A Role of Yeast Adhesin Amyloids in Force-Dependent Adhesion and Biofilm Formation

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by

Cho Xiao Juan Chan

A dissertation submitted to the Graduate Center Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

A Role of Yeast Adhesin Amyloid in Force-Dependent Adhesion and Biofilm Formation

by
Cho Xiao Juan Chan
Advisor: Professor Peter N. Lipke

_Candida albicans_ adhesins have amyloid-forming sequences (Ramsook et al. 2010, Otoo et al. 2008). Similarly, Tango and Waltz predicted that amyloid-forming sequences are also present in _Saccharomyces cerevisiae_ flocculins, Flo1p and Flo11p. The cell surface of Flo1p- and Flo11p-expressing cells have ordered domains that are thioflavin T fluorescent and Congo red birefringent, two hallmarks of amyloids. Flo1p and Flo11p amyloids were important for activities of the flocculins including cell-to-cell adhesion, cell-to-substrate adhesion, and agar invasion. In addition, amyloid-perturbing dyes thioflavin S and Congo red inhibited aggregation, biofilm formation and agar invasion.

Force-induced formation and propagation of adhesion nanodomains in Als5p-expressing cells were demonstrated with single-molecule atomic force microscopy (Alsteens et al. 2010). Because amyloid formation can be triggered by force, we investigated whether laminar flow and mechanical stress could activate amyloid formation and therefore increase adhesion. Shearing _S. cerevisiae_ cells expressing Als5p or _C. albicans_ at 0.8 dyne/cm² increased quantity and strength of cell-to-surface and cell-to-cell binding, compared to 0.02 dyne/cm². Mechanical stress from vortex-mixing at 2500 rpm also increased the aggregation of _S. cerevisiae_ cells expressing Als5p
or *C. albicans*. Similarly, cells expressing Flo1p and Flo11p also showed shear- and mechanical stress-dependent binding, and biofilm formation.

I report here for the first time that catch bonding behavior in yeast cells was mediated by amyloid formation. Amyloids mediate both sensing and response in the presence of force. Adhesin-expressing cells binding to surfaces under shear stress were less likely to be washed off from the substrate than cells that were not stressed. This is characteristic of catch bonding. Catch bonding behavior was accompanied by the formation of amyloid nanodomains through the clustering at homotypic amyloid sequences. Thus, these nanodomains increased binding avidity of the adhesin-expressing cells to other cells (flocculation and aggregation assays) and to substrate surfaces.

Furthermore we have devised ways of quantifying forces needed to activate aggregation, cell adhesion, and amyloids on the surface of yeast cells. Two different types of force, mechanical stress from vortex-mixing and shear stress from laminar flow increased adhesion and biofilm formation. Additionally, we quantified amyloid formation in live whole cell yeast suspensions in response to force. Fluorescent confocal microscopy and flow cytometry were used to quantify surface amyloids. Force-activated cells had punctate nanodomains with increased thioflavin T staining. Collectively, the assays can now be used to quantify amyloids in other fungal adhesins.

These results demonstrate that 1. there are functional amyloids present in fungal adhesins Flo1p and Flo11p from *S. cerevisiae*, 2. amyloid formation mediates adhesion, agar invasion and biofilm, 3. amyloid nanodomains mediate force-sensitive catch-bonding, and 4. force-sensitive amyloid formation on the yeast cell wall surface can be quantified.
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Chapter I

General Introduction
A. Amyloid and diseases

Pathological protein amyloids are implicated in neurodegenerative diseases such as Alzheimers’ (AD), Parkinsons’ (PD), and prion-associated spongiform encephalopathies (Barten, Albright 2008, Elgersma et al. 2007, Huang et al. 2007, Sorrentino, Bonavita 2007). Amyloid diseases are characterized by the presence of misfolded proteins in the brain. The protein aggregates form plaques and fibrous tangles. Aggregates accumulate in brain tissue and are associated with dying neurons, leading to senility and memory loss.

Protein amyloids β (aβ) and Tau are the culprits indicated in AD. The peptide form of aβ 1-40 and aβ 1-42 are the major components of senile plaques found in the brains of afflicted patients (Forloni et al. 2013). The oligomeric form of aβ is responsible for neural cell loss in the brain leading to memory loss (Cleary et al. 2005, Lesne et al. 2006). aβ is generated from amyloid precursor protein (APP). Mutations in genes encoding APP were shown to cause familial AD (Guerreiro, Gustafson & Hardy 2012). The neurofibrillary tangles of AD patients contain Tau protein. Tau protein is a microtubule-associated protein expressed mainly in axons. Hyperphosphorylated Tau characterized intracellular aggregates (Buee et al. 2000). There is growing consensus that these pathological structures are not the direct cause of AD but a result of the process of toxic oligomeric formation (Jucker, Walker 2013). The neurotoxicity is proposed to arise from aggregated protein perturbing cellular and intracellular membranes, forming amyloid pores such as ion channels, and interacting with membrane receptors (Forloni et al. 2013).

In PD, the amyloid fold of α-synuclein is associated with the pathology of the disease. α-Synuclein is abundant in the brain and found mainly at presynaptic termini, which are
specialized structures of nerve cells. α-Synuclein in the soluble state has well-defined secondary and tertiary structure. In abnormal aggregates found in nerve cells, called Lewy bodies, there is a large amount of aggregated fibrillar α-synuclein, a hallmark indicator of PD. The fibril core of α-synuclein fibers spans residues 20-100 with residues 39-41 adopting the cross β structure (Chen et al. 2007, Qin et al. 2007, Vilar et al. 2008, McGlinchey, Yap & Lee 2011).

Spongiform encephalopathies also known as prion diseases are fatal neurodegenerative diseases that results from cellular prion protein converting to an aggregated, β-sheet rich amyloidogenic form (scrapie prion protein). Prions deviate from their normal biological role and become infectious via a misconformed β-sheet rich protein. In humans an example of prion disease is Creutzfeldt-Jakob disease (CJD). CJD results from the deposit of amyloid aggregates in the brain that lead to injury and death of neurons (Poggiolini, Saverioni & Parchi 2013). Human prion diseases arise from a 23 kDa constitutive prion protein, PrP (Renner, Melki 2014). The normal state prion protein of the cellular form PrP\textsuperscript{C} is converted to a proteolysis resistant state PrP\textsuperscript{Res} through refolding and aggregation (Colby, Prusiner 2011).

B. Functional amyloids

Historically, amyloids are associated with the aforementioned diseases, but there has been mounting evidence that shows evolutionarily conserved amyloid proteins play a functional role in microbial organisms, and mammalian cells (Blanco et al. 2012, Fowler et al. 2006, Fowler et al. 2007, Ramsook et al. 2010, Wickner et al. 1999). Some of these functional roles range from adhesion and biofilm formation in microbes to protection from the sun’s ultraviolet rays in mammals (Chapman et al. 2002, Olsen et al. 1998, Ramsook et al. 2010, Garcia et al. 2011, Fowler et al. 2006). Because amyloids are ubiquitous in disease and nondisease states, it is
therefore of interest to predict, detect, inhibit, and understand the functional roles of these proteins.


In mammals, a pigment cell-specific protein with amyloid-like characteristics, Pmel is responsible for the formation of fibers in the melanosome. The melanosome is an important organelle that stores pigments found in melanocytes in the skin, hair, and eye. The Pmel protein is well-conserved. Sequences of the Pmel from rat, horse, cow, dog, chicken, quail, frog, and telost fish are highly homologous (Theos et al. 2005). These Pmel fibers have amyloid properties such as insolubility and stability in detergents (Berson et al. 2003). Fibers of Pmel have an important functional role in protecting the melanocytes from oxidative damage. Animals that lack Pmel are characterized with hypopigmentation and melanocyte inviability (Schonthaler et al. 2005, Spanakis, Lamina & Bennett 1992, Kerje et al. 2004). The β-sheets formed by Pmel serve as a scaffold for the polymerization of melanin (Watt et al. 2013). The polymerization of reactive small molecules to make melanin is a critical step in protection against UV damage (Fowler et al. 2006).

In the melanosome, Pmel is processed and cleaved into luminal fragment Mα. The Mα fragment is released, and it is further processing through proteolysis and glycosylation to form
the melanosomal matrix fibrils (McGlinchey, Yap & Lee 2011). The detection of Mα fibers are
done by electron microscopy, and staining with amyloid dye thioflavin S (ThS) and Congo red
(CR). The cross-β sheet structure is seen with X-ray diffraction and circular dichroism
spectroscopy (Fowler et al. 2006). In highly pigmented cells, the fibers grow 2-fold faster
reaching lengths of 14-20 nm (McGlinchey, Yap & Lee 2011).

i. The role of functional amyloids in adhesion

Microbial functional amyloids play a critical role in the adhesion to surfaces and structural integrity during establishment and persistence of biofilms in bacteria and fungi (Blanco et al. 2012, Chapman et al. 2002, Garcia et al. 2011, Chan, Lipke 2014, Ramsook et al. 2010). A critical step in forming the micro-communities of pathogenic organisms is to establish contact with the host cells and substrate surfaces. This contact is mediated by adhesion proteins found on the pathogen’s cell surface. Als adhesins and flocculins from fungi and curli and TasA from bacteria mediate the host-cell, and cell-to-cell contacts. These adhesion proteins have amyloid-forming ability that was determined to be critical for adhesion. Therefore, it is of interest to elucidate how amyloids mediate adhesive interactions in order to offer future therapeutics in remediating adhesion and biofilm formation.

a. Bacterial amyloids

1. Gram negative bacteria

Functional amyloids are found in gram negative bacteria *Escherichia coli* and *Salmonella* species. Curli are functional amyloids in these microbes. A well-ordered extracellular matrix made of the curli protein surrounds bacterial cells in a biofilm as analyzed by NMR (McCrate et al. 2013). Amyloid curli fibers are visible by
transmission electron microscopy of *E. coli* biofilms grown on agar plates (Evans, Chapman 2013, Chapman et al. 2002). There is a β-sheet rich secondary structure with a CD minimum peak at 218nm. Curli increases the absorbance of Congo red and causing a redshift the absorbance (Chapman et al. 2002). The fibers also increase thioflavin-T fluorescence (Chapman et al. 2002). There are functional amyloids in gram negative bacteria curli that are amyloid property positive.

Functions of curli include binding of the bacteria to surfaces such as fibronectin, forming the major component of biofilm extracellular matrix, and interacting with host factors such as plasminogen and host immune system (Olsen, Jonsson & Normark 1989, McCrate et al. 2013, Sjobring, Pohl & Olsen 1994). Adhesion is a critical first step in the formation of bacterial biofilm formation. Curlin is the major protein component of *E. coli* biofilms (McCrate et al. 2013). The biofilm matrix is made up of polysaccharides and the amyloid fibrils, curli. Biofilms from different habitats such as seawater, freshwater, drinking water, and activated sludge can be stained with thioflavin T showing that amyloid adhesins from bacteria are present in all biofilms and that it is a major and important component of the extracellular matrix (Larsen et al. 2007).

The major subunit of curli is CsgA. CsgA can self-polymerize in vitro and the fibers are indistinguishable from fibers formed in vivo (Wang et al. 2007). CsgA is secreted across the outer membrane as an unstructured soluble peptide and then templated into an amyloid on the cell surface by the nucleator, minor curli subunit CsgB (Gibson et al. 2007, Hammar, Bian & Normark 1996, Hammer, Schmidt & Chapman 2007). These curli amyloid fibers are restricted to the cell surface and are 6-
12 nm in length (Chapman et al. 2002). CsgA has five repeating units that arrange in a cross-β structure by aligning the glutamine and asparagine residues in each repeating unit in stacks thus stabilizing the amyloid fold (Wang, Chapman 2008). The side chain interactions of glutamine residues at the 49th and 139th position of CsgA promotes amyloid formation (Wang, Chapman 2008). A replacement of those residues with Asn disrupted curli assembly. Therefore, amyloids play a structural role in the development of a stable biofilm in gram negative bacteria. In biofilm formation, it is important to have a strong scaffolding material, and the stable curli satisfy this property. The strong scaffolding material aids microbial adhesion to surfaces and to other cells in changing environments. Compounds that prevent the in vitro polymerization of the major subunit of the curli protein CsgA resulted in the inhibition of biofilm formation (Cegelski et al. 2009).

2. Gram positive bacteria

Functional amyloids are also found in gram positive bacteria such as Bacillus subtilis and Streptococcus mutans. In Streptococcus mutans an adhesin P1 forms a fuzzy fibrillar coat on the surface of the bacterial cells as seen with electron microscopy (Ayakawa et al. 1987). P1 is an adhesion protein that mediates binding of bacteria with salivary constituents, host matrix proteins and other oral bacteria (Brady et al. 2010). The amyloid is detectable in human dental plaque. S. mutans biofilm formation is inhibited by known inhibitors of amyloid fibrillation (Oli et al. 2012). P1 has amyloid properties in vitro and in vivo such as binding of Congo red, apple-green birefringence under cross polarized light, and increased ThT fluorescence (Oli et al. 2012). The P1 protein has a β-sandwich arranged in two sheets and has three β-

Biofilm formation by *S. mutans* was inhibited by amyloid perturbing compounds Congo red (CR), thioflavin S (ThS), and epigallocatechin-3-gallate (EGCG) (Oli et al. 2012). EGCG is the principal polyphenol present in green tea. EGCG inhibits amyloid fibrillization of extracellular proteins from *S. mutans*. Extracellular protein shed into the bacterial cultures were amyloidogenic material.

In *Bacillus subtilis*, the biofilm matrix is mainly composed of exopolysaccharides and an amyloid fiber-forming protein, TasA. *B. subtilis* is a soil-dwelling bacterium that produces highly structured surface biofilms (Branda et al. 2001). The absence of TasA or the exopolysaccharide resulted in a residual matrix, while the absence of both results in complete failure to form the complex multicellular biofilms (Branda et al. 2006). The biofilm colony of cells lacking TasA loses its normal wrinkled topography and has less extracellular material, whereas the wild type cells have thick sheets of extracellular material (Branda et al. 2006). TasA formed extracellular fiber-like structures radiating out from the cell surface forming this biofilm matrix (Romero et al. 2010). TasA fibers range from 10-15nm in width and are amyloid dye positive. Also, fibers are resistant to harsh detergent treatments. When TasA is added back to a tasA mutant it restores wild-type biofilm morphology, indicating the purified protein retained biological activity (Romero et al. 2010). TasA forms amyloid fibers that bind cells together in the biofilm (Romero et al. 2010).
b. Fungal amyloids

1. Prions

\textit{Saccharomyces cerevisiae} have prion traits: [PSI$^+$], [URE3$^+$], and [RND$^+$]. These yeast phenotypes are transmitted to daughter cells in a non-Mendelian manner via an amyloid version of the yeast proteins Sup35p, Ure2p and Rnlq1p, respectively. The yeast prions self-perpetuate. The fibrillar aggregates manifest as dominant traits and spontaneously assemble into fibrils \textit{in vitro} (Kabani, Melki 2011). The infectious state of the protein increases phenotypic diversity through conformational changes in protein and not through amino acids changes (Kabani, Melki 2011).

Sup35p is a component of the eukaryotic release factor, and is required for suppressing of tRNAs in translational termination and stop codon recognition. In the prion [PSI$^+$] state, cells have a reduction of function in translational termination activity, and ribosome read-through naturally occurs at stop codons (Namy, Duchateau-Nguyen & Rousset 2002). The [PSI$^+$] prion can adopt different amyloid conformations (Westergard, True 2014). The glutamine and asparagine amino acid-rich prion domain is 123 residues long and is located at the N terminus (Ter-Avanesyan et al. 1994). The generation of [PSI$^+$] is dependent on the presence of [RNQ$^+$] prion (Stein, True 2011). [PSI$^+$] allows yeast to adapt and survive in changing environments. This trait governs the evolution of new traits (True, Berlin & Lindquist 2004, True, Lindquist 2000).

Ure2p is a regulator of nitrogen catabolism, repressing genes for the enzymes and transporters needed for using poor nitrogen sources, when a rich source is
available (Wickner, Masison & Edskes 1995, Wickner et al. 1999). In the presence of ammonia and glutamine, Ure2p binds to Gln3p and Gat1p and preventing translocation to the nucleus (Beck, Hall 1999, Blinder, Coschigano & Magasanik 1996, Cox et al. 2000, Hardwick et al. 1999, Magasanik, Kaiser 2002). Gln3p and Gat1p are GATA-type transcriptional activation factors that allows budding yeast to respond to environmental nitrogen levels and available nitrogen sources by regulating the expression of genes involved in utilization and transport. Ure2p is genetically antagonistic to Gln3p. Gln3p controls the expression of genes encoding enzymes and transporters required when only poor nitrogen sources are available (Kabani, Melki 2011, Coschigano, Magasanik 1991, Cox et al. 2000, Kulkarni et al. 2001). During nitrogen starvation or the presence of poor nitrogen sources, Gln3p accumulates in the nucleus and activates nitrogen catabolite repression genes. The native soluble form Ure2p is unstructured. The prion domain of Ure2p is 65-89 amino acids in length and is Q and N rich at the N-terminus (Masison, Wickner 1995a, Pierce et al. 2005). The prion domain is required for induction of [URE3+] trait and the amyloid forming capacity (Masison, Wickner 1995). Without this domain the [URE3+] prion state cannot be attained.

