{-1}$ cm$^{-1}$</td>
<td>550 nm exactly</td>
</tr>
<tr>
<td>Qdot® 800 nanocrystals</td>
<td>1,700,000 M$^{-1}$ cm$^{-1}$</td>
<td>550 nm exactly</td>
</tr>
</tbody>
</table>

(Adopted from Invitrogen)
Figure 3.1. Flowchart of antibody-quantum dot conjugation procedure (Invitrogen)
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Evaluation of Wheat Flour Dough by Farinograph

A characteristic hockey stick shaped Farinograms for the dough is shown in Figure 4.1 and relevant properties are shown in Table 4.1. Flour moisture content was found to be 11.62% (w/w). Water absorption of wheat flour was found to be 57.8%. The arrival time where the curve reached 500 Brabender Units) was found to be 75s (arrival time); peak time, was 12 minutes. The departure time where the curve went below the 500 BU line was 23 min. The dough sample was further mixed 10 minutes after the departure time. The final mixing time used in this study was 33 minutes. Stability of the dough sample was calculated to be 22 min. The Farinogram suggests that flour used is a strong flour because of the long stability time observed.
4.2 Microstructural Characteristics

Confocal Laser Scanning Microscopy (CLSM) was used to visualize the distribution of gliadin in bread dough at 4 different mixing times (arrival time, peak time, departure time and 10 min after departure time).

Confocal laser scanning micrographs are shown in figures 4.2, 4.4, 4.6, 4.8, 4.10, 4.12, 4.14, 4.16 in reflection, transmission and the overlay of transmission and reflection in arrival time (Figures 4.2 and 4.10), peak time (Figures 4.4 and 4.12), departure time (Figures 4.6 and 4.14), and 4.10 min after departure time (figures 4.8 and 4.16) respectively. The two sets of figures correspond to fixing the sample in acetone and in paraformaldehyde. Acetone preserves the antigens very well compared to paraformaldehyde and paraformaldehyde enables the protection of the structure compared to acetone because the paraformaldehyde crosslinks the amine groups of antigens. The acetone fixing method results in more accurate binding between the gliadin antibody and the epitopes on gliadin. The paraformaldehyde fixing method on the other hand fixes the molecular structure in place due to crosslinking but at the expense of less accurate gliadin antibody binding. The reflection signal is shown in red (top left images), the transmission in black and white (top right images), and the overlay of transmission and reflection in combination of red, black and white (bottom left images) in figures 4.2, 4.4, 4.6, 4.8, 4.10, 4.12, 4.14, and 4.16. The fluorescence intensities of gliadin at the characteristic mixing times as a function of location on the sample are illustrated in 3D figures 4.3, 4.5, 4.7, 4.9, 4.11, 4.13, 4.15, 4.17 and 4.19 respectively. The dough sample sections shown in figures 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, and 4.8, 4.9 were fixed in acetone, and the dough sample sections shown in Figure 4.10, 4.11, 4.12, 4.13, 4.14, 4.15, and 4.16 were fixed in 4% paraformaldehyde.
It is clearly seen that gliadin molecules were not distributed homogenously and were present as large compact clusters illustrated in Fig 4.2(a) and both large and small starch molecules existed (fig 4.2(b)) in arrival time. Gliadin molecules were partially interconnected and there are still some unoccupied areas between protein filaments. There were various large starch molecules that were not embedded in gliadin molecules. Most of the small starch molecules were surrounded by gliadin molecules and attached to them. The fluorescence intensities of gliadin molecules in arrival time are illustrated in 3D images (fig. 4.3). The result illustrated that most of the gliadin molecules were clustered in several specific areas representing higher intensities in the bulk dough. While the lack of homogenous networking for the gluten protein at arrival time has been known for more than three decades through Moss’ classic and elegant study using dyes, a specific understanding for gliadin was not available until this study and a parallel study in our laboratory (Ansari et al, 2014). Clearly gliadin behavior is not very different than gluten behavior but knowing the specific distribution is in the dough might offer insights on the role of this distribution on the quality of the dough. Li et al (2004) studied the distribution of added gliadin using dyes but again the intrinsic gliadin in dough is being studied for the first time.