In [URE3+] cells, Ure2p is sequestered within prion aggregates. The loss of interaction with Gln3p renders the cells capable to metabolize poor nitrogen sources such as allantoate, and ureidosuccinate even in the presence of good nitrogen sources (Wickner 1994, Kabani, Melki 2011). [URE3+] cells express an allantoate transporter, Dal5p (Lacroute 1971). Dal5p is an allantoate permease which utilizes the presence of poor nitrogen sources.
The prion form [RNQ+] helps facilitate the *de novo* formation of other prions. The function of the non-prion state of Rnq1 is unclear, but a prion conformational change enhances [PSI+] generation (Stein, True 2011). [RNQ+] variants induce [PSI+] at high frequencies. It is hypothesized that the presence of an efficient [RNQ+] template primes the cell for [PSI+] formation by overproducing Sup35p and its self-propagating amyloid of Rnq1p (Derkatch et al. 1997).

These amyloids states of the yeast protein are important for epigenetic mechanisms of inheritance (Shorter, Lindquist 2005). The self-propagating protein allows for inheritable phenotypes to promote survival in wild yeast strains under different growth conditions (Halfmann et al. 2012).

2. **Als proteins (adhesins)**

The cell surface of *Candida albicans* has agglutinin-like sequences (Als) adhesins that mediate adhesion, a critical first step in infection. *Candida albicans* is an opportunistic pathogen that causes candidiasis in immunocompromised people such as infants, elderly and HIV patients. There are eight members of the Als proteins: Als1-7 and Als9 (Hoyer 2001, Zhao et al. 2004). *ALS* genes are expressed in different growth phases of *C. albicans*. Als1p is expressed maximally when an inoculum of cells is placed into fresh yeast-peptone-dextrose media, and Als3p expression is maximal when germ tubes appear (Zhao et al. 2004). Als5p is expressed in some strains of *C. albicans* and not in others (Hoyer 2001, Hoyer, Hecht 2000). Als proteins functions include adhesion to various substrates such as fibronectin,
laminin, type IV collagen, gelatin, and host cells (Sheppard et al. 2004, Zhao et al. 2005).

Als proteins have an N-terminal secretion signal sequence that is cleaved, immunoglobulin (Ig) -like N terminal domains, a conserved threonine rich region (T), a mid-region containing threonine-rich tandem repeats (TR), a long Ser/Thr-rich glycosylated stalk, and a C-terminal glycophosphatidylinositol (GPI) anchor (Hoyer 2001, Gaur, Klotz 1997, Dranginis et al. 2007) (Figure 1). During cell wall biogenesis, the GPI anchor is cleaved in the glycan, and the remnant covalently attached to cell wall polysaccharide (Gonzalez et al. 2010).

The different domains found in the Als protein family perform various functions. S. cerevisiae cells expressing Als5p protein have the ability to adhere to ECM proteins, human cells, and heat denatured-bovine albumin serum-coated beads (Gaur, Klotz 1997a, Rauceo et al. 2004). The N-terminus of the adhesin is Ig-like because it can bind peptides with broad specificity and structurally similar to immunoglobulins (Salgado et al. 2011). Within a cavity is the region where peptides interact with the hydrophobic residues. In the absence of peptides the N-terminus can recognize their own C termini as ligands (Salgado et al. 2011). The TR domain of the Als proteins is highly conserved. Ab initio modeling show there are three-stranded antiparallel β-sheet domains in the tandem repeat region of yeast adhesins (Frank et al. 2010). In Als5p, the TR domain consists of six copies of a 36-mer repeat. Protein constructs that lack the TR region showed a decrease in binding affinity to fibronectin than constructs with the six copies of the repeat (Rauceo et al. 2006).
Within the mid-threonine rich region of many adhesins including Als5p is a 5 amino acid sequences predicted by Tango (http://tango.crg.es/) to form amyloids (Otoo et al. 2008, Ramsook et al. 2010, Fernandez-Escamilla et al. 2004). The Tango predicted sequence is IVIVA. S. cerevisiae expressing Als5p is positive for amyloid properties. These include binding thioflavin T and S, a red shifted absorbance in the presence of Congo red, and apple-green birefringence under crossed polarized light. The soluble protein forms amyloids fibrils under electron microscopy, and has a CD spectrum characteristic of β-sheets (Rauceo et al. 2004, Garcia et al. 2011, Gilchrist, Bradshaw 1993).

Amyloids play an important role on the cell surface of yeast, mediating adhesion, nanodomain formation and biofilm formation. In the presence of amyloid-perturbing dyes Congo red (CR), thioflavin S (ThS) and thioflavin T (ThT), Als5p mediated adhesion was inhibited (Garcia et al. 2011, Ramsook et al. 2010). To further look at the role of amyloids in adhesion, an amyloid mutant of Als5p was made with site-directed mutagenesis. A valine at the 326th position was mutated to asparagine (Garcia et al. 2011). A single site mutation in the amyloid region of Als5pV326N abrogated amyloid formation (Garcia et al. 2011). Cells expressing the V326N mutant protein exhibit a decrease in aggregation. Cells expressing wild type Als5p form an overnight biofilm on polystyrene. Amyloid mutant Als5pV326N cells had a decrease in biofilm formation on polystyrene (Garcia et al. 2011). The amyloid mutant also shows a decrease in fluorescence of the amyloid-reporting dye thioflavin T (Garcia et al. 2011). Synthetic anti-amyloid peptide V326N also decreased the aggregation of
wild-type Als5p expressing cells. Amyloid sequence IVIVA plays a functional role in Als5p-mediated adhesion and biofilm formation.

The amyloid-forming sequence of Als5p also mediates the formation of clusters of Als5p, nanodomains, on the cell surface (Alsteens et al. 2010, Garcia et al. 2011). V5-epitope tagged Als5p-expressing cells were immobilized onto a membrane and specific detection of Als5p was made with an atomic force microscope (AFM) tip derivatized with anti-V5 antibodies (Alsteens et al. 2010). Force maps show an evenly distributed Als5p molecules on the cell surface when the AFM tip probes an undisturbed target area. However, when that same area was reprobed with the AFM tip the protein density increased 1.55 fold and was no longer evenly distributed (Alsteens et al. 2010). Clusters of Als5p formed ranging in size from 100-500 nm, were called forced-induced nanodomains. Similarly, nanodomains were observed in the remote areas similar to the ones formed in the target area, indicating mechanical stimuli from the pull of an AFM tip resulted in the formation of Als5p clusters across the entire cell surface. The clustering of the Als5p on the cell surface was abolished with the V326N single site mutation. Als5p cells clustered, stained with thioflavin-T and have surface amyloid nanodomains. The aforementioned characteristics were abolished with the nonamyloid mutant-expressing cells (Garcia et al. 2011).

The amyloid sequence of Als5p has a functional role. It mediates cell-to-cell binding, and cell-to-substrate binding. It plays a role in biofilm formation. It also mediates force-induced clustering of Als5p molecules on the cell surface.
C. Structure and properties of amyloid

Amyloids are highly-ordered protein aggregates with filaments that are β-sheet rich in secondary structure. The highly ordered cross-β structures form what is called a cross β-fold where the β strands are aligned perpendicular to the fibril axis (Dobson 1999, Pedersen, Otzen 2008, Sipe, Cohen 2000) (Figure 2). Amyloid formation results from the breaking of intramolecular interactions as a result of local or global unfolding of proteins (Salvatella 2013). The conformational changes expose hydrogen bonds and hydrophobic residues. These elements form intermolecular bonds and aggregate the protein. Hydrogen bonding between β-strands stabilizes the formation of amyloid fibers (O’Nuallain et al. 2004, Pulawski et al. 2012, Petkova et al. 2002). The resulting stable fibers are insoluble in detergents, bind to specific thioflavin dyes and Congo red, and are resistant to proteolytic activity (Chiti, Dobson 2006). These amyloid fibers can be formed from proteins or small peptides after nucleation step.

The binding of small molecules such as Congo red and thioflavin to amyloids has been well studied and used to characterize amyloid proteins. The β-sheet secondary structure of amyloids has a strong affinity for the dye Congo red. Congo red bound to amyloids result in a characteristic apple-green birefringence under polarized light (Howie et al. 2008, Howie, Brewer 2009, Howie, Owen-Casey 2012). Simulations show that the dye binds to one site parallel to the fibril axis (Klunk, Debnath & Pettegrew 1994) and protofibrils (Wu et al. 2008).

Thioflavin T is another small molecule that binds to amyloids. It is a benzothiazole-based dye (Naiki et al. 1989, Vassar, Culling 1959) which results in an increase in fluorescence when bound to amyloids (LeVine 1993). Naiki et al. 1959 noted that upon binding to the fibrils there is a shift in the excitation maximum from 385nm to 450nm and in emission maximum from 445nm.
to 482 nm. Thioflavin T acts as a molecular rotator in solution with the benzylamine and benziathiole rings freely rotating therefore quenching benziathiole fluorescence. When thioflavin T is bound to amyloid fibrils, free rotation is restricted and therefore results in an increase in fluorescence (Stsiapura et al. 2007, Stsiapura et al. 2008).

**D. Prediction of amyloid-forming sequences using programs Tango, Waltz, Aggrescan, FoldAmyloid, and ZipperDB**

A wide array of proteins with various sequences can adopt an amyloid fold. The amino acid sequence of a protein determines the structural conformation. Predicting the propensity of a protein to aggregate from its primary structure is important for studying the deposit of unwanted protein in disease states. Proteins rich in hydrophobic, aromatic, and β-branched amino acids have a high amyloid-forming propensity (Pawar et al. 2005). With an emerging importance of amyloids in natural roles, it is important to have the ability and ease of predicting amyloid forming sequences in proteins. There are many amyloid prediction programs available on the internet that is free to use. All one has to do is input the amino acid sequence and with a push of a button, a profile of amyloid-forming sequences will be highlighted in the protein. Examples of amyloid-predicting algorithms and software available to predict the aggregation potential of your favorite proteins include Tango, Waltz, Aggrescan, FoldAmyloid and ZipperDB. Some of these programs try to differentiate amyloid fibrils predictions from amorphous aggregate predictions (Hamodrakis 2011).

**i. Tango**

Tango (http://tango.crg.es/) is a statistical algorithm that predicts the propensity for β sheet-aggregation in peptides and proteins. The tendency for β sheet-aggregation is
calculated from three energy contributions. The three energy contributions are from the entropic energy of immobilizing residue i in a β-strand and side chain-side chain interactions of residue i with residues at positions i+1 and i+2 (Fernandez-Escamilla et al. 2004, Linding et al. 2004, Rousseau, Schymkowitz & Serrano 2006). A peptide will have aggregation tendency if there are at least five consecutive residues fixed in the β sheet-aggregated conformation. The main secondary structure of the amino acid sequence is assumed to be β-strand. Tango takes into account the effects of pH, ionic strength and temperature of the protein.

Tango positive amyloid predictions are found in many C. albicans adhesins and are also present in S. cerevisiae adhesins (Ramsook et al. 2010). These predictions have been shown to be true in adhesins expressed by yeast. The Als family of proteins has amyloid positive sequences as predicted by Tango. These sequences are threonine, valine and isoleucine rich. In Als1p and Als5p, the predicted amyloid sequence is IVIVA. These proteins form amyloid fibrils under electron microscopy (Ramsook et al. 2010). A mutation in the amyloid forming region of Als5p as predicted by Tango or treatment with anti-amyloid dyes resulted in decrease adhesion function of the protein (Ramsook et al. 2010, Alsteens et al. 2010, Garcia et al. 2011).

**ii. Waltz**

Another program that is able to predict amyloid aggregation is the Waltz algorithm (http://waltz.switchlab.org/). This tool works by using a position-specific scoring matrix of the amino acids in predicting amyloid forming sequences (Maurer-Stroh et al. 2010). Waltz program is able to distinguish amyloid-forming sequences from amorphous β-sheet
aggregates. Waltz predicted short peptides of 5 or 6 residues in length (HYFNIF, RVFNIM, VIYKI), to form amyloid-like fibrils as seen with transmission electron microscopy (Morris et al. 2013). Fourier transform infrared spectroscopy absorption bands of 1626-1633 and 1666-1676 cm\(^{-1}\) were observed for the three peptides that was consistent with β-sheet structure.

iii. Aggrescan

Aggrescan (http://bioinf.uab.es/aggrescan/) predicts the hotspots for aggregation in a polypeptide sequence (Conchillo-Sole et al. 2007). These calculations look at the aggregation-propensity values per amino acid in the sequence over a sliding window of 5, 7, or 11 residues (Conchillo-Sole et al. 2007). The program Aggrescan has validated the already amyloid folding regions in Aβ-42 peptide at residues 17-22 (de Groot et al. 2006, Conchillo-Sole et al. 2007). The Aβ-42 core which contains the leu17-val18-phe19-phe20-ala21 is important for peptide aggregation (de Groot et al. 2006). The wild-type peptide has a 220 nm minimal in the CD spectra. A substitution of F19D resulted in CD spectra absent of the signature dip (de Groot et al. 2006). The mutant exhibited a decrease in thioflavin T binding and lack of fibril formation on electron micrograph images (de Groot et al. 2006). Biofilm associated proteins present from Staphylococcus species, Acinetobacter baumanii and Salmonella enterica have amyloidogenic peptides as predicted by Aggrescan. The peptides increase in thioflavin T fluorescence and fiber formation (Lembre, Vendrely & Martino 2014).
iv. FoldAmyloid

FoldAmyloid (http://bioinfo.protres.ru/fold-amyloid/oga.cgi) predicts amyloid folding potential based on its amino acid sequence. The amylogenic regions of a protein are predicted on the basis of packing densities (contacts) or probability of forming hydrogen bonds for each residue (Garbuzynskiy, Lobanov & Galzitskaya 2010, Galzitskaya, Garbuzynskiy & Lobanov 2006). Regions that have a high probability of forming backbone-backbone hydrogen bonds as well as regions with high packing densities are responsible for fibril formation.

v. ZipperDB

ZipperDB (http://services.mbi.ucla.edu/zipperdb/) is an amyloid prediction database that computes the propensity of amyloids fibril formation based on crystal structure of the peptide NNQQNY from Sup35p prion protein (Nelson et al. 2005). This database analyzed many protein sequences with high fibrillation propensity to form a “steric zipper” which is two self-complementary β-sheets (Thompson et al. 2006). The β-sheets for the base structure of the protofibril with the side chains interdigitate like a zipper. The resulting predicted hexapeptide segments of the protein conform to the Rossetta design energy contributions calculated from hydrogen bonds, and steric overlaps. Protein sequences from lysozyme, αβ-42 and tau had predicted amyloid fibril forming sequences that was consistent with what is already known.

Amyloid forming sequences in proteins can be predicted with readily available online programs. The amyloids that were predicted by these programs show experimental data that are
characteristics of amyloids. These include amyloid dye thioflavin T binding and the formation of amyloid fibers under electron microscopy.
E. Aim of Thesis Research

Amyloids are ubiquitous and play a functional role in mediating adhesion in Als5p. In this study, we look to find similar behavior of amyloids in Als5 and in unrelated flocculins Flo1p and Flo11p. Similar to Als5p, flocculins Flo1p and Flo11p have amyloid predicted sequences by the program Tango. We look to see if force can be used to activate adhesin mediated aggregation in an amyloid dependent manner. Flocculation increased when cells are exposed to the force from vortex-mixing and shear from laminar flow. Since force is important for aggregation and adhesion and the first step of biofilm formation entails that, we look to see how force plays a key role in the formation of biofilm formation.
G. Figures

**Figure 1. Domains of Als adhesins and flocculins.** The tandem repeat region of the proteins is predicted with Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html). The GPI anchor is predicted with PredGPI (http://gpcr.biocomp.unibo.it/predgpi/). Amyloid sequences are in red predicted by Tango (http://tango.crg.es/).
**Figure 2. Model for amyloid formation.** In parallel β–strands form continuous β–sheets. Amyloids are formed from the interaction of the β–sheets perpendicular to the growing axis. Adapted from (Shewmaker, McGlinchey & Wickner 2011).
Chapter II

Functional role of amyloids in yeast flocculins Flo1p and Flo11p
A. Introduction

Yeast cell surface adhesins mediate cell-to-cell aggregation and cell-to-surface adhesion, including for example, the Candida albicans adhesin Als5p (Gaur, Klotz 1997) and Saccharomyces cerevisiae flocculins Flo1p and Flo11p (Dranginis et al. 2007). Although many yeast adhesins have little or no homology, they have similar architecture: an N-terminal secretion signal sequence, a globular ligand binding region, a mid-region containing threonine-rich tandem repeats (which are not homologous between the proteins), a long Ser/Thr-rich glycosylated stalk, and a C-terminal GPI anchor (Figures 1 and 3) (Dranginis et al. 2007). During cell wall biogenesis, the GPI anchor is cleaved in the glycan, and the remnant covalently attached to cell wall polysaccharide. Both the flocculins Flo1p and Flo11p share these similar regions within the protein.

The mid-region of Als5p has a 5-amino acid sequence predicted by Tango (http://tango.crg.es/) to form amyloids (Otoo et al. 2008, Ramsook et al. 2010, Fernandez-Escamilla et al. 2004). This sequence is IVIVA has a 93% propensity for β-aggregation. Because of the similarity in structure and function of flocculins to Als5p we looked to see if there are amyloid-forming sequences and if whether these amyloids play a functional role as well.
B. Materials and Methods

Strains and media

*Saccharomyces cerevisiae* variant *diastaticus* *MATα* {*ura3 leu2-3, 112 his4*} expressing Flo11p and the deletion strain *MATα* {*ura3 leu2-3, 112 his4 flo11:URA3*} were kindly gifted by Dr. Anne Dranginis (St. John’s University). Cells were grown in yeast-extract-dextrose (YPD) at 30\(^\circ\)C at 170 rpm for 24 hours.

*Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα* {*FLO1 gal1*} was purchased from ATCC (Manassas, VA). Cells were grown in YPD at 30\(^\circ\)C at 170 rpm for 24 hours.

*Saccharomyces cerevisiae* W303-1B strain *MATα* {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15*} was grown in YPD at 30\(^\circ\)C at 170 rpm for 24 hours.

Flocculation assays. Assays for flocculation mediated by Flo1p and Flo11p were carried out as described by Lo and Dranginis, using cells prewashed with EDTA to inhibit flocculation before assay. Flo11p cells were washed three times and resuspended in 20 mM sodium acetate buffer with 1 mM EDTA, pH 5.5. Flo1p-expressing cells were resuspended in 20 mM sodium acetate buffer with 200 µM EDTA, pH 5.5. Flocculation was initiated by addition of 0.67 mM CaCl\(_2\) for Flo1p expressing cells and 0.33 mM CaCl\(_2\) for Flo11p expressing cells. The cell suspension was vortexed for 10 s, and the optical density at 600 nm was monitored at 5-s intervals for the first 2 minutes and then every 30 s for 3 minutes in a Spectronic 20 D+ spectrophotometer.

Amyloid-binding dyes assays. Amyloid dyes were added to Flo1p and Flo11p-expressing cells before the addition of CaCl\(_2\). 1.0 mM ANS, 0.5 mM CR, 0.190 mM ThS, and 5 mM ThT were used. A dose response for different concentrations of ThS and CR were used to determine the
half maximal inhibitory concentration of flocculation. Cells were vortex-mixed on a low setting for 10 seconds to suspend the dyes with the cells, then CaCl₂ was added to the cell suspensions and OD₆₀₀ₙₘ was monitored.

**Thioflavin T fluorescence assays.** Cells and cellular aggregates were treated with thioflavin T at 30 µM in sodium-acetate buffer (20mM each, pH 5.5), washed twice in the same buffer, and observed at 480 to 540 nm, with excitation at 425 to 440 nm.

**Birefringence of cells expressing flocculins.** *S. cerevisiae* cells were analyzed between polarizing filters with a 20× objective under bright-field conditions. Flocculation was initiated by addition of CaCl₂ as described above.

**Growth inhibition assay.** Serial dilutions of the indicated strains were grown on the indicated agar plates. Plates were made with 1% yeast extract, 2% dextrose, 1% peptone, 40mg/L of adenine, and 2% agar. A final concentration of 186 µM ThS, 30 µM CR, and 5 mM ThT was added. Images were taken of the plates after 3 days.

**Agar invasion assays.** Agar plates were prepared as mentioned above with yeast extract + peptone + dextrose + adenine. A final concentration of 200 µM ThS or 30 µM CR was added to the agar suspension before cooling. The plates were poured in sterile conditions and allowed to cool overnight. A 50 µl of 1 million cells were plated in the center of the plate. The plate was set in a 30°C incubator and images were taken after 14 days. Images were taken before washing and after washing of the colony.
C. Results

**Amyloid sequences in flocculins.** The protein sequences of Flo1p and Flo11p were analyzed with Tango and each was predicted to have several sequences with high propensity for β–aggregation (Table 1). These predicted β–aggregation sequences are rich in β–branched aliphatic amino acids Ile, Thr, and Val, similar to the amyloid-forming sequence of Als5p. A soluble form of Flo11p purified from *S. cerevisiae* forms amyloid fibers, has increased thioflavin T fluorescence, and increased absorbance and red-shifted Congo red assays (Ramsook et al. 2010). The synthetic peptides of Flo1p also have a circular dichroism spectra characteristic of beta-aggregation, and Congo red absorbance was slightly higher and red-shifted (Ramsook et al. 2010). Thus flocculins have Tango predicted high β–aggregation sequences that are characteristic of amyloid proteins.

There are numerous amyloid sequences in flocculins. There are 22 Tango positive amyloid sequences in Flo1p and 3 in Flo11p (Figure 3). The Tango positive sequences of Flo11p are found in the N-terminus, whereas the Tango positive sequences for Flo1p are found throughout the whole protein. There are 4 Waltz positive sequences in Flo1p and 7 in Flo11p. These sequences are found near both the N and C terminus. In Flo1p, a majority of the amyloid sequences are found in the repeat region of the protein, and the amyloid sequences TIIVI are repeated 9 times and TVIVI repeated 7 times. There are no amyloid positive sequences in the repeat region of the Flo11p. In Flo1p, there is one amyloid sequence TVVTI at position 1299 that is both Tango and Waltz positive.

**Cell adhesion amyloids in vivo.** To look for amyloids *in vivo*, we used intact cells expressing Flo1p and Flo11p. On the cell surface of these yeast cells we saw birefringence which
is a characteristic of ordered structures like amyloids (Figure 4). Cells expressing the flocculin, Flo1p were slightly more birefringent than W303-1B which is not expressing any flocculin. The adhesin-expressing cells became more birefringent after flocculation was initiated with the addition of Ca$^{+2}$. In cells that were expressing Flo11p, birefringence was also increased after flocculation was initiated with the addition of Ca$^{+2}$. This increase in birefringence indicates an ordered amyloid cell surface as a result of the presence of flocculin molecules similar to that was seen with Als5p-expressing cells (Rauceo et al. 2004).

Amyloids bind thioflavin T, and increase its fluorescence, but the dye does not inhibit amyloid formation at low concentrations (Eisert, Felau & Brown 2006). Since there are Tango-predicted amyloid forming sequences in Flo1p and Flo11p, we therefore wanted to see if there was cell surface fluorescence in the presence of thioflavin T. Intact yeast cells expressing the flocculins were stained with thioflavin T and inspected with fluorescence microscopy (Figure 5). Thioflavin T stained the cells, and the staining was brighter for the Ca$^{+2}$-induced flocculating cells than for the nonflocculating samples. The flocculin-expressing cells were more fluorescent than cells that were not either flocculins (Figure 5) (Ramsook et al. 2010). Therefore, expression of Flo1p or Flo11p lead to greater ThT binding, indicating amyloid is present on the cell surface.

Effects of amyloid-binding dyes on cellular aggregation. We looked to see whether the predicted amyloid-forming sequences play a functional role in intact yeast cells. We tested the effect of amyloid-binding dyes on flocculation of *S. cerevisiae* expressing Flo1p or Flo11p. These adhesin expressing cells flocculate or form large aggregates in the presence of Ca$^{+2}$ ions (Dranginis et al. 2007, Lo, Dranginis 1998, Lo, Dranginis 1996). Congo red binds to and disrupts amyloids (Feng et al. 2008); therefore we tested whether this dye can inhibit flocculation. Inhibition of Flo1p or Flo11p mediated flocculation in the presence of Congo red was seen at
concentrations as low as 30 µM, with the half-maximal inhibition at 0.5mM (Table 2). Thioflavin S was similarly potent, and completely inhibited the flocculation reaction for both proteins (Table 2 and Figures 6 and 7). Both Congo red and thioflavin S reduced both the rate at which the cells flocculated (the initial slope) and the amount of flocculation (final decrease in OD$_{600\text{nm}}$) (Table 2 and Figures 6 and 7). The half-maximal inhibitory concentrations of thioflavin S were 45 µM for Flo11-mediated flocculation and 100-200 µM for the Flo1p-mediated reaction (Figures 6 and 7 and Table 2). 1-Anilinonaphthalene-8-Sulfonic Acid (ANS) had little effect on flocculation, and high concentrations of thioflavin T mediated a more rapid and extensive aggregation (Table 2). Adhesins Flo1p and Flo11p mediate the function of flocculation, and in the presence of amyloid-binding dyes this was inhibited. This shows that amyloids play an important role in mediated adhesion of yeast cells. It is not understood why thioflavin T at high concentrations potentiated flocculation.

Congo red has an effect on the yeast cell wall assembly, because the dye interferes with the formation of polysaccharide fibrils and chitin synthase (Kopecka, Gabriel 1992, Roncero, Duran 1985). It was unlikely that the effect of Congo red was due to cell wall perturbation because the effective inhibitory concentrations for flocculation were low and the dyes were present only during the flocculation assay itself, and not during wall biogenesis. To see whether Congo red and thioflavin dyes have an inhibitory effect on flocculation and not on growth, cells were grown in the presence of the two dyes. 30 µm Congo red and 5 mM ThT inhibited growth of the flocculating strains in cell dilution grown assays (Figure 8) (Ramsook et al. 2010). In contrast, 200 µm Thioflavin S was not growth inhibitory (Figure 8). Therefore, thioflavin S did not affect growth but had potent antiflocculation effects for interactions mediated by Flo1p and Flo11p. In general, there was no correlation between growth inhibition of the tested dyes and
their effects in flocculation assays: some dyes were cytotoxic but did not inhibit flocculation, and another inhibited flocculation but was not cytotoxic.

**Agar invasion.** A phenotype exhibited by the expression of Flo11p on the cell surface of yeast cells is the ability to confer agar invasion (Lo, Dranginis 1996). We therefore wanted to test whether amyloids play a functional role in the invasive properties of the flocculins Flo1p and Flo11p. Agar plates were prepared in the presence amyloid dyes 30 µM Congo red and 200 µM thioflavin S. The concentrations of dyes used did not affect the growth of the yeast cells in these assays because the colony size was the same after 2 week growth (Figure 9). Cells expressing flocculins Flo1p or Flo11p after washing of the colony after two weeks of growth on the plates have invasion inclusions in the agar (Figure 10). A Δflo11 mutant did form very small inclusions, and W3031B had inclusions but not as much as the flocculin-expressing cells. In the presence of Congo red the size and the number of invasion inclusions decreased. Thioflavin S was the most potent in preventing invasion; no strains showed any inclusions. Amyloid dyes Congo red and thioflavin S prevented the yeast cells from invading into the agar (Figure 10). Thus, Congo red and thioflavin S were able to inhibit the inclusions of yeast cells in the agar.
D. Discussion

Our results support a functional role of amyloid formation in yeast cell adhesion mediated by flocculins. Yeast cells show surface birefringence and binding of thioflavin T, both characteristics of amyloids (Teertstra et al. 2009). Flo1p and Flo11p expressing cells have both characteristics. Surface birefringence and thioflavin T staining increased in aggregated cells relative to nonaggregated cells.

We also show that amyloid binding dyes inhibited or potentiated aggregation. Thioflavin S was very potent at inhibiting aggregation at µM concentrations and was not toxic or growth inhibitory to the cells. Thioflavin T, which is used to monitor amyloid formation because it often does not inhibited amyloid formation (Eisert, Felau & Brown 2006, Feng et al. 2008), actually potentiated flocculation at high concentrations. Therefore, amyloid-binding compounds affected the aggregation reactions at low concentrations and can have potential future therapeutic consequences.

Here we demonstrated that flocculins Flo1p and Flo11p contain amyloid-forming sequences that are Ile, Val, Thr rich in composition. The sequences form amyloids under native conditions at low concentrations. Flo1p and Flo11p, the amyloids play a functional role in mediating flocculation, and amyloid inhibitors decreased aggregation. Amyloid formation and having a functional role is widespread and is a mechanism for cell-to-cell interaction and are well characterized role in bacteria (Barnhart, Chapman 2006, Blanco et al. 2012, Fowler et al. 2007). We show for the first time that amyloids are also present in baker’s yeast and mediate adhesion.
E. Tables

Table 1. Amyloid positive sequences from *S. cerevisiae* adhesins. Tango was used to predict the amyloid-forming sequences in Flo1p and Flo11p. The settings were at pH of 5.5, temperature of 298.15°K, ionic strength of 0.02, and with no N-terminal and C-terminal protection. The sequence is ran with a 100 amino acid overlaps. Flo1p NCBI accession number NP_009424. Flo11p GenBank number ABS87372.1. Sequences are predicted by Tango of >30% are included.

<table>
<thead>
<tr>
<th>Protein</th>
<th>β-aggregation sequence</th>
<th>β-aggregation (%)</th>
<th>Ile, Val and Thr content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flo11p</strong></td>
<td>VTTVVSTTVV</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>IITTFV</td>
<td>53</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>LVTTAVTTTVV</td>
<td>90</td>
<td>82</td>
</tr>
<tr>
<td><strong>Flo1p</strong></td>
<td>VYMYAGY</td>
<td>32</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>TVIVI</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TIIVI (repeat 9 times)</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TVIVI (repeat 6 times)</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TVIVV</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TLVTVT</td>
<td>37</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>AIVSTATVTVT</td>
<td>48</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>TVVVTI</td>
<td>39</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TLVTVT</td>
<td>41</td>
<td>83</td>
</tr>
</tbody>
</table>
Table 2. Effect of amyloid-binding dyes on flocculation of Flo1p- and Flo11p-expressing S. cerevisiae cells. Saccharomyces cerevisiae variant diastaticus MATa {ura3 leu2-3, 112 his4} expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University).

Saccharomyces cerevisiae Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). Flocculation assays were carried out as described by Lo and Dranginis, 1996 with modifications. Briefly, $3 \times 10^7$ cells/ml were prewashed with EDTA to inhibit flocculation before assay. Flocculation was initiated by addition of CaCl₂, and the suspension was vortexed for 5 s, the optical density at 600 nm (OD₆₀₀) was monitored at 5-s intervals in a Spectronic 20 D+ spectrophotometer.

<table>
<thead>
<tr>
<th>Dye and flocculin expressed</th>
<th>Rate of flocculation (% of control)</th>
<th>Extent of flocculation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS (1.0 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flo1p</td>
<td>109</td>
<td>107</td>
</tr>
<tr>
<td>Flo11p</td>
<td>205</td>
<td>122</td>
</tr>
<tr>
<td>Congo red (0.50 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flo1p</td>
<td>49</td>
<td>59</td>
</tr>
<tr>
<td>Flo11p</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Thioflavin S (0.19 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flo1p</td>
<td>49</td>
<td>45</td>
</tr>
<tr>
<td>Flo1p</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Thioflavin T (5 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flo1p</td>
<td>384</td>
<td>239</td>
</tr>
<tr>
<td>Flo11p</td>
<td>227</td>
<td>191</td>
</tr>
</tbody>
</table>
F. Figures

Figure 3. Domains and amyloid forming sequences of Flo1p and Flo11p. The tandem repeat region of the proteins is predicted with Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html). The GPI anchor is predicted with PredGPI (http://gpcr.biocomp.unibo.it/predgpi/). Amyloid sequences are in red predicted by Tango (http://tango.crg.es/), and purple by Waltz (http://waltz.switchlab.org/). N-glycosylation was predicted with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/)
Figure 4. Birefringence of cells expressing Flo1p and Flo11p. *Saccharomyces cerevisiae* variant *diastaticus* *MATa* {ura3 leu2-3, 112 his4} expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα* {FLO1 gal1} was purchased from ATCC (Manassas, VA). *Saccharomyces cerevisiae* W303-1B strain *MATα* {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15} was purchased. Yeast cells were analyzed between polarizing filters with a 20X objective. The outer images between each pair are the bright field images and the center images are the crossed polarized images. The left pair is taken in the absence of Ca$^{+2}$ and the right in the presence of 0.67mM Ca$^{+2}$.
Figure 5. Thioflavin T fluorescence of cells expressing flocculins Flo1p and Flo11p.

*Saccharomyces cerevisiae* variant *diastaticus MATα {ura3 leu2-3, 112 his4}* expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα {FLO1 gal1}* was purchased from ATCC (Manassas, VA). *Saccharomyces cerevisiae* W303-1B strain *MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15}* was purchased. Cells were stained with thioflavin T and then visualized with bright field and fluorescence microscopy. Flocculation was induced with the addition of 0.67mM Ca$^{+2}$. 