Figure 4.4 shows the change in gliadin microstructure at peak time for the same wheat dough. Gliadin strands still form a compact and coarse network in which some large starch particles were surrounded by gliadin and many small starch particles were embedded in them(fig 4.4a, 4.5). There were fewer gliadin free regions present in the dough section as a result of the distribution of gliadin in the dough. It can be seen that gliadin strands became thinner compared to arrival time because of elongation and distribution of gliadin strands. Gliadin proteins were still observed to be agglomerated. However a smoother protein matrix was formed compared to
arrival time (fig. 4.5). The hydrophobicity of gliadin proteins clearly prevent the hydrophilic starch to successfully mix with gliadin. The starch granules tend to be attracted with one another and the protein despite the intense energy introduced during mixing is still in the form of aggregates.

The protein network uniformity was enhanced with prolonged mixing time. Changes in gliadin microstructure in departure time are shown in figure 4. 6. Uniformity was related to distribution and dispersion of gliadins across the section. Prolonged mixing led to formation a more continuous relatively evenly distributed protein matrix. Even though most of the space between starch granules was occupied by gliadin strands, a high number of relatively small gliadin strands were formed. Starch molecules were evenly distributed within the gliadin matrix and the protein film was distributed around them. Clearly the additional mechanical energy during mixing hydrated the gliadin to its fully extent and helped the formation of a relatively well formed gliadin film. The presence of water facilitated the contact and distribution between the hydrophilic starch granules and the now better mixed and hydrated protein film.

The most uniform gliadin distributions were observed at 10 min. after departure time. Gliadin strands formed airy sponge-like structure partly because of the incorporation of air into the dough and partly by surrounding adjacent starch molecules (fig. 4.8). Starch molecules located in the dough matrix were observed to be uniformly distributed.

It can be seen that gliadin proteins are more abundant around compact protein rich domains formed due to aggregation of hydrated gluten during mixing and less around starch molecules in arrival times illustrated in Figures 4.10. Gliadin is the more viscous of the two major classes of proteins, gliadins and glutenins and flows best among the two protein classes. Glutenins because of their high elasticity and high hydrophobicity are not likely to disperse as well as gliadins.
There were some regions that did not show a lot of gliadin but the density and size of these regions is a lot smaller compared to arrival time and peak time. The protein network is not well formed yet and gliadins were not properly dispersed around starch in these regions. This is consistent with earlier work about development of gluten. While in these studies the distribution of gluten has been studied, this is the first such study specifically focusing on distribution of gliadin. Therefore, black regions shown in the fig. 4.10 show no gliadin molecules and only starch molecules are observed. In the fig. 4.10 gliadin free regions are encircled with green ellipsoid indicators, and compact protein masses were marked with blue ellipsoid indicators. At this mixing time there is insufficient dispersion and distribution of gliadin protein. These findings were supported by other researchers. Dough sheared at small strains resulted in the formation of heterogeneous protein structures on the scale of more than 100 µm and protein rich and protein poor regions (starch rich) were observed. (Peighambardoust et al., 2006). This was also in agreement with the findings of Paredes-Lopez and Bushuk (1982), Moss et al. (1987) and more recently those of Calderon-Dominguez et al. (2003), who presented that coarse protein filaments were formed at the early stages of dough mixing. As mixing time increased, gliadins were more uniformly distributed in the dough matrix and began to uniformly surround starch granules. Compared to dough samples at arrival time, gliadin was uniformly distributed in the dough matrix at peak time, as shown in figure 4.12. Figure 4.13 illustrated that gliadin filaments spread across the dough matrix because they became more stretched and various starch granules were attached to the gliadins. In figures 4.14 and 4.15, we show some protein dense regions drawn with green ellipsoid markers and a few protein poor regions encircled in blue ellipsoid markers. At departure time, starch granules were homogenously bound to the thinly stretched red-colored gliadin strands and starch molecules were surrounded by gliadin filaments.
Schluenz et al. (1999) and Lee et al. (2001) works showed that the energy input during mixing increases the amount of developed protein matrix up to the optimum mixing time where a protein network forms. Another observation that follows is that the formation of a protein structure resulted in a smoother structure as observed by microscopy. During dough development, water diffuses into flour particles resulting in hydration and swelling of the starch and proteins. In the early stages, the swollen proteins start to interact and form aggregates because they are predominantly hydrophobic and the presence of water promotes hydrophobic interactions that progressively lead to an entangled polymer network.