![Image of fluorescence microscopy](image-url)
Figure 6. Amyloid dye thioflavin S inhibits flocculation of Flo11p- and Flo1p-expressing cells. *Saccharomyces cerevisiae* variant *diastaticus MATα {ura3 leu2-3, 112 his4}* expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα {FLO1 gal1}* was purchased from ATCC (Manassas, VA). Cells were treated with different concentrations of thioflavin S and then flocculation was initiated with the addition of calcium. (A) Flo11p-expressing cells. (B) Flo1p-expressing cells.
Figure 7. Flocculation rates of Flo11p- and Flo1p-expressing cells. *Saccharomyces cerevisiae* variant *diastaticus MATa {ura3 leu2-3, 112 his4}* expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα {FLO1 gal1}* was purchased from ATCC (Manassas, VA). Cells were treated with different concentrations of thioflavin S and then flocculation was initiated with the addition of calcium. (A) Flo11p-expressing cells. (B) Flo1p-expressing cells.
**Figure 8. Growth inhibition assay.** *Saccharomyces cerevisiae* variant *diastaticus MATa* \{ura3 leu2-3, 112 his4\} expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα* \{FLO1 gal1\} was purchased from ATCC (Manassas, VA). *Saccharomyces cerevisiae* W303-1B strain *MATα* \{leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15\} was purchased. Serial dilutions of Flo1p and Flo11p expressing cells grown in YPD and YPD + 200 μM ThS.
Figure 9. Growth assay for agar invasion assays. *Saccharomyces cerevisiae* variant diastaticus MATα {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain MATα {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). *Saccharomyces cerevisiae* W303-1B strain MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} were purchased. Cells were grown in the presence of dye or without dye for 2 weeks. The images were taken with an inverted dissecting microscope at 15X total magnification. The cells were not washed off from the plate.
Figure 10. Agar invasion assays. *Saccharomyces cerevisiae* variant diastaticus MATα {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain MATα {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). *Saccharomyces cerevisiae* W303-1B strain MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15} was purchased. Cells were grown on agar plates with or without dye. The cells were grown for 2 weeks and then washed. The images were taken with an inverted dissecting scope at 15X total magnification.
Chapter III

Role of force-sensitive amyloid-like interactions in fungal catch-bonding and biofilms
A. Abstract

The *Candida albicans* Als adhesin Als5p has an amyloid-forming sequence that is required for aggregation and formation of model biofilms on polystyrene. Because amyloid formation can be triggered by force, we investigated whether laminar flow could activate amyloid formation and therefore increase binding to surfaces. Shearing *Saccharomyces cerevisiae* cells expressing Als5p or *C. albicans* at 0.8 dyne/cm$^2$ increased quantity and strength of cell-to-surface and cell-to-cell binding, compared to 0.02 dyne/cm$^2$. Thioflavin T fluorescence showed that the laminar flow also induced adhesin aggregation into surface amyloid nanodomains in Als5p-expressing cells. Inhibitory concentrations of amyloid-dyes thioflavin S, Congo red, or a sequence-specific anti-amyloid peptide decreased binding and biofilm formation under flow. Shear-induced binding also led to formation of robust biofilms. There was less shear-activated increase in adhesion, thioflavin fluorescence, and biofilm formation in cells expressing the amyloid-impaired V326N-substituted Als5p. Similarly, *S. cerevisiae* cells expressing Flo1p or Flo11p flocculins also showed shear-dependent binding, amyloid formation, biofilm formation, and inhibition by anti-amyloid compounds. Together these results show that laminar flow activated amyloid formation and led to enhanced adhesion of yeast cells to surfaces and to biofilm formation.
B. Introduction

Biofilms are communities of microorganisms that form on surfaces. They are ubiquitous and exist in locations as diverse as the mouth, on indwelling catheters, and in fast-moving streams (Hall-Stoodley, Costerton & Stoodley 2004, Stewart, Franklin 2008, Vlamakis et al. 2013). Biofilms influence the spread of infections and can clog medical tubing. In formation of biofilms, adherence of microbes to a surface is followed by cell division and/or capture of free flowing microbes into the growing biofilm, and production of an extracellular matrix of macromolecules. There are functional amyloids present in biofilms made by bacteria and yeast (Blanco et al. 2012, Otzen, Nielsen 2008, Larsen et al. 2007, Garcia et al. 2011). These functional amyloids play roles in cell adhesion and in biofilm matrices (Otzen, Nielsen 2008, Romero et al. 2010, Blanco et al. 2012).

In yeasts, adherence to substrate and cell-to-cell aggregation is mediated by cell surface glycoprotein adhesins. The Candida albicans Als adhesins and the Saccharomyces cerevisiae Flo flocculins are examples of adhesins that have little or no homology, but they have similar architecture. Each has an N-terminal secretion signal sequence, a globular ligand-binding region, a mid-region containing threonine-rich tandem repeats (which are not homologous between the proteins), a long Ser/Thr-rich glycosylated stalk, and a C-terminal GPI anchor (Dranginis et al. 2007). During cell wall biogenesis, the GPI anchor is cleaved in the glycan, and the remnant covalently attached to cell wall polysaccharide (Gonzalez et al. 2010). Within the mid-regions of Als5p, Flo1p, and Flo11p are 6-7-amino acid sequences predicted by Tango to form amyloids (http://tango.crg.es/) (Otoo et al. 2008, Ramsook et al. 2010). The amyloid sequence in Als5p is important for cell-to-cell aggregation and cell-to-substrate adhesion (Garcia et al. 2011). A single site amino acid substitution in the amyloid region of Als5p^{V326N} decreases in vitro cell-to-cell
aggregation, cell-to-substrate adhesion, and fluorescence of the amyloid-reporting dye thioflavin T (Garcia et al. 2011). Results with cells expressing the *S. cerevisiae* flocculins Flo1p and Flo11p are consistent with this model: anti-amyloid dyes Congo red and thioflavin S decrease the rate and extent of flocculation of cells expressing either flocculin (Ramsook et al. 2010).

Tensile forces present in the environment often increase the strength of bonds formed between microbes, between microbes and surfaces, and between leukocytes and endothelia. These strengthened bonds, called “catch-bonds,” result in enhanced interactions (Isberg, Barnes 2002, Marshall et al. 2003, Thomas 2008). Cell adhesion proteins such as mammalian selectins and *Escherichia coli* FimH form catch bonds. Leukocytes sheared at 0.3 dyne/cm$^2$ or higher switch from a freely moving state to an immobilized state (Alon et al. 1998) and *E. coli* sheared at 20 dyne/cm$^2$ or higher switch from rolling adhesion to stationary adhesion (Thomas et al. 2004). To our knowledge, catch bonding has not been reported in fungi, so we looked for similar behavior. We have carried out parallel experiments to determine the role of Als5p, Flo1p, and Flo11p, three unrelated adhesins, in fungal biofilm formation. We report here that yeast cell adhesion shows similar behavior, and that such whole-cell catch bonding is dependent on force-sensitive amyloids.
C. Materials and Methods

Strains and media S. cerevisiae strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p^WT or Als5p^V326N were grown in complete synthetic medium (CSM) lacking tryptophan with galactose as carbon source (Garcia et al. 2011). Cells were grown at 24°C at 170 rpm for two days. S. cerevisiae variant diastaticus MATα ura3 leu2-3,112 his4 expressing FLO11 and the deletion flo11 MATα ura3 leu2-3, 112 his4 flo11:URA3 were kindly gifted by Dr. Anne Dranginis (St. John’s University). S. cerevisiae strain BX24-2B FLO1 MATα FLO1 gal1 was purchased from ATCC (Manassas, VA). These cells and C. albicans SC5314 were grown in yeast-extract-peptone-dextrose (YPD) at 30°C at 170 rpm for 24 hours. Overnight cultures of C. albicans SC5314 were diluted 10-fold into fresh YPD and incubated for 45 minutes at 30°C 170 rpm to induce increased expression of Als1p (Coleman et al. 2010, Hoyer et al. 1995a). Yeast were washed 3 times with TE (10 mM Tris with 1 mM EDTA) pH 7.0 and then resuspended in TE at OD_{600nm} of 0.07 before binding to channels in the laminar flow device.

Shear flow experiments Flow experiments were carried out in a Bioflux 200 laminar flow device and cells were visualized on an Olympus inverted phase microscope. 0-20 dyne/cm^2 48-well plates were used at room temperature. In some experiments channels were pre-coated with heat denatured BSA, 1 mg/ml for 1 hour, then washed in TE before introduction of cells. Cell counts were done with Image J software using the cell counter analysis tool. Counts were done for images after 2 hours of shearing, and after 10 minutes of shearing at 20 dyne/cm^2. Each cell was counted whether in a cluster or not. Cells with small buds were counted as one cell. Clusters were binned as 2, 3-9 or >10 cells. Error bars represent s.d. for triplicate determinations.
**2 Hour assays with cells in coated channels.** 850 ul of each strain of cells at 1.4 X $10^6$/ml were pumped from the inlet well of the plate to the outlet well for 2 hours at each respective shear flow into the coated channel surface. These cells were not allowed to settle under stationary conditions. After 2 hours, the flow rate was increased to shear at 20 dyne/cm$^2$ for 10 minutes.

**ThT staining of cells in channels.** Cells were seeded onto the uncoated channel surface from the outlet well. They were allowed to adhere without flow for 15 minutes. TE buffer with 1 uM ThT was added to the inlet well and flowed over the cells at specified rates. Images were taken with a 405/450 nm filter set one per minute for 1 hour. The images shown are negatives.

**Biofilm growth** Cells suspended at 6 X $10^6$/ml were allowed to adhere to non-coated channel surfaces for 15 minutes without flow. Then growth medium was pumped through the channels for 48 hours at the shear stress specified. To assay the effects of anti-amyloid dyes on biofilm growth, 0.2 mM ThS or 10 μM CR was added to the medium during growth.

Peptides were purchased from the Rockefeller University Proteomics facility, and were previously described (Garcia et al. 2011, Ramsook et al. 2010).
D. Results

Effect of shear on cell-to-substrate binding of Candida albicans. The Als adhesins of C. albicans mediate both cell-to-substrate and cell-to-cell binding (Hoyer et al. 2008, Gaur, Klotz 1997b). When an atomic force microscope is used to apply an extending force to cell-surface attached Als5p, the adhesins cluster on the cell surface through amyloid-like interactions (Alsteens et al. 2010, Garcia et al. 2011). Therefore, we determined whether there was similar activation under laminar flow conditions similar to those the fungi encounter in vivo. C. albicans strain SC5314 cells were flowed over the surface of a channel coated with heat-denatured BSA in a laminar flow device. Flow of buffer with the same number of cells (1.4 X 10^6/ml) continued for two hours at rates yielding shear stresses of 0.02-2.6 dyne/cm^2. At the lowest applied force, about 30 cells remained bound within the observed field (Figure 11). When the cells were subjected to higher shear, 0.2 to 1.6 dyne/cm^2, there were an increasing number of cells bound to the surface, up to 18-fold greater (Figure 11). At the highest stress tested, 2.6 dyne/cm^2, few cells remained bound. To make sure that flow rate did not affect the number of cells bound to the surface, the samples sheared at 0.02dyne/cm^2 were monitored for 4 additional hours. There was no increase in the binding of the cells to the channel. Similarly, when the cell density was increased 4-fold to 5.6 X 10^7/ml, there was no significant increase in the number of cells bound at low shear stress (data not shown). Time-lapse analyses showed that cells moved along the substrate under low shear, but not under high shear (Figure 12). These results demonstrated enhanced cell-to-substrate binding under moderate flow.

Shear stress also increased the formation and retention of clusters of cells. Cells subjected to force of 0.2-1.6 dyne/cm^2 formed clusters, with clusters of maximal size formed at 0.8 dyne/cm^2 (Figure 13). At 0.8 dyne/cm^2 there was a slight decrease in the number of cells bound
in comparison to the 0.2 dyne/cm$^2$, but the clusters were larger. Therefore, increased force mediated both cell-to-substrate and cell-to-cell binding. This increased binding under stronger shear is characteristic of catch-bonding.

**Als5p mediates catch-bonding.** *C. albicans* expresses many adhesins, so it is difficult to attribute a specific behavior to any specific cell surface component (Gaur, Klotz 1997b, Lipke et al. 2012, Sheppard et al. 2004). Therefore we studied *C. albicans* Als5p in a *S. cerevisiae* surface-display model. Als5p-expressing *S. cerevisiae* cells were allowed to settle on a surface coated with heat-denatured BSA and sheared at 0.02, 0.8 or 1.6 dyne/cm$^2$ for 2 hrs. Fewer than five Als5p-expressing cells in the whole image field remained on the surface at 0.02 dyne/cm$^2$ after 2 hours, but at 0.8 dyne/cm$^2$ more than $10^3$ cells bound (Figure 11). At 1.6 dyne/cm$^2$ shear, about one third as many cells bound. The bound cells also formed clusters, as the *C. albicans* cells did (Figures 13 and 14). There were more large clusters formed at 0.8 dyne/cm$^2$ than at 0.02 dyne/cm$^2$ and 1.6 dyne/cm$^2$. Cells transformed with an empty vector did not bind well at any shear stress (Figure 11).

After 2 hours, we assessed the resistance to flow by increasing shear stress to 20 dyne/cm$^2$ for 10 minutes (Figure 15). No cells were retained that had been exposed to low shear (0.02 dyne/cm$^2$). About 20% of the cells that adhered at 0.8 dyne/cm$^2$ remained bound after this high-shear washing. Of the cells that were bound after washing at 1.6 dyne/cm$^2$, about 50% remained after washing at 20 dyne/cm$^2$. Therefore expression of Als5p on the surface of *S. cerevisiae* led to stronger binding after the cells had been sheared at moderate stress, *i.e.* catch-bonding behavior similar to that of *C. albicans*. 

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**Catch-bonding increases biofilm formation.** To assess effects of shear on biofilm growth and attachment, Als5p-expressing *S. cerevisiae* cells were allowed to adhere without flow on an uncoated surface, then non-adherent cells were removed, and the adherent cells sheared at 0.01 dyne/cm² or 0.1 dyne/cm² for 48 hours in growth medium. Als5p-expressing cells remained bound to the channel and proliferated into a biofilm. The biofilm was denser and more extensive at the higher shear than at lower shear (Figure 16). The cells transformed with empty vector did not bind well to the channel and did not form a biofilm. Results were similar for *C. albicans*: the biofilm was thicker if the cells were sheared in medium at 0.1 dyne/cm² than when sheared 0.01 dyne/cm² (Data not shown).

**Amyloid dependence of catch-bonding and biofilm formation.** This force-dependent activation prompted us to determine whether shear stress induced increased binding through formation of amyloid nanodomains under laminar flow conditions. Cells expressing the non-amyloid mutant Als5p\(^{V326N}\) have impaired formation of surface amyloid nanodomains after stimulation by AFM, and are poorly activated in aggregation assays (Lipke et al. 2012, Alsteens et al. 2010, Garcia et al. 2011). Such cells did not bind as well to the channels, the clusters that formed were smaller than the Als5p-expressing cells, and the amyloid-impaired mutants were more easily removed by washing at 20 dyne/cm² (Figures 11, 17, 14, and 15). Similarly, cells expressing Als5p\(^{V326N}\) formed biofilms that were sparser and less flow-activated than cells expressing the wild-type Als5p (Figure 16).

If fungal catch-bonding behavior is dependent on formation of surface amyloids, then treatments that disrupt amyloids should inhibit the shear-dependent strengthening of adhesive bonds. The amyloid-perturbing dye thioflavin S (ThS, 0.2 mM) or Congo red (CR, 0.5 mM), was added to the flowing buffer. ThS and CR each inhibited binding of Als5p-expressing cells to
BSA-coated surfaces, with ThS being more effective (Figure 17). Similarly, CR or ThS inhibited binding of _C. albicans_ cells (Figure 17). ThS and CR did not affect binding of cells harboring the empty vector. With cells expressing the amyloid-reduced mutant Als5p\(^{V326N}\), CR and ThS also inhibited the binding to channel surface. Therefore, amyloid-perturbing dyes inhibited the shear-activated adhesion of the cells.

To assay for the effects of amyloid perturbing dyes on biofilm formation, Als5p-expressing _S. cerevisiae_ or SC5314 was seeded onto channel surfaces, then subjected to flow at 0.1 dyne/cm\(^2\) for 24 hours in growth medium with or without 0.2 mM ThS or 10 \(\mu\)M CR. Biofilms developed in the control channels, but not in ThS-treated channels. In channels treated with CR, there was a significant decrease in the size of the biofilm (Figure 18).