As the dough gradually develops and becomes increasingly homogenous with energy addition, the protein strands are prolonged into a continuous network surrounding most of the starch granules (Bloksma et al, 1990). The role of native gliadin in this networking process is not well understood and has not been studied by prior researchers because of unavailability of molecular tools to specifically probe gliadin behavior until now. What is well known however is that gliadin has better flow properties and is more viscous compared to glutenin which is much more elastic. The most uniform gliadin distributions was found in 10 min after departure time where the dough is overmixed compared to arrival time, peak time and departure time presented in figures 4.16 and 4.17 as expected because gliadin can flow better than the rest of gluten and can distribute the most in the dough compared to other protein components. Gliadins were evenly distributed and surrounded starch granules by enclosing them and there was no region that did not have gliadin at a mixing time 10 min after departure time. This is consistent with observations in the SEM study by Amend et al. (1991) of over-mixed dough. They reported that substantial extending perforates led to formation of an interconnected network of fine gluten strands, which surround all starch particles.
Mixing helps the formation of a uniform and fine gluten structure. Surrounding starch granules and addition of energy input increases the amount of protein matrix formation as dough transitions from a nondeveloped dough to a developed dough.

Both large lenticular and small spherical granules were observed in all dough sections illustrated in figures 4.2, 4.4, 4.6, 4.8, 4.10, 4.12, 4.14, and 4.16. Because of its birefringence property under polarized light, starch granules can be discriminated from air bubbles or lipids.

We also utilized from Image J program to be able to analyze and process the images from different sections of dough samples (Table 4.2 and 4.3). Particle count (ΣP), average size ΦA (µm²), % area, average intensity and perimeter (µm) values were calculated. The highest particle counts were observed in the both acetone and 4 % paraformaldehyde fixed samples mixed for 23 min representing 10 min after departure time. The particle counts were 452±91. for acetone fixed samples whereas 491±129 was obtained from paraformaldehyde fixed samples. The smallest particle counts were found in both acetone and paraformaldehyde fixed dough samples. Particle counts were calculated to be 261.4±17.5 and 280±16 respectively. These numbers are reasonable because the formation of a large coarse protein strands at the early stages of mixing process is expected since there is not energy and mixing time to effectively disperse the protein and gliadin more specifically. The sections obtained from samples mixed for 75 seconds related to arrival time gave the smallest surface area. There was an increase in the % total area of detectable proteins with prolonged mixing. The highest intensity values (mean value) were found at peak time in both acetone and paraformaldehyde fixed sections. 10 min after departure time gave similar results.
Figure 4.20 illustrated binary CLSM micrographs of wheat dough at different mixing times. At arrival time (fig 4.20 a), mixing led to the formation of coarse protein domains in the dough structure. Discontinuous protein rich domains and areas with mainly starch granules are visible, demonstrating structural inhomogeneity in the dough at arrival time.

At peak time (fig 4.20 b) mixing transformed the discontinuous protein domains into interconnected gliadin domains. Amend et al. (1991) stated that the mixing process prolongs the gluten structures, results in making the surface area larger and the depth smaller. Thus, numerous extended gliadin filaments transform into films, a continuous gluten phase.

More mechanic energy applied lead to Prolonged mixing of dough (Fig. 4.20 c and d) resulted in formation of a fine gliadin structure with maximum homogeneity. The finest gliadin strands formation was observed in fig 4.20d.

It was also observed that gliadin is not only found surrounding starch granules in dough, but also are located on the boundaries of gas cells or `cell walls`. Figures 4.19 and 4.20 show the distribution and the florescence intensity of gliadin in the gas cell walls. The round structures with sharp interfaces observed in dough correspond to gas pores formed by air inclusions during mixing. This is consistent with the work of (Li et al., 2004) who observed that gliadin was found not only in strands of dough, but was also seen related the gas cell walls. It was noted that compared to a fermented bread dough (with yeast) the gas cells are rather small (largest air void approx.45 mm) and composed of air instead of CO₂ as in bread dough.

Gliadin could be very important to the rheology of bulk dough and also suggests that it could get involved in formation of gas cell walls, which supports the expansion capacity (Ornebro et al., 2000).
In order to develop a good understanding of the distribution of gliadin throughout the depth of the dough section Z-stack images were obtained at 0.75 µm intervals. These images complement the previous images for the four mixing times by giving an understanding of the three dimensional distribution of gliadin in dough samples. 3D images were obtained by reconstructing images captured at different focal planes at sequential depths, 0.75 µm apart in the Z stack in a single display. 3D images of are illustrated in figures 4.21, 4.22, 4.23, 4.24, 4.25, 4.26, and 4.27 respectively for arrival time, peak time, departure time and 10 minutes after departure time fixed in acetone and paraformaldehyde.

The results showed that antibody-quantum dots conjugates successfully diffused into the 3D matrix and were bound to gliadins. Distribution and location of gliadin at different focal planes in each section were found to show similar patterns for a given mixing time. Gliadins were evenly dispersed in dough sections and typically localized in the center of the sections. This supports the observation and hypothesis that the mobility of gliadin due to its lower viscosity enables gliadin to diffuse to the inner sections of the dough along with all other parts of the sample. Intensities of gliadins at top and bottom stacks were relatively low compared to ones located at center. It might be because of optical sectioning of starch molecules found at top and bottom surfaces of sections play a dominant part in the imaging process.
4.3 Figures and Tables

Table 4.1. Physical properties of wheat flour dough obtained from farinograph

<table>
<thead>
<tr>
<th>Wheat flour</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% moisture content of flour(%w/w)</td>
<td>11.62</td>
</tr>
<tr>
<td>Water Absorption (%)</td>
<td>57.8</td>
</tr>
<tr>
<td>Arrival time (sec)</td>
<td>75</td>
</tr>
<tr>
<td>Peak time (min)</td>
<td>12</td>
</tr>
<tr>
<td>Departure time (min)</td>
<td>23</td>
</tr>
<tr>
<td>Stability (min)</td>
<td>22</td>
</tr>
</tbody>
</table>
Table 4.2. Parameters associated with protein properties after image processing of CLSM images as affected by mixing time during dough preparation. Results are illustrated as mean±standard deviation(n=5) (acetone fixed samples)

<table>
<thead>
<tr>
<th>Mixing time</th>
<th>Particle Count (ΣP)</th>
<th>Average size(µm²)</th>
<th>% Area</th>
<th>Mean (average intensity)</th>
<th>Perimeter (P) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>261.4±17.49</td>
<td>156.8085±19.99237</td>
<td>41.643±2.709281</td>
<td>16.1606±3.829772</td>
<td>24.053±5.914597</td>
</tr>
<tr>
<td>10 min. after</td>
<td>452±91.87383</td>
<td>81.10471429±35.84722959</td>
<td>38.632±5.236603</td>
<td>26.036±11.33719</td>
<td>29.93157±3.9499</td>
</tr>
</tbody>
</table>
Table 4.3 Parameters associated with protein properties after image processing of CLSM images as affected by mixing time during dough preparation. Results are illustrated as mean±standard deviation(n=5) (4 % paraformaldehyde fixed samples)