**Anti-amyloid peptide decreased cell adhesion.** An anti-amyloid peptide (SNGINIVATTTRTV) specifically inhibits nanodomain formation and activation of adhesion in Als5p-expressing cells (Garcia et al. 2011, Lipke et al. 2012). We tested whether this peptide would inhibit shear-activated binding of cells to the substrate in BSA-coated channels. Als5p-expressing cells were flowed through channels at 0.8 dyne/cm\(^2\) in the absence or presence of the peptide (200 \(\mu\)g/ml). After two hours the cells were sheared at high force (20 dyne/cm\(^2\)). The anti-amyloid peptide reduced both the number of cells bound and the number retained after high shear (Figure 19). A peptide with identical composition but random sequence (VITGVTNIRTSVA) did not prevent attachment or retention of the Als5p cells. For cells expressing amyloid-reduced Als5p\(^{V326N}\), there was reduced attachment to the channel, much less flow resistance, and the two peptides were similar in facilitating removal of the attached cells. Therefore, the anti-amyloid peptide caused sequence-specific inhibition of binding and retention of Als5p-expressing cells.
Effect of shear on surface amyloids. We assayed formation of surface nanodomains by real-time staining with the amyloid dye thioflavin T (ThT). Als5p-expressing S. cerevisiae cells were allowed to adhere to the surface in the laminar flow device, then subjected to shear stress at 0.02 dyne/cm² or 0.2 dyne/cm² in TE containing 1 μM ThT, a concentration that does not affect cellular binding (Garcia et al. 2011). Als5p-expressing cells subjected to higher shear showed bright ThT fluorescence after 7 minutes relative to cells sheared at low force (Figure 20). The non-amyloid mutant Als5pV326N and cells with the empty vector did not show increased fluorescence. This result showed that amyloid nanodomain formation accompanied force-dependent activation and catch-bonding for Als5p.

Role of S. cerevisiae flocculins in amyloid-mediated catch-bonding and biofilm formation. The S. cerevisiae flocculin proteins Flo1p and Flo11p are not homologous to each other, nor to the Als adhesins. Nevertheless they also contain Tango-predicted amyloid sequences (Ramsook et al. 2010). Therefore we tested whether high shear could activate the cell-to-surface binding. Flo1p or Flo11p-expressing S. cerevisiae were allowed to adhere on the surface of the flow channel for 2 hours in flowing buffer. More Flo11p-expressing cells remained attached to the channel surface after shear at 0.8 dyne/cm² than at 0.02 dyne/cm² (Figure 21). Similar results were seen with Flo1p-expressing cells: at 0.8 dyne/cm² more cells remained bound than at 0.02 dyne/cm². As with Als5p-mediated binding, cells washed at 0.8 dyne/cm² were more resistant to dislodgement during washing at 20 dyne/cm². When flocculin-expressing cells were sheared in the presence of 0.2 mM ThS or with 0.5 mM CR, ThS decreased the binding of cells expressing either flocculin. CR inhibited the binding of Flo11p-expressing cells, but not Flo1p-expressing cells (Figure 21).
Flo11p-expressing cells also showed development of ThT surface fluorescence during shear at 0.5 dyne/cm² after 35 min. Neither cells sheared at 0.05 dyne/cm² nor *S. cerevisiae* var. *diastaticus* with a deletion of *FLO11* showed an increase in fluorescence (Figure 22 and data not shown).

Flo11p-and Flo1p-expressing cells formed thicker biofilms after overnight shearing at 0.05 dyne/cm² than cells that were sheared at 0.01 dyne/cm² (Figure 23). (Higher shear stress led to formation of biofilms that blocked the channels.) *S. cerevisiae* var. *diastaticus* cells with the genomic deletion for the flocculins did not form a thick biofilm. Therefore Flo1p and Flo11p showed properties similar to Als5p under flow, and formed similarly robust biofilms.
E. Discussion

Our data support several findings on shear activation of yeast adhesins. First, laminar flow promoted increased cell binding to surfaces and increased cell aggregation. Second, this activation was dependent on shear-induced formation of functional surface adhesin amyloids. Third, this shear-dependent activation led to formation of robust biofilms. A model for the mechanism is cartooned in Figure 24. Shear flow, like other extension forces, leads to unfolding of T domains. This unfolding exposes the amyloid-forming sequences, which then associate strongly to form surface amyloid nanodomain patches that bind to surfaces and to other fungi with high avidity (Alsteens et al. 2010, Lipke et al. 2012, Garcia et al. 2011). We tested three unrelated yeast adhesins, Als5p, Flo1p, and Flo11p. Each adhesin was activated by forces greater than 0.02 dyne/cm², and for each, activation was accompanied by formation of surface amyloids. Thus, formation of adhesin amyloid nanodomains led to increased binding under shear, behavior that characterizes catch bonding (Alon et al. 1998, Thomas et al. 2004, Thomas 2008, Isberg, Barnes 2002, Marshall et al. 2003). To our knowledge, catch bonding has not been previously reported in yeast or other fungi.

Shear activation of yeast adhesins. Single molecule Atomic Force Microscopy shows that pulling on individual Als5p molecules on the surface of a live cell leads to clustering of the adhesins into amyloid nanodomains patches (Alsteens et al. 2010, Garcia et al. 2011). As a result, the macroscopic dissociation rate is reduced as an exponential function of the number of arrayed adhesins, a phenomenon known as avidity. Thus, surface nanodomains mediate strong binding through increased avidity for ligand. The binding becomes extremely strong, despite relatively low affinity of an individual adhesin molecule for its ligand (Lipke et al. 2012).
Our results here show similar behavior in cells subjected to laminar flow. Such flow activated *C. albicans* or *S. cerevisiae* cells expressing Als5p, Flo1p, or Flo11p to bind to coated and uncoated surfaces and to aggregate as well. Some of the cells bound at 0.8dyne/cm$^2$ were resistant to dislodgment from the channel surface at 20 dyne/cm$^2$ shear stress. As a comparison, forces in the circulatory system can be in the range of 5-20 dyne/cm$^2$ for large arteries, and less in capillaries. In this range, bacteria change from rolling along the surface to stable binding as catch-bonding is activated. The adhesin-expressing yeasts show similar behavior. Thus the fungal systems responded to forces of 0.1-1.6 dyne/cm$^2$, similar to or slightly lower than other catch-bonding systems (Wilson, Hube 2010, Walshe et al. 2005, Resnick et al. 2003, Sheppard et al. 2004, Lipke et al. 2012, Alsteens et al. 2010). Therefore *C. albicans* and *S. cerevisiae* show catch-bonding behavior similar to bacteria and leukocytes.

**Amyloid formation in shear-activated yeast adhesins.** AFM-induced nanodomains are ThT fluorescent and birefringent, and their formation depends on the presence of amyloid-forming sequences in the adhesins (Alsteens et al. 2010, Garcia et al. 2011, Rauceo et al. 2004, Ramsook et al. 2010). Similarly, laminar-flow-induced activation had the characteristics of amyloid formation. Activation was accompanied by development of ThT surface fluorescence (Figures 19 and 22). Anti-amyloid compounds including ThS, CR, and an Als5p sequence-specific amyloid-blocking peptide inhibited activation (Figures 13, 19, 18, and 21). There was also reduced activation of cells that expressed an amyloid-impaired Als5p$^{V326N}$ form of the adhesin. Together these data imply that the mechanism of activation under flow is the same as the AFM: formation of amyloid nanodomains with increased avidity.

The remaining activity of Als5p$^{V326N}$ reflected the normal functions of other domains of the adhesin. The peptide-binding Ig-like invasin domains and the tandem repeats, which mediate
hydrophobic effect interactions, remain intact and functional (Garcia et al. 2011, Lipke et al. 2012, Frank et al. 2010). Therefore, this amyloid-impaired form bound effectively to the surface, but the binding was weaker, and was inhibited by both specific and non-specific inhibitors. Such cells were also easily dislodged at high shear, and formed reduced biofilms.

This shear-induced amyloid nanodomain activation appears to be general for yeast adhesins. The behavior of the S. cerevisiae Flo adhesins and C. albicans Als5p was similar, including flow-induced activation, sensitivity to CR and ThS, and development of surface fluorescence (Figures 11, 17, 20, 21, and 22). C. albicans itself also showed similar behavior, probably reflecting amyloid-like properties of other Als and non-Als adhesins (Figures 11 and 21) (Ramsook et al. 2010, Garcia et al. 2011, de Groot et al. 2013). Additionally, C. albicans and other pathogenic fungi in abscesses in human tissue also display surface amyloids (Gilchrist et al. 2012, Garcia-Sherman et al. 2014), Garcia-Sherman et al unpublished). Thus, the activation by force appears to be a conserved mechanism for fungal adhesins (Dranginis et al. 2007, Ramsook et al. 2010, de Groot et al. 2013).

**Biofilm formation increased by shear stress.** Consequences of amyloid-dependent fungal catch-bonding included larger aggregates and robust biofilms (Figures 13, 14, 16, 20, and 21). The amyloid-impaired Als5pV326N mutant did not form biofilms as thick or extensive as the wildtype, and showed reduced sensitivity to ThS inhibition. This result supports the idea that biofilm formation begins with the initial binding of cells to a surface, and is dependent on amyloid-forming adhesion proteins on the cell surface. Bacterial biofilms show similar force dependence. The thickness of the Pseudomonas strain CT07 gfp biofilms is reduced when flow is reduced from 0.95 dyne/cm² to 0.09 dyne/cm² (Bester et al. 2013). Seven Listeria monocytogenes strains had initial adhesion rates that were significantly greater for shear stress at
11 dyne/cm$^2$ than at 1 dyne/cm$^2$ on stainless steel (Skovager et al. 2012). Biofilms of marine and bacteria and water-supply bacteria show increased cohesion and durability as the shear stress is raised (Salta et al. 2013), (Paris, Skali-Lami & Block 2007). Thus the behavior of the fungal biofilms under flow was similar to that of bacterial biofilms.

In summary, shear-flow-mediated activation of yeast adhesins illustrates several novel findings. First, the behavior is that of catch-bonding, the strengthening of bonds under tension. Second, in yeast the catch-bonding behavior is explainable by formation of amyloid-interacting arrays of cell adhesion molecules. To our knowledge, such a mechanism has not been previously reported. Third, amyloid nanodomain formation is a response to force in at least three different yeast adhesins in two species, and circumstantial evidence supports an even broader occurrence. Finally, this amyloid-dependent catch-bonding is important in formation of robust and flow-resistant biofilms.
Figure 11. Effect of shear stress on adhesion. *S. cerevisiae* strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p^{WT} or Als5p^{V326N} were used. *C. albicans* SC5314 or *S. cerevisiae* cells were allowed to adhere to surface under flow. Top: micrographs of representative fields enlargement of the channel surface at 0.8 dyne/cm^2 for each strain. Scale bar: 5 μm. Bottom: adherent cells were photographed after 2 hours, and quantified with Image J.
Figure 12. Movement of yeast cells over a surface at different shear forces. Representative clusters of *C. albicans* SC5314 cells were imaged after 99 min at the indicated shear stresses. Note the cells moving between frames at the lowest shear stress (*).
Figure 13. Effect of shear on clustering of *C. albicans*. *C. albicans* SC5314 cells were adhered to channels under continuous flow at different rates for 2 hours. A) Micrographs of representative fields. B) Cell counts binned by cluster size. Increased shear led to increased cluster size.
BSA-coated channels

A, B, C, D: Cells were pumped through at designated shear stress for 2 hours

B and D: Shear flow increase to 20 dyne/cm² for 10 minutes

A

![Bar chart for Als5p](image)

B

![Bar chart for Als5p](image)

C

![Bar chart for Als5p^V326N](image)

D

![Bar chart for Als5p^V326N](image)
Figure 14. Effect of shear on clustering of *S. cerevisiae*. *S. cerevisiae* strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p<sup>WT</sup> or Als5p<sup>V326N</sup> were used. *S. cerevisiae* cells expressing Als5p (A and B) or Als5pV326N (C and D) were adhered to channels under continuous flow at different rates. Cells in representative micrographs were analyzed with Image J and counts binned by cluster size. A and C: Left two panels were analyzed after 2 hours under shear stress as designated. Cells expressing empty vector did not bind to the channel surface (data not shown). B and D (right two panels): The stress was then increased to 20 dyne/cm<sup>2</sup> for 10 min, and the remaining cells analyzed.
Figure 15. Resistance of surface-bound cells to high shear. *S. cerevisiae* cells harboring empty vector (EV), or expressing Als5p or Als5p$_{V326N}$ or *C. albicans* SC5314 cells were bound to BSA-coated flow channels under the designated shear stress. After 2 hours, the flow was increased to 20dyne/cm$^2$ for 10 minutes. The number of cells remaining bound was determined with Image J.
Figure 16. Biofilm growth for *S. cerevisiae* strains at two shear stresses. *S. cerevisiae* strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5pWT or Als5pV326N were used. Adherent cells were grown under laminar flow for 48 hours at the designated shear stress. Scale bar: 25 µm.
Figure 17. Effects of high shear and amyloid dyes on binding of yeast to BSA-coated surfaces. *S. cerevisiae* strain W303-1B *MATα leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p$_{WT}$ or Als5p$_{V326N}$ were used. Designated strains were allowed to adhere to BSA-treated channels 2 hours under laminar flow at 0.8 dyne/cm$^2$ before imaging (left column). In the second column, adherent cells were then washed at high shear (20 dyne/cm$^2$). In the third and fourth columns anti-amyloid compounds were added to the flow buffer during adherence. Micrographs are representative fields enlargement of the channel surface. Scale bar: 5 µm.
Figure 18. Effects of amyloid-perturbing dyes thioflavin S and Congo red on biofilm formation of Als5p-expressing and SC5314 cells. *C. albicans* and *S. cerevisiae* strain W303-1B *MATα leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) expressing Als5pWT was used. Micrographic images of channel surfaces with cells grown 24 hours at 1 dyne/cm² in media without or with amyloid-perturbing dyes.
Figure 19. Effects of peptides on binding of yeast to BSA-coated surfaces. *S. cerevisiae* strain W303-1B *MATα leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) expressing Als5p<sup>WT</sup> or Als5p<sup>V326N</sup> were used. Micrographs and cell counts of channel surfaces with cells expressing Als5p or Als5p<sup>V326N</sup> sheared at 0.8 dynes/cm<sup>2</sup> for 2 hours. Micrographs are representative fields enlargement of the channel surface. Scale bar: 5 µm. A. Cells were sheared in the absence or presence of anti-amyloid V326N peptide or a scrambled peptide of the same composition (200µg/ml). B. Channels after an additional 10 min. high shear at 20dyne/cm<sup>2</sup>. 


Figure 20. Effect of shear stress on thioflavin-T fluorescence of Als5p-expressing S. cerevisiae cells. S. cerevisiae strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p\textsuperscript{WT} or Als5p\textsuperscript{V326N} were used. Bright-field and corresponding fluorescence micrographs of Als5p-expressing cells. Micrographs are representative fields enlargement of the channel surface. Fluorescence micrographs are photographic negative representations. Scale bar: 5 µm.
Figure 21. Effects of shear and anti-amyloid compounds on flocculin-dependent adhesion.

*Saccharomyces cerevisiae* variant diastaticus *MATa* (*ura3 leu2-3, 112 his4*) expressing Flo11p and the deletion strain *MATa* (*ura3 leu2-3, 112 his4 flo11:URA3*) were kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATα* (*FLO1 gal1*) was purchased from ATCC (Manassas, VA). *S. cerevisiae* expressing Flo1p or *S. cerevisiae v. diastaticus* expressing Flo11p or its congenic *flo11* deletion strain were adhered to uncoated channels for 2 hours at the designated shear stresses. The third column shows effects of subsequent 10 min. washes at 20 dyne/cm². The 4th and 5th columns show the effects of anti-amyloid compounds present during shear at 0.8 dyne/cm².

<table>
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<tr>
<th>Shear stress (dyne/cm²)</th>
<th>0.02</th>
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<tr>
<td>Additional treatment</td>
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<td>20 dyne/cm²</td>
<td>ThT (0.2 mM)</td>
<td>CR (0.5 mM)</td>
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<td>Flo1p</td>
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<td>None (Δflo11)</td>
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Figure 22. Effect of shear stress on thioflavin-T fluorescence of Flo11p-expressing cells.