<table>
<thead>
<tr>
<th>Mixing time</th>
<th>Particle Count (ΣP)</th>
<th>Average size(µm²)</th>
<th>% Area</th>
<th>Mean (average intensity)</th>
<th>Perimeter (P) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT</td>
<td>396±132.9361</td>
<td>124.873±54.012</td>
<td>48.055±6.733</td>
<td>17.6385±4.8465</td>
<td>20.609±0.709</td>
</tr>
<tr>
<td>10 min after</td>
<td>491.6667±129.6238</td>
<td>118.95375±33.4639456</td>
<td>49.71525±6.390554</td>
<td>30.90125±3.163943</td>
<td>23.217±10.88407</td>
</tr>
</tbody>
</table>
Figure 4.1. A Farinogram of wheat flour dough. AT, PT and departure time were indicated with arrows.
Figure 4.2. Microstructure of dough section fixed in Acetone at Arrival time. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm and emission wavelength of 615nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Blue indicator illustrates a region where gliadin strands were aggregated whereas green ellipsoids illustrated the gliadin free region. Scale bar:20 µm.
Figure 4.3. The fluorescence intensity of gliadin in dough sample at Arrival time (acetone fixed section) scanned at different depths with an objective with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm and emission wavelength of 615 nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak.
Figure 4.4. Microstructure of dough section fixed in Acetone at Peak time. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (\(\lambda\)) and emission wavelength of 615 nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. (X=300\(\mu\)m, Y=300\(\mu\)m)
Figure 4.5. The fluorescence intensity of gliadin in dough sample at departure time (acetone fixed section) scanned at different depths with an objective with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (405 nm) and emission wavelength of 615 nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak.
Figure 4.6. Microstructure of dough section fixed in Acetone at Departure time. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (λ) and emission wavelength of 615 nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Scale bar: 20 μm
**Figure 4.7.** The fluorescence intensity of gliadin in dough sample at departure time (acetone fixed section) scanned at different depths with an objective with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (?) and emission wavelength of 615nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak.
Figure 4.8. Microstructure of dough section fixed in Acetone at 10 min after Departure time. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm and emission wavelength of 615nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Scale bar:20
Figure 4.9. The fluorescence intensity of gliadin in dough sample at departure time (acetone fixed section) scanned at different depths with an objective of Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (\(\lambda\)) and emission wavelength of 615 nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak.
Figure 4.10. Microstructure of dough section fixed in 4% paraformaldehyde at arrival time using a Brabender Farinograph. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (λ) and emission wavelength of 615 nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Blue indicator illustrates a region where gliadin strands were aggregated whereas green ellipsoids illustrated the gliadin free region. Scale bar: 20
Figure 4.11. The fluorescence intensity of gliadin in dough sample at departure time (4 paraformaldehyde fixed section) scanned at different depths with an objective with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (?) and emission wavelength of 615nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak.
Figure 4.12. Microstructure of dough section fixed in 4% paraformaldehyde at peak time using a Brabender Farinograph. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (\( \lambda \)) and emission wavelength of 615nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Blue indicator illustrates a region where gliadin strands were aggregated whereas green ellipsoids illustrated the gliadin free region. Scale bar: 20\( \mu \)m
Figure 4.13. The fluorescence intensity of gliadin in dough sample at departure time (4 paraformaldehyde fixed section) scanned at different depths with an objective Carl Zeiss Plan-Apochromat 20x/0.8 M27 with excitation wavelength of 405 nm (?) and emission wavelength of 615nm. Fluorescence intensity is proportional to the height of the observed peaks and the whiteness of the peak. (x and y axis indicate horizontal plane, z illustrates intensity)
Figure 4.14. Microstructure of a dough section fixed in 4% paraformaldehyde at departure time using a Farinograph. A, Quantum dot fluorescent intensity conjugated with the gliadin protein matrix under 633 nm laser light; B, starch granules under polarized light; C overlay of gliadin fluorescent intensity in figure A and polarized starch in B showing the distribution of gliadin around starch. The green ellipse shows a region where there is no gliadin.
Figure 4.15. The Fluorescence intensity of quantum dot conjugated gliadin at dough departure mixing time using a Farinograph (4% paraformaldehyde fixed section) The image represents a dough section 300 micronsX300 microns and the peaks represent the fluorescence intensity of quantum dots conjugated with gliadin. The regions where there are no gaps represent starch granules which take up that space.
Figure 4.16. Microstructure of dough section fixed in 4% paraformaldehyde at 10 min after departure time using a Brabender Farinograph. A (top left), Protein molecules bound to quantum dots scanned with an objective Carl Zeiss Plan-APOCHROMAT 20x/0.8 M27 with excitation wavelength of 405 nm (?) and emission wavelength of 615nm; B (top right), starch granules under polarized light; C (bottom left), overlay of A and B showing the distribution of gliadin in the dough matrix and around starch. Scale bar: 20 μm.
Figure 4.17. The Fluorescence intensity of quantum dot conjugated gliadin at dough departure mixing time using a Farinograph (4% paraformaldehyde fixed section) The image represents a dough section 300 micronsX300 microns and the peaks represent the fluorescence intensity of quantum dots conjugated with gliadin. The regions where there are no gaps represent starch granules which take up that space
Figure 4.18. Distribution of gliadin around gas cells representing higher intensity and bulk dough (acetone fixed section, departure time) The image represents a dough section 300 micronsX300 microns and the peaks represent the fluorescence intensity of quantum dots conjugated with gliadin.
Figure 4.19. Structural details of distribution of gliadin around gas cells (4 paraformaldehyde fixed section, departure time) Magnified CLSM images
Figure 4.20. Binary CLSM micrographs of wheat dough at arrival time A (top left), Peak time B (top right), departure time C (bottom left), 10 min after departure time D (bottom right) (acetone fixed samples) Proteins are illustrated in black. The image represents a dough section 300 microns X 300 microns.
Figure 4.21 A 3D image of acetone fixed section at arrival time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between focal planes were set up 0.75 µm.
Figure 4.22 A 3D image of acetone fixed section at peak time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between two focal planes were set up 0.75 µm.
Figure 4.23 A 3D image of acetone fixed section at departure time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between focal planes were set up 0.75 µm.
**Figure 4.24** A 3D image of acetone fixed section at 10 min after departure time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between focal planes were set up 0.75 µm.
Figure 4.25 A 3D image of 4% paraformaldehyde fixed section at arrival time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between focal planes was set up 0.75 μm.
Figure 4.26 A 3D image of 4% paraformaldehyde fixed section at peak time. Red color illustrates the location and distribution of gliadin at each focal plane. Interval between focal planes were set up 0.75 µm.
Figure 4.27 A 3D image of 4% paraformaldehyde fixed section at 10 min after departure time. Red color illustrates the location and distribution of gliadin at each focal plane. Intervals between focal planes were set up 0.75 µm
CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE WORK