Saccharomyces cerevisiae variant diastaticus MATa {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain MATa {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). Biofilm formation of S. cerevisiae var. diastaticus expressing ScFlo1lp and S. cerevisiae Bx24-1B strain expressing ScFlo1lp were imaged after 24 hours at shear stress of 0.01 dyne/cm² (top row) and 0.05dyne/cm² (bottom row). Micrographs are representative fields enlargement of the channel surface. Fluorescence micrographs are photographic negative representations. Scale bar: 5 µm.
Figure 23. Effect of shear stress on flocculin-mediated biofilm formation. *Saccharomyces cerevisiae* variant diastaticus MATα {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain MATα {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). Flo11p- or Flo1p-expressing *S. cerevisiae* were seeded on surfaces, and then grown under flow at the designated shear stress for 24 hrs. Scale bar: 25 µm.
**Figure 24. Model for shear stress activation of Als5p.** Top left, model showing the domains of Als5p on the cell surface; the amyloid forming sequences (red) are buried inside the folded T domains (yellow). Top right: under strong shear stress, the T domains unfold, the amyloid-forming sequences are exposed, and surface amyloid nanodomains form. Bottom: if the amyloid-forming sequence is absent as in Als5p$^{V326N}$ (left), or if the shear stress is weak, the nanodomains cannot form.
Chapter IV

Force-sensitivity of *Saccharomyces cerevisiae* flocculins and *Candida albicans* Als adhesins
**A. Abstract**

*Candida albicans* Als5p and Als1p adhesins and *Saccharomyces cerevisiae* Flo11p and Flo1p flocculins have amyloid-forming sequences. In Als5p, these amyloid sequences aggregate the adhesins to form high avidity surface nanodomains (Alsteens et al. 2010. Proc Natl Acad Sci USA 107:20744-9). Here we report use of quantitative cell adhesion assays, supported by confocal microscopy and flow cytometry to quantify formation of surface amyloid nanodomains on live cells. Mechanical stress from vortex-mixing induced formation of amyloid nanodomains and activated cell adhesion for Als5p, Flo1p, and Flo11p. Vortex-mixing cells for 60 seconds increased the rates and numbers of aggregating cells for all three adhesins; 1.7-fold for cells expressing Als5p, 1.5-fold for cells expressing Flo1p, and 1.2-fold for cells expressing Flo11p. There was no activation in cells expressing amyloid-impaired Als5p$^{V326N}$. Activation was also blocked by inhibitory concentrations of anti-amyloid dyes. Activation was accompanied by similar fold increases in amyloid-dependent surface fluorescence and in size and frequency of amyloid nanodomains visualized by confocal microscopy. Vortex-mixing also activated Flo1p- and Flo11p-mediated flocculation even in the absence of added Ca$^{+2}$. The effects of vortex-mixing were replicated in heat-killed cells as well. Together these results demonstrated quantitative effects on force-dependent activation of cell surface amyloid nanodomains in yeast expressing three different adhesins.
B. Introduction

Yeast cell surface adhesins mediate cell-to-cell aggregation and cell-to-surface adhesion, including for example, the *Candida albicans* adhesin Als5p (Gaur, Klotz 1997b) and *Saccharomyces cerevisiae* flocculins Flo1p and Flo11p (Dranginis et al. 2007). Although many yeast adhesins have little or no homology, they have similar architecture: an N-terminal secretion signal sequence, a globular ligand binding region, a mid-region containing threonine-rich tandem repeats (which are not homologous between the proteins), a long Ser/Thr-rich glycosylated stalk, and a C-terminal GPI anchor (Dranginis et al. 2007). During cell wall biogenesis, the GPI anchor is cleaved in the glycan, and the remnant covalently attached to cell wall polysaccharide (Gonzalez et al. 2010). Within the mid-regions of many adhesins including Als5p and the *Saccharomyces cerevisiae* flocculins Flo1p and Flo11p are 6-7-amino acid sequences predicted by Tango (http://tango.crg.es/) to form amyloids (Otoo et al. 2008, Ramsook et al. 2010, Fernandez-Escamilla et al. 2004). A single site mutation in the amyloid region of Als5p\textsuperscript{V326N} decreases cell-to-cell aggregation, cell-to-substrate adhesion, and fluorescence of the amyloid-reporting dye thioflavin T (Garcia et al. 2011). Results with the *S. cerevisiae* Flo1p and Flo11p are consistent with this model: anti-amyloid dyes Congo red and thioflavin S decrease the rate and amount of flocculation (Ramsook et al. 2010).

Extension forces cluster the adhesins into amyloid-dependent surface patches, and consequently activate Als-protein-mediated adhesion (Alsteens et al. 2010, Garcia et al. 2011). Mechanical extension force applied with an AFM tip activates both the clustering of Als5p (wild type) molecules into nanodomains and propagation of the clusters along the cell surface. This clustering is mediated by the amyloid-forming sequence, because the clustering response is absent from a non-amyloid-forming mutant form of the protein Als5p\textsuperscript{V326N} (Garcia et al. 2011,
We have proposed that the pulling on the surface protein results in exposure of the amyloid regions of the protein, which then interact through amyloid stacking to cluster with neighboring Als5p molecules in 100-500nm diameter surface nanodomains (Garcia et al. 2011, Lipke et al. 2012). These nanodomains are bright in thioflavin T fluorescence (Alsteens et al. 2010, Garcia et al. 2011). Als5p clusters take minutes to form and propagate slowly around the cell surface at a rate of ~20 nm/min. Similarly, hydrodynamic shear activates the yeast surface amyloids to increase surface binding, cell-cell aggregation, and robust biofilm formation (Chan, Lipke 2014).

_In vitro_ many proteins can be partially unfolded, leading to exposure of amyloid-forming sequences, and their subsequent aggregation into β-sheet rich forms that assemble in a cross-β structure, characteristic of amyloid fibril formation (Calamai, Chiti & Dobson 2005); (Harper, Lieber & Lansbury 1997, Sunde, Blake 1998, Serpell 2000, Dobson 1999, Fandrich et al. 2006). For instance, conformational changes in proteins resulting from partial unfolding from their native state facilitate amyloid formation in transthyretin (McCutchen et al. 1995) and lysozyme (Merlini, Bellotti 2005). Therefore, forces that unfold proteins can lead to amyloid formation.

Indeed, amyloid formation itself may be triggered by shear force. When Aβ-peptide is stirred there is an increase in thioflavin T fluorescence as well as growth of amyloid fibers that are not seen with quiescent peptides (Dunstan et al. 2009). Dunstan et al. hypothesized that the stirring allows the protein to unfold and results in a faster growing fiber by the reduction of the energy barrier for amyloid formation or the β-sheet structures. Dunstan et al. hypothesized that a possible mechanism of the effect of shear is the alignment of the aggregates, to facilitate assembly into fibrils. This idea is supported by observations that protein aggregates align under flow (Hill et al. 2006). Shear flow from a Couette cell produces amylogenic precursors in β-
lactoglobulin, and enhances fibril formation as well through the alignment and further unfolding of the protein under shear flow thus resulting in the formation of amyloid precursors and or their maturation into fibers (Hill et al. 2006, Dunstan et al. 2009).

We have previously demonstrated force-induced formation of surface amyloid nanodomains of yeast adhesins (Alsteens et al. 2010, Chan, Lipke 2014). Nevertheless, we have not previously been able to make quantitative comparisons of rates or extent of amyloid formation in vivo. Therefore we set out to induce and measure activation in populations of cells. We reasoned that if force were important for activation of yeast adhesins, shear stress from vortex-mixing would activate formation of amyloid nanodomains of the surface-bound yeast adhesins. Nanodomain formation would then activate cell adhesion (Lipke et al. 2012). We find that this approach is effective. Quantitative comparisons show that amyloid formation responds to shear stress, and acts as both force sensor and effector. Thus we can measure effects of shear stress on activation of cell adhesion.
C. Materials and Methods

Strains and media

*Saccharomyces cerevisiae* strain W303-1B MATα leu2 ura3 ade2 trpl (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p or Als5p<sup>V326N</sup> were grown in complete synthetic medium (CSM) lacking tryptophan with galactose as carbon source (Garcia et al. 2011). Cultures were grown for 48 hours at 24°C at 170 rpm.

*Saccharomyces cerevisiae* variant diastaticus MATα ura3 leu2-3, 112 his4 expressing Flo11p and the deletion flo11 MATα ura3 leu2-3, 112 his4 flo11:URA3 were kindly gifted by Dr. Anne Dranginis (St. John’s University). Cells were grown in yeast-extract-dextrose (YPD) at 30°C at 170 rpm for 24 hours.

*Saccharomyces cerevisiae* strain BX24-2B FLO1 MATα FLO1 gal1 was purchased from ATCC (Manassas, VA). Cells were grown in YPD at 30°C at 170 rpm for 24 hours.

*Candida albicans* strain SC5314 grown overnight in YPD at 30°C at 170 rpm. Activation of Als1p expression was induced by placing an aliquot of cells in fresh YPD medium and shaking at 170 rpm at 30°C for 45 minutes.

Tango prediction

Predictions of Tango positive protein sequences were done by uploading sequences to the website http://tango.crg.es. Overlaps of 100 amino acids were used if the protein sequence was over 500 amino acids. The pH setting of Als5p<sup>WT</sup> was set at 7.0 and for the flocculins Flo1p and Flo11p it was set at 5.5. No N terminal or C terminal protection was used. The temperature was
298.15 K and the ionic strength at 0.02. A \( \beta \)-aggregation potential > 20\% was considered positive for amyloid formation.

**Aggregation assays**

Aggregation assays were carried out as previously described (Gaur, Klotz 1997b, Rauceo et al. 2004) with modifications. Briefly, cells were centrifuged at 4000 rpm for 3 minutes to remove culture media. The cells were then washed gently twice with 10mM tris, 1mM EDTA (TE) pH 7.0 and then resuspended in the same buffer. The OD\(_{600nm}\) of the cell suspension was determined with a Spectronic 21 D+ spectrophotometer, and the suspension was adjusted to 10\(^8\) cells/ml. Cells were either left stationary on the lab bench or vortex-mixed at 2500 rpm using a Fisher Scientific multi-tube vortexer. Cell suspension (1ml) was mixed with 10\(^6\) BSA-coated magnetic beads. The suspensions were gently agitated at 170 rpm at 24\(^\circ\)C for 10 minutes. After 10 minutes the assay tubes were placed on a magnet. The unbound cells were gently removed with a pipet and the beads with the cell aggregates were washed once 500 \(\mu\)l of TE buffer. For microscopic viewing, cells were resuspended in 100 \(\mu\)l of TE buffer and 4 \(\mu\)l applied to a glass slide. Microscopic observations were made with an Olympus microscope using a 60X oil objective. For quantification, the aggregates and beads were resuspended in 300 \(\mu\)l 1 M NaOH and shaken gently on an orbital shaker for 20 minutes. The beads were then separated on a magnet, and the OD\(_{600nm}\) determined on a 200 \(\mu\)l aliquot in a 96-well plate with a Spectronic Genesys plate reader. In this assay OD\(_{600nm}\) of 1.0 corresponds to 8.8 \(\times\) 10\(^7\) cells/ml. Unless otherwise stated, all assays were done on at least two independent cultures, in triplicate for each.

M-280-tosylactivated-magnetic Dynabeads (Invitrogen, Carlsbad, CA) covalently derivatized with 1mg/ml heat-denatured bovine serum album (BSA) overnight according to the manufacturer’s protocol were added to a final volume of TE buffer of 1ml,
When desired, cells were heat killed in a water bath at 60° C for 15 minutes and then incubated at room temperature for 1 hour before activation and assay.

**Flocculation assays**

Assays for flocculation mediated by ScFlo11p were carried out as previously described (Lo, Dranginis 1996b), using 2 x 10^7 cells/ml prewashed with EDTA to inhibit flocculation before assay. Flo11p cells were washed three times and resuspended at 2 x 10^7 cells/ml in 20 mM sodium acetate buffer with 1mM EDTA, pH 5.5. Flo1p expressing cells were washed and resuspended to 6 x 10^7 cells/ml in 20 mM sodium acetate buffer with 200 μM EDTA, pH 5.5.

Cells were vortex-mixed or gently resuspended and flocculation was initiated by addition of 670 μM CaCl₂ for ScFlo1p expressing cells and 330 μM CaCl₂ for ScFlo11p expressing cells, the suspension was gently vortexed for 10 s on a low setting and the OD₆₀₀nm was monitored at 5-s intervals in a Spectronic 21 D+ spectrophotometer. Flocculation rates were calculated as previously described (Ramsook et al. 2010). Unless otherwise stated, all assays were done on at least two independent cultures, in triplicate for each.

For visualization of flocs, 4 μl samples were placed onto a slide and viewed with an Olympus bright field microscope with a 60X oil objective.

**Dye inhibition**

Als-expressing *S. cerevisiae* cells or *C. albicans* cells were vortex-mixed or left quiescent for 5 minutes and then ThS (200 μM) was added. Ligand-coated beads were added to a final volume of 1ml. The cells were placed for an aggregation assay of 10 minutes at 170 rpm at 24°C. Then the cells were treated as above.
Flo1p and Flo11p-expressing cells were vortex-mixed or left quiescent for 5 minutes and then ThS (200 μM) or CR (500 μM) was added to the cell suspension and corresponding concentrations of Ca\(^{2+}\) was added as mention above and the OD\(_{600\text{nm}}\) was monitored.

**Confocal microscopy**

Confocal imaging was done with a Nikon confocal microscopy. 10\(^8\) cells were stained with ThT (500 nM) in a final volume of 1ml immediately after vortex-mixing. The cells were vortex mixed on a low setting with the dye for 5 seconds to resuspend the dye, and then 4 μl of the suspension was placed onto a glass slide for imaging. The stained cells were not washed prior to microscopy. The gain of the microscope was set at 7.75 with the phase at 162. The excitation was at 408nm with an emission detector at 450 ± 35 nm. Pictures were taken at 2048 x 2048 quality.

**Flow Cytometry**

Flow cytometry was done with BD Biosciences FACSCanto with an excitation of 405 nm and an emissions filter of 450 ± 50nm. 10\(^6\) cells were in 12mm x 75 mm tubes with or without vortex-mixing and then brought to a final concentration of 1 μM ThT in a total volume of 1 ml in their respective buffer as mentioned above. The cells were filtered with a 70 μm filter before analysis. 20000 cells were monitored for each assay.

**Staining protocols**

Stock concentrations of thioflavin S and T were made with deionized water and filtered with a 2 μm filter. The concentration was then determined with a spectrophotometer, using Beer’s law, using and extinction coefficient of 2.66 x 10\(^3\) L/mol*cm in 100% ethanol. Amyloid
staining of the adhesion expressing cells was done with thioflavin T and thioflavin S (5 µM unless otherwise noted). Vortex-mixed or quiescent cells were slowly mixed with the added dye for 5 seconds. Aliquots (4 µl) of the cells were then added to a glass slide for microscopy.
D. Results

**Effect of vortex-mixing on adhesion of Als5p-expressing S. cerevisiae cells.** To obtain evidence for force-mediated activation of adhesion in whole cell suspensions, we looked for increases in cell-to-bead adhesion and cell-to-cell aggregation of Als5p-expressing cells with ligand-coated beads (Gaur, Klotz 1997). Suspensions of cells expressing Als5p were vortex-mixed for 5 minutes at 2500 rpm. The initial adhesion activity was determined by monitoring size of aggregates in the first 10-15 minutes of aggregation assays. (These brief assays minimize induction of nanodomains that occurs during standard 45 min assays (Gaur, Klotz 1997, Garcia et al. 2011). Cells that had been vortex-mixed formed bigger initial aggregates than cells that were not vortex-mixed (Figure 25A). To quantify the number of cells bound to BSA-coated beads we washed the aggregates with NaOH and then determined optical density at 600nm. There was an average 1.6-fold increase in the number of cells bound to the ligand-coated beads when vortexed mixed 10-minute aggregation assays (Figure 25B).

If force-induced cell adhesion is amyloid-dependent, then turbulent mixing should not activate aggregation for the non-amyloid mutant Als5p$^{V326N}$ adhesin. As predicted, there was no increase in the size of the aggregates of the amyloid mutant protein (Figure 25A, B). There was also no activation of aggregation in the cells expressing empty vector when vortex-mixed (Figure 25A, B).

We determined the vortex-mixing time needed to activate the cells. Mixing for 60 seconds increased the number of cells bound to the beads from $(8.1 \pm .4) \times 10^6$ to $(1.4 \pm .03) \times 10^7$. There was no additional increase with mixing times up to 5 min (Figure 25C). Therefore
under these conditions 60 seconds of vortex-mixing was sufficient to increase the adhesion and aggregation of the cells to ligand-coated beads.

**Inhibition of activation with thioflavin S.** At concentrations above 30 μM, the amyloid-binding dye thioflavin S (ThS) binds to and disrupts amyloids, therefore decreasing adhesion (Rauceo et al. 2004, Ramsook et al. 2010, Garcia et al. 2011). If amyloids mediate the vortex-induced activation of aggregation, then amyloid-binding dyes should inhibit this increase. This was indeed the case: ThS (200 μM) added during vortex-mixing inhibited the binding of vortex-mixed Als5p-expressing cells to the ligand-coated beads by 6.3-fold (Figure 25D). In the presence of ThS, there were no aggregates formed with Als5p V326N-expressing cells or cells with empty vector, nor was there any effect of vortex-mixing (data not shown).

**Effects of vortex-mixing on flocculation of Flo1p- and Flo11p-expressing S. cerevisiae cells.** Because the *S. cerevisiae* flocculins Flo1p and Flo11p also contain Tango positive sequences and were inhibited by amyloid-binding dyes, we hypothesized that the flocculins would also show force-dependent activation and inhibition of activation by the anti-amyloid dyes (Ramsook et al. 2010). We examined the effects of vortex-mixing on *S. cerevisiae* var. diastaticus cells expressing their native adhesin Flo11p. Suspensions of flocculin-expressing cells were vortex-mixed for 5 minutes or not, and then Ca^{2+} was added to initiate flocculation (Bidard et al. 1994, Lo, Dranginis 1996). Figure 26 shows the results of flocculation assays with vortex-mixed and quiescent cells. Vortex-mixing the cells before flocculation led to increases in the number of cells flocculated (lower final O.D.600nm: Figure 26A) and the initial rate of flocculation (Figure 26B). Specifically, Flo11p-expressing cells had flocculation rate 1.5-fold increased relative to cells that had not been vortex-mixed. A flo11-deleted *S. cerevisiae* var. diastaticus mutant strain did not flocculate (Lo, Dranginis 1996) and had no increase in
flocculation when vortex-mixed (Fig 26A and 26B). Surprisingly, vortex-mixing also increased the size of cell clusters (flocs), even in the absence of added Ca$^{+2}$ (Figure 26C). The cells that were not vortex-mixed had mean floc sizes of 5-7 cells. Vortex-mixing increased the mean cells per floc 9-fold, as determined by image analysis of three microscopic fields (Figure 26C). The vortex-mixing time effective in activation of Flo11p-expressing cells was similar to that for Als5p$^{WT}$-expressing cells (Fig. 26D). Thirty seconds of mixing increased the flocculation rate 1.3-fold from (2.6 ± 0.04) x 10$^{-3}$ to (3.5 ± 0.4) x 10$^{-3}$. The rate increased to 4.2 x 10$^{-3}$ with 5 minutes of mixing.

In a test of a third adhesin, the S. cerevisiae mannose-binding lectin Flo1p, vortex-mixing also led to 1.2-fold increases in the fraction of cells flocculating and in the flocculation rate (Figure 27A). We see activation of Flo1p-expressing cells with vortexing at 30s and a maximum rate at 120s (Figure 27B). Similarly to Flo11p-expressing cells, vortex-mixing increased the size of the flocs of Flo1p-expressing cells in the absence of Ca$^{+2}$ (Figure 27C). The cells that were not vortex-mixed had on average, 3-4 cells per floc but most of the cells were single cells or 2 cells per floc. Vortex-mixing increased the number of cells per floc 20-fold.

**Effects of amyloid-binding dyes on flocculation of Flo11p-expressing cells.** ThS (200 μM) or 500 μM Congo red inhibits the flocculation of Flo11p- and Flo1p-expressing cells (Ramsook et al. 2010). We therefore determined whether these dyes would inhibit the increase in flocculation rate and the extent of flocculation of Flo11p-expressing cells that were vortex-mixed (Figure 26A and B). Cells treated with 500 μM CR had a 2.3-fold decrease in the flocculation rate, and cells treated with 200 μM ThS had a 33-fold decrease in the flocculation rate. These concentrations of dye do not affect growth, but are potent at preventing cell aggregation (Ramsook et al. 2010). Similarly CR and ThS also inhibited force-activated flocculation in
Flo1p-expressing cells. Treatment with 500μM CR decreased the flocculation rate of vortex activated cells by 1.2-fold. Thioflavin S decreased the rate by 1.4-fold (Figure 27A).

**Effect of vortex-mixing on cell surface thioflavin-T fluorescence.** Sub-inhibitory concentrations of thioflavin T (ThT) stain amyloids *in vitro* and on yeast cell surfaces (Calamai, Chiti & Dobson 2005, Hill et al. 2006, Ramsook et al. 2010, Garcia et al. 2011). To assay whether the increase in adhesion was accompanied by the formation of amyloids on the surface of cells expressing yeast adhesins, we stained quiescent and vortex-mixed cells with 1 μM ThT, and analyzed them by flow cytometry. For Als5p-expressing cells, vortex-mixing caused a 31% increase in the mean cellular fluorescence (Table 3). Control cells expressing EV or Als5p V326N did not show similar increases. Vortex-mixing did not induce detectable changes in the forward or side scatter of the cell suspensions (data not shown). There were similar results with Flo11p and Flo1p-expressing cells: vortex-mixing increased the number the mean ThT fluorescence and the number and intensity of surface amyloid nanodomains (Table 3). For Flo11p-expressing cells, the mean fluorescence intensity of cells increased 31% after vortex mixing. Flo1p-expressing cells showed a 55% increase in mean fluorescence. Therefore, vortex-mixing cells expressing any of the three adhesins led to significant increases in surface fluorescence and nanodomains frequency and intensity with the amyloid-staining dye ThT.

**Effects of vortex-mixing on cell surface amyloid nanodomains.** Extension of individual Als5p molecules by single molecule AFM leads to formation of surface amyloid nanodomains that migrate around the cell surface (Alsteens et al. 2010). These nanodomains are stained effectively with ThT (Alsteens et al. 2010). Cells were vortex-mixed for 5 minutes, and then stained by the “Instant Stain” protocol described in Methods. The results showed increased density and fluorescence of ThT-stained nanodomains on cells expressing Als5p, Flo1p, or
Flo11p (Figure 28). This increase was not seen with the non-amyloid mutant Als5p$^{V326N}$, cells with empty vector, or the S. cerevisiae var. diastaticus strain with a deletion in flo11. These data confirmed that vortex-mixing induced formation of ThT-fluorescent surface nanodomains in cells expressing a yeast adhesin.

**Effects of vortex-mixing in C. albicans.** We assayed increases of aggregation in live C. albicans cells. To maximize expression of Als1p and perhaps other adhesins, cells were diluted in fresh YPD media for 45 minutes before the assays (Rauceo et al. 2004, Gaur, Klotz 1997a, Hoyer et al. 1995b). When C. albicans cells were vortex-mixed at 2500 rpm for 5 min., the initial aggregates were larger than in cells not vortex-mixed (Fig. 29A). The mixing time for maximal activation was similar to that for Als5p-expressing S. cerevisiae; there was a 20% increase within 60 seconds of vortex-mixing (Figure 29B). Activation was accompanied by a slight increase in surface ThT fluorescence, and a more uniform distribution of surface amyloid puncta (Fig. 29C). As with Als5p, Flo11p, and Flo1p, ThS (200 μM) or CR (500 μM) inhibited the aggregation and adhesion of cells to ligand-coated beads (Fig. 29D). Quantification of the number of cells bound showed that there was a 32-fold decrease in cells bound to bead following treatment with ThS and 9-fold decrease with CR treatment.

**Effects of vortex-mixing of heat-killed adhesin-expressing cells.** Heat-killed cells aggregate effectively, and are able to form surface nanodomains (Alsteens et al. 2010, Garcia et al. 2011). Therefore, if vortex-mixing-induced nanodomain formation is independent of cellular metabolism, it should also be apparent in heat-killed cells. Cells expressing Als5p, Als5p$^{V326N}$, Flo1p, or Flo11p were heat-killed for 20 min at 60°C. As expected, there was an increase in punctate nanodomain fluorescence on the surface of heat-killed Als5p cells when vortex-mixed (Figure 30). In contrast, heat-killed Als5p$^{V326N}$ non-amyloid mutant cells showed only
intracellular staining with ThT without the characteristic annular pattern. Similarly, vortex-mixing activated flocculation for heat-killed cells expressing either Flo1p or Flo11p in the presence of Ca\(^{+2}\) (Figures 31A and 31B). Significantly, heat killed Flo11-expressing cells flocculated well in the absence of added Ca\(^{+2}\) (Figure 31C). The addition of Ca\(^{+2}\) initiated additional flocculation, thus increasing the size of flocs (data not shown).
E. Discussion

There are three major conclusions from the current study of effects of vortex-induced activation. First, the results demonstrate that the adhesins are force sensors, and that they respond to force by activating nanodomains formation autonomously. This remarkable activity takes place on cell surfaces without need for a signaling or metabolic response in the cell. Secondly, there is now analytic data to show that shear stress from vortex-mixing can partially substitute for Ca^{+2} in activation of Flo1 and Flo11. Finally, the shear stress necessary for activation of amyloid nanodomains is similar to that in standard lab protocols for washing and resuspending yeasts, and below the maximum speed for many lab vortex mixers. Therefore cell preparation procedures can inadvertently affect aggregation behavior.

In this study, we describe approaches to relative quantification of the effects of shear stress on activation of fungal adhesins. This analysis requires the ability to control induction of surface amyloid nanodomain formation in a population of cells, and we find that vortex-mixing can rapidly initiate this response. Quantification of adhered cells was supplemented with flow cytometry and confocal microscopy with thioflavin T. The results demonstrated similar shear stress-induced clustering and activation in three unrelated yeast adhesins, and support our findings of similar activation under laminar flow (Chan, Lipke 2014).

**Generality of force activation.** A major finding is that *S. cerevisiae* flocculins showed the same behavior as *C. albicans* as well as the *C. albicans* Als adhesins after vortex-activation as well as under laminar flow (Chan, Lipke 2014). Although the flocculin protein domains are non-homologous to Als and to each other, they were also activated by vortex-mixing or laminar flow, and also formed surface nanodomains in response to the applied force. Thus, in all tested
yeast systems vortex-mixing increased adhesion activity and simultaneously increased cell surface fluorescence with the amyloid reporting dye ThT (Figure 28, 29, and 30). The results confirm relevance of the *S. cerevisiae* surface display system to *C. albicans in vivo* (Gaur, Klotz 1997, Rauceo et al. 2004).

**Fungal adhesins as force sensors.** Our results support a model in which the adhesins themselves respond to force (Alsteens et al. 2010, Chan, Lipke 2014). This autonomy is supported by findings of nanodomain formation and increased adhesion activity in heat-killed cells (Figures 30 and 31). Vortex-mixing of either live or dead cells led to clustering the molecules to form nanodomains and activation of fungal cell aggregation (Figures 28 and 30) (Garcia et al. 2011, Alsteens et al. 2009). A simple mechanism is illustrated in Figure 24 of the accompanying paper (Chan, Lipke 2014), in which amyloid sequence-containing T domains in Als5pWT and Als1p are unfold to expose the amyloid core sequence, leading to amyloid interactions with neighboring molecules to form cell surface nanodomains within minutes (Alsteens et al. 2010, Alsteens et al. 2012, Beaussart et al. 2012, Garcia et al. 2011, Lipke et al. 2012, Alsteens et al. 2013). The clustering of adhesins increases avidity, because there are localized patches with many arrayed adhesion molecules. The clustering results in very high local concentrations of adhesins, and subsequent reduction in macroscopic $k_{off}$ values (Lipke et al. 2012).

In all three adhesins, nanodomain formation followed one minute of vortex mixing. This time is significantly shorter than the 25 min interval observed after single molecule stimulation for Als5p in AFM experiments (Alsteens et al. 2010). On the other hand, Als-bearing cells start to become globally cell surface activated within 10 minutes in adhesion assays (Gaur, Klotz 1997, Rauceo et al. 2004). These differences in activation rate are consistent with differences in
the frequency of molecular stretching in the three different scenarios. A few molecules are individually stretched in the AFM. In adhesion assays, cells are gently mixed, usually at 170-200 rpm. This process results in random collisions, adhesions and subsequent stretching of adhesins, so many areas of the cell surface are stimulated in the course of a few minutes. Our unpublished data shows that aggregation in fact happens faster and to greater extent as the mixing speed in the assay becomes faster. Under the rapid mixing employed here, the stimulation was much faster, within a minute for Als5p, Flo1p, and Flo11p. Therefore, the speed of mixing affects both rate and extent of aggregation and adhesion.

**Role of Ca\(^{2+}\) in flocculin activity.** Also novel is our finding that Ca\(^{2+}\) was not absolutely required for activation of flocculation the flocculins Flo1p and Flo11p (Figure 26C). Historically, flocculation assays for both flocculins have been initiated by addition of mM Ca\(^{2+}\) followed by brief vortex mixing (Mill 1964, Taylor, Orton ). We were surprised to see flocculation immediately following vortex-mixing, before addition of the Ca\(^{2+}\). We also observed increased cell surface fluorescence and nanodomain formation followed mixing even in the absence of added Ca\(^{2+}\). These results are consistent with a previous study which showed the addition of Ca\(^{2+}\) to a standing cell suspension did not initiate flocculation but subsequent agitation at 80 rpm resulted in floc formation (Stratford, Keenan 1987). (Our unpublished data confirmed this observation.) Under our current protocol of vortex-mixing at 2500 rpm for 5 minutes, the mixing-induced flocs were smaller than the flocs that formed after the addition of Ca\(^{2+}\). Therefore we can speculate that the role of Ca\(^{2+}\) in flocculin activation is similar to its role in mammalian cadherins, i.e. to induce or maintain an adhesion-active conformation (Takeichi 1990, Hyafil, Babinet & Jacob 1981). In the flocculins shear-induced conformational changes must also induce and maintain the active state.
Practical considerations. The forces needed to activate cell adhesion are comparable to those the yeast encounter in vivo and in the lab. Unfolding of the amyloid-containing T domains of Als5p or Als1p for single molecules in the AFM followed application of forces in the range of 50-100 pN (Alsteens et al. 2009, Alsteens et al. 2010, Beaussart et al. 2012). This force is similar to that encountered in flowing blood, or under flow in the natural environment (Charm, Kurland 1969). Physiological shear rates in vivo are in the range of 100-8000 s\(^{-1}\) in blood vessels and the extracellular matrix (Charm, Kurland 1969). Such a force is also similar to that applied by vortex mixing, where shear rates range from 209-6905 sec\(^{-1}\) (Bai et al. 2012) are applied to the cell surface in air-liquid interface. The product of shear rate and viscosity (8.9 x 10\(^{-3}\) dyne-sec-cm\(^{-2}\)) yields a resulting shear force of ~10-100 dyne-cm\(^{-2}\), or 1-10 pN-\(\mu\)m\(^{-2}\). Although this force appears less than the instantaneous force applied in AFM, its application over a minute’s time would lead to high T domain unfolding probability (Alsteens et al. 2009). Thus, Als proteins show activation under forces such as vigorous vortex mixing, which we have often used to resuspend cell pellets. The Flo adhesins were even more susceptible to activation by vortex-mixing (Figures 26 and 27). Therefore, cell preparation procedures will affect results of cell adhesion assays, and cells will need to be treated gently to achieve baseline aggregation ability (the equivalent of the quiescent cells in our assays). This increase in aggregation will allow us to study the fast kinetics of amyloid nanodomain formation with the use of time course confocal microscopy.

In summary, vortex-mixing measurably and reproducibly activated surface amyloid nanodomain formation to increase avidity of fungal adhesins. We have demonstrated similarity in activation of unrelated adhesins from two species of yeast. Bioinformatic analyses show that similar amyloid-forming sequences are common in fungal adhesins, as well as some bacterial
adhesins (Ramsook et al. 2010, Otoo et al. 2008). Indeed the importance of amyloid interactions has also been demonstrated in several other fungal and bacterial adhesion systems, including assembly of gram negative curlins (Ben Nasr et al. 1996, Olsen, Jonsson & Normark 1989, Olsen et al. 1998, Sjobring, Pohl & Olsen 1994), and gram positive adhesins including *Streptococcus mutans* P1(Oli et al. 2012), and *Bacillus subtilis* TasA(Romero et al. 2010, Romero et al. 2011). It remains to be seen if any of these other systems also show force-induced clustering and activation.
F. Table

Table 3. Fluorescence FACS analysis of thioflavin stained force activated cells.

Saccharomyces cerevisiae variant diastaticus MATa {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain MATa {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). Saccharomyces cerevisiae Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). S. cerevisiae strain W303-1B MATα leu2 ura3 ade2 trp1 (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p^WT or Als5p^V326N were used. Flow cytometry was done with BD Biosciences FACSCanto with an excitation of 405 nm and an emissions filter of 450 ± 50nm. 10^6 cells were in 12mm x 75 mm tubes with or without vortex-mixing and then brought to a final concentration of 1 μM ThT in a total volume of 1 ml in their respective buffer as mentioned above. The cells were filtered with a 70 μm filter before analysis. 20000 cells were monitored for each assay. The table presents the geometric mean of the fluorescence from thioflavin filter.