In this study we brought a novel perspective for the application of QDs in food science by using them as fluorescent probes which are more stable and brighter compared with organic dyes to tag and identify gliadins in wheat flour dough. We tagged gliadin proteins with water soluble, biocompatible amine derivatived polyethylene glycol functionalized quantum dots to increase the clarity and specificity of imaging and then investigate the effect of different mixing conditions on their distribution and their role in building dough structure.

Mixing led to considerable changes in the distribution of gliadins in dough microstructure. CLSM images obtained from dough sections were very bright and clear and allowed us distinguish gliadin easily. The amount of protein was least in arrival time as compared with the amounts present in peak time, departure time and 10 min after departure time. The highest protein content was observed in 10 min after departure time. It was presumed that the intensity of quantum dots was not only related to the amount of protein matrix but also the area occupied by the protein in the sample. The proteins were more compact in arrival times due to fact that gliadin proteins were not aligned. The most uniform gliadin distribution were observed in 10 min after departure time. Addition of energy input increases Uniformity of protein matrix formation from arrival time to 10 min after departure time increased with the increase of energy input during mixing. The QDs were found not only to be localized around the air cells representing higher intensities but also bulk of dough.
In the future work, quantum dots can be employed to label specific gliadin and glutenin fractions of dough sample simultaneously and this will offer an opportunity to obtain detailed structural information of wheat flour dough systems together with rheological measurements.
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