<table>
<thead>
<tr>
<th>Expressed Adhesin</th>
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<th>Vortex-mixed</th>
<th>Ratio</th>
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</thead>
<tbody>
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<td>142</td>
<td>161</td>
<td>1.13</td>
</tr>
<tr>
<td>Als5p^V326N</td>
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<td>1367</td>
<td>1.44</td>
</tr>
</tbody>
</table>
G. Figures

A

No vortex-mixing  Vortex-mixed
Empty Vector
Al5p
Al5p

B

Number of cells bound to beads (x10^7)

No vortex-mixing  Vortex-mixed
Empty vector  Al5p-WT  Al5p-V328N

C

Number of cells (x10^7)

Vortexing time (s)

D

Vortex-mixed  Vortex-mixed + 0.2mM TSA
Figure 25. The effects of vortex-mixing on adhesion and aggregation of *S. cerevisiae*. *S. cerevisiae* strain W303-1B *MATα leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p<sup>WT</sup> or Als5p<sup>V326N</sup> were used. Cells carrying an empty vector or expressing Als5p<sup>WT</sup> or Als5p<sup>V326N</sup> were vortex-mixed or not, then aggregated for 10 minutes with heat-denatured BSA-coated magnetic beads: (A) Bright-field micrographs of the cells. The dark-colored beads are 1 μm in diameter. Scale bars represent 20μm; (B) Quantification of cells adhering to beads. Error bars represent s.d. for n=4. A student t-test was performed. *** represents p<0.001. (C) Time course for activation of Als5p-expressing cells by vortex mixing at 2500 rpm. (D) Effects of anti-amyloid dye on vortex-activated adhesion and aggregation of *S. cerevisiae* cells expressing Als5p<sup>WT</sup>. A 10- minute aggregation assay in the absence or presence of 0.2 mM thioflavin S. Error bars represent s.e.m. of n=3.
Figure 26. Effects of turbulent flow from vortex-mixing on flocculation extent and rate of \textit{S. cerevisiae var. diastaticus} cells expressing \textit{Flo11p}. \textit{Saccharomyces cerevisiae} variant diastaticus \textit{MATa} \{\texttt{ura3 leu2-3, 112 his4}\} expressing Flo11p and the deletion strain \textit{MATa} \{\texttt{ura3 leu2-3, 112 his4 flo11:URA3}\} were kindly gifted by Dr. Anne Dranginis (St. John’s University). Cells were vortex-mixed or not, then flocculation was initiated by addition of 0.33 mM CaCl$_2$. Error bars represent s.e.m. (A) Flocculation of Flo11p-expressing cells with no vortex-mixing (n=6) and cells that were vortex mixed for 5 minutes (n=5); for \textit{flo11} deletion mutant, n=3. (B) Flocculation rates were calculated between 40s-80s. Inhibition by anti-amyloid ThS (0.2 mM) and CR (0.5mM) (n=3). * indicates a student T test p<0.05 , ** p<0.001 . (C) Effect of vortex-mixing on floc size in the absence of Ca$^{+2}$. Scale bar represent 20µm. (D) Kinetics of activation by vortex-mixing. Error bars represent s.e.m. for n=3.
Figure 27. The effects of turbulent flow from vortex-mixing on the flocculation of *S. cerevisiae* cells expressing Flo1p. *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B MATα {FLO1 gal1} was purchased from ATCC (Manassas, VA). Flo1p-expressing cells were vortexed mixed or not, then flocculation was initiated by addition of 1mM CaCl$_2$. (A) Flocculation rates in absence or presence of anti-amyloid dyes. Error bars represent standard deviation of triplicates. A student t-test was performed with *p<0.01* p value for vortex-mixed and no vortex-mixing cells has a value of 0.007524. The p value for vortex-mixing and the vortex-mixing in the presence of ThS is 0.003813. The p value for vortex-, mixing and vortexing mixing in the presence of Congo red is 0.06005. (B) Effect of vortex mixing time on activation of flocculation. (C) Effect of vortex mixing on floc size in the absence of CaCl$_2$. Scale bars represent 20µm.
Figure 28. Effects of vortex mixing on surface amyloid nanodomains. *Saccharomyces cerevisiae* variant *diastaticus MAT*a {ura3 leu2-3, 112 his4} expressing Flo11p and the deletion strain *MAT*a {ura3 leu2-3, 112 his4 flo11:URA3} were kindly gifted by Dr. Anne Dranginis (St. John’s University). *Saccharomyces cerevisiae* Flo1p-expressing strain BX24-2B *MATa* {FLO1} was purchased from ATCC (Manassas, VA). *S. cerevisiae* strain W303-1B *MATa leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) harboring the empty vector (pJL1-EV) or expressing Als5p$^{WT}$ or Als5p$^{V326N}$ were used. Confocal micrographs of cells stained with 500 nM ThT without vortex mixing (top row) or after 5 min vortex mixing (bottom row). Scale bars represent 5µm.
Figure 29. Effect of vortex-mixing on aggregation and surface nanodomains on *C. albicans* SC5314 cells. (A) Number of cells bound to beads after vortex mixing for different times. Error bars represent standard deviation for n=3. (B) ThT staining of control and vortex-mixed SC5314 cells. Scale bars represent 5µm; C. Inhibition of aggregation with amyloid dyes 0.2mM ThS and 0.5mM Congo red after vortex-mixing; scale bars represent µm (D) quantification of effects of vortex mixing on initial aggregation. Scale bars represent 100µm
**Figure 30. Effects of vortex-mixing on heat killed Als5p-expressing cells.** *S. cerevisiae* strain W303-1B *MATα leu2 ura3 ade2 trp1* (Rodney Rothstein, Columbia U.) expressing Als5p\textsuperscript{WT} or Als5p\textsuperscript{V326N} were used. Cells were vortex-mixed or not then stained with ThT (500 nM). Scale bars represent 5µm.
Figure 31. Effects of vortex mixing on heat-killed flocculin-expressing cells. *Saccharomyces cerevisiae* variant diastaticus MATΔ {ura3 leu2-3, 112 his4} expressing Flo11p was kindly gifted by Dr. Anne Dranginis (St. John’s University). (A) Effects of vortex-mixing on the flocculation rate of Flo11p-expressing cells after heat-killing. (B) Effects of vortex-mixing on the flocculation rate of Flo1p-expressing cells after heat-killing. Error bars represent standard deviation. (C) Floc size in heat-killed Flo11p-expressing cells. No CaCl₂ was added. Scale bars represent 100 µm.
Chapter V
Summary/Conclusions
Four new findings are supported by my data: 1. there are functional amyloids present in fungal adhesins Flo1p and Flo11p from *S. cerevisiae*, 2. amyloid-forming sequences in the flocculins mediate flocculation, 3. the amyloid nanodomains mediate force-sensitive catch-bonding, and 4. this force-sensitive amyloid formation on the yeast cell wall surface can be quantified.

**Amyloid-forming sequences in Flo1p and Flo11p.** *Candida albicans* adhesins have amyloid-forming sequences (Ramsook et al. 2010, Otoo et al. 2008). Yeast cells expressing *C. albicans* adhesins Als1p and Als5p are thioflavin T fluorescent and Congo red birefringent. Similarly, I have shown that the cell surface of Flo1p- and Flo11p-expressing *Saccharomyces cerevisiae* cells have ordered domains that are thioflavin T fluorescent and Congo red birefringent, two hallmarks of amyloids. This suggests that sequences in the Flo1p and Flo11p form amyloids.

Two different amyloid prediction programs, Tango and Waltz, predicted many different amyloid positive sequences in Flo1p and Flo11p. Tango positive sequences were isoleucine, threonine, and valine rich. The Waltz predicted sequences have various amino acid compositions, and are not necessarily isoleucine, threonine, and valine rich. One sequence from Flo1p was positive for both amyloid predictor programs. Though there were 2.5 times more amyloid positive sequences in Flo1p than Flo11p, both proteins responded the same way to amyloid binding dyes thioflavin T, thioflavin S, and Congo red. We do not know how many of these predicted sequences play a functional role.

**Flocculin amyloids mediate adhesion and agar invasion.** Amyloid-forming sequences in flocculins of *S. cerevisiae* mediated flocculation, agar invasion, and biofilm formation.
Aggregated cells were more birefringent and thioflavin T fluorescent suggesting an increase in ordered domains on the cell surface which is expected if flocculin amyloids mediate adhesion. Cell aggregation and biofilm formation decreased in the presence of amyloid-perturbing dyes. Additionally, amyloid-perturbing dyes inhibited agar invasion. Therefore Flo1p and Flo11p amyloids were important for activities of the flocculins including cell-to-cell adhesion, cell-to-substrate adhesion, and agar invasion.

**Amyloids mediate catch-bonding in adhesin-expressing cells.** We report here for the first time that catch bonding behavior in yeast cells was mediated by amyloid formation. In adhesin-expressing cells (Als1p, Als5p, Flo1p, and Flo11p) mechanical or shear stress increased aggregation in an amyloid-dependent manner. Cell binding to surfaces under shear stress were less likely to be washed off from the substrate than cells that were not stressed. This is characteristic of catch bonding. Catch bonding behavior was accompanied by the formation of amyloid nanodomains. Thus, environmental forces cause adhesin molecules to cluster at homotypic amyloid sequences. These nanodomains increased binding avidity of the adhesin-expressing cells to other cells (flocculation and aggregation assays) and to substrate surfaces (channel surface). These results show that amyloids mediate both sensing and response in the presence of force.

**Quantification of amyloids on force-activated adhesin-expressing cells.** We have devised ways of quantifying forces needed to activate aggregation, cell adhesion, and amyloids on the surface of yeast cells. Two different types of force, mechanical stress from vortex-mixing and shear stress from laminar flow increased adhesion and biofilm formation. Mechanical vortexing at 2500 rpm at around a minute was enough to increase adhesion. Shear rate of 0.8
dyne/cm² or greater increased yeast cell binding and biofilm formation. Different forces elicit a similar response of increasing adhesion in adhesin-expressing cells.

Additionally, we quantified amyloid formation in live whole cell yeast suspensions in response to force. Fluorescent confocal microscopy and flow cytometry were used to quantify surface amyloids. Force-activated cells had punctate nanodomains with increased thioflavin T staining. Collectively, the assays can now be used to quantify amyloids in other fungal adhesins. The quantification is fast and cost efficient. Different types of microbes can readily be screened in a short time frame for cell surface amyloids.

In conclusion, bioinformatic analyses have shown that amyloids are ubiquitous in yeast adhesins (Otoo et al. 2008, Ramsook et al. 2010). The studies in this dissertation show that amyloid formation mediates adhesion, agar invasion and biofilm formation. As amyloid proteins sense a change in flow rate; adhesion molecules cluster as a response to force. The nanodomains formed will increase binding avidity. Cells will bind tighter to each other and to surfaces in order not to be swept away, a characteristic of catch bonding. These roles highlight the importance of a cellular response under changing environments. Amyloids sense change and mediate a response mechanism beneficial to the microbial organism’s energy and resources.

Understanding the role of amyloids in microbial adhesion is important to the medical community and the billion dollar beer brewing and oil industries. Microbial biofilm formation is widespread, occurring in hospital catheter tubing and offshore oil pipelines. Biofilms forming in catheters cause infections. Catheters can be coated with anti-amyloid compounds to inhibit adhesion, thus biofilm formation. Biofilms can also develop in pipelines that carry oil and gas, causing expense and time for removal. Pipe surfaces can be designed to have anti-amyloid
properties to prevent clogs. More efficient force activated adhesion can be time- and cost-effective for the beer brewing industry. After the fermentation process, cells are separated from the ethanol-containing liquid by centrifugation. Cells that flocculate faster will decrease the need for buying and maintaining centrifugation equipment. These examples highlight the importance of studying the role of yeast adhesin amyloids in force-dependent adhesion and biofilm formation.
Chapter VI
Supplementary Information/Miscellaneous
A. Results

I. Amyloid binding dyes inhibit biofilm formation on polystyrene

Flo11p-expressing cells formed a 24 hour biofilm in polystyrene plates. Δflo11 cells did not form a dense biofilm in the polystyrene plates. Wells pretreated with 2 mM thioflavin S, 10 µM Congo red and 5 µM thioflavin T inhibited the cells from adhering and forming a biofilm on the plates (Figure 32). Alternatively, cells were allowed to grow and form a biofilm in the presence of dyes. 200 µM ThS, 10 µM CR and 5 µM ThT inhibited for formation of biofilm on the polystyrene plates (Figure 33). Pretreatment and treating cells at the same time with amyloid-perturbing dyes inhibited biofilm formation on polystyrene plates.

II. Vortex-mixing increases aggregation of C. albicans strain Day 185

Vortex-mixing for 5 minutes at 2500 rpm increases the aggregate size of C. albicans strain Day 185. Vortex mixing increased adhesion in 15 minute aggregation assays (Figure 34).

III. Constructing amyloid mutants of Flo11p

To generate amyloid mutants the plasmid a gift from Dr. Anne Dranginis lab of Yeplac181+FLO11+GPI was used. Restriction enzymes Bstz171 and SalI were used to cut the FLO11 gene out of the plasmid (Figure 35). The FLO11 fragment generated will be 4.237 kb. A small plasmid pBR322 is used to house the FLO11 gene with the same unique sites. This will make PCR-site directed mutagenesis easier in a small plasmid. The ligated FLO11 gene and the pBR322 plasmid are 7.005kb. The DNA was then transformed into E. coli,
purified and transformed into yeast. Colonies were screened for phenotypic characteristics. Transformed cells exhibited agar invasion (Figure 36).

PCR-site directed mutagenesis was carry out to generate the Flo11p amyloid mutants F1054N, V1032N and V1137N (Figure 37). Primers were designed and purchased from Invitrogen (Table 4). The DNA was then transformed into bacteria and subsequently purified and transformed into yeast cells of strain BY4742. There was only one colony in the cells transformed with F1054N, about 13 colonies for cells transformed with V1032N and no colonies grew for cells transformed with V1137N. The amyloid mutant F1054N showed a decrease in agar invasion (Figure 38). The inclusions of cells in the agar of F1054N mutant does not go as deep as the wildtype Flo11p-expressing cells. 200µM ThS did not inhibit the amyloid mutant form invading into the agar, however 30µM CR decreased the invasion.

IV. **Rhodamine labelled anti-amyloid Flo1p and Flo11p peptides.**

Synthetic anti-amyloid peptides of Flo1p and Flo11p were purchased from Rockefeller University (Table 5). The peptides were labelled with rhodamine. 200µg/ml of peptide was allowed to incubate with wildtype Flo1p and Flo11p expressing cells to assess the binding of the peptides to the cell surface. Fluorescence microscopy the rhodamine labelled-peptides AA-40, AA-41, and AA-43 of Flo11p bound to the cell surface. The anti-amyloid peptides show specificity to the Flo11p amyloid peptide. Interestingly, peptide AA-40 formed a haze around the cells, what I hypothesized as staining of extracellular matrix that might have amyloid properties. However the Flo1p anti-amyloid peptides did not stain the Flo1p expressing cells as well (Figure 39 and 40). AA-44 did not show any fluorescence while AA-45 and AA-46 show a haze around the cells.
B. Tables

**Table 4. Primers for site-directed mutagenesis of FLO11.** DNA sequence of *FLO11* was inputted into Invitrogen primer design server.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer sequence</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1032N</td>
<td>CCTACCACACCRGTAACTACAGTTAACTCAACCACCCGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GACGGTGGTTGAGTTAACTGTAGTTACAGGTGTGGTAGG</td>
<td>Antisense</td>
</tr>
<tr>
<td>F1054N</td>
<td>CAAAACCAGGTGGTAATTACAACCTACAAATGTCACCAAAAAACATTCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGAATGTTTTTGGTGCATTTGTAGTTGTAATTTACCACCACCTGGTTTTG</td>
<td>Antisense</td>
</tr>
<tr>
<td>V1137N</td>
<td>CACCCCTGTAAACAGCTAACCAACCACCGTTATTAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGTAACACCGGTGGTTGTTAGCTGTGTGTAACAGGGGTG</td>
<td>Antisense</td>
</tr>
</tbody>
</table>
Table 5. Anti-amyloid peptides of Flo1p and Flo11p. Anti-amyloid peptides for Flo1p and Flo11p were screened by one amino acid substitution of Tango and Waltz positive amyloid sequences. These peptides are <1% for β-aggregation as predicted by Tango and 0% as predicted by Waltz. Anti-amyloid peptides are synthesized by Rockefeller University. ++ presence of ring on the surface of cells, and - no ring.

<table>
<thead>
<tr>
<th></th>
<th>Anti-amyloid peptide sequence</th>
<th>Fluorescence image observations-presence of halo</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-40 Flo11p V1032N</td>
<td>PVTVPNSTTVV</td>
<td>+</td>
</tr>
<tr>
<td>AA-41 Flo11p F1054N</td>
<td>EITTNVTK</td>
<td>+</td>
</tr>
<tr>
<td>AA-42 Flo11p Q55N</td>
<td>DNQNIMQYTL</td>
<td>-</td>
</tr>
<tr>
<td>AA-43 Flo11p Y196N</td>
<td>DFPGFNNW</td>
<td>+</td>
</tr>
<tr>
<td>AA-44 Flo1p V356etc</td>
<td>ETIINIR</td>
<td>-</td>
</tr>
<tr>
<td>AA-45 Flo1p Y211N</td>
<td>EGTVYMAGYYYP</td>
<td>-</td>
</tr>
<tr>
<td>AA-46 Flo1p Y215N</td>
<td>EGTVYMAGYN</td>
<td>+</td>
</tr>
</tbody>
</table>
C. Figures

Figure 32. Effects of pretreating polystyrene wells with anti-amyloid dyes on biofilm formation. Wells were pretreated with amyloid binding dyes for 24 hours and then washed 3 times with sodium acetate buffer. Then an inoculum of cells were added to each well and allow to grow in YPAD media for twenty hours. The wells were then washed and stained with crystal violet. Images were taken with an inverted microscope with 10x objective.
Figure 33. Effects of anti-amyloid dyes on biofilm formation. Untreated wells were inoculated with cells and grown for 24 hours in YPAD media. The wells were then washed and stained with crystal violet. Images were taken with an inverted microscope with 10x objective.
Figure 34. Vortex-mixing increases aggregation of *Candida albicans* strain Day 185 cells. 15 minutes aggregation assays were done with Day 185 after vortex-mixing. Images were taken with Nikon microscope using 20x objective.
Figure 35. Restriction digest of YePlac1181+*FLO11*+GPI. Gel electrophoresis of digesting FLO11 with Bstz171 and SalI restriction enzymes. Dark bright band in ladder refers to 5kb.
Figure 36. Agar invasion of a colony of BY4742 transformed with \textit{FLO11}+GPI anchor with the pBR322 plasmid. Images were taken with a Nikon dissecting scope at a total magnification of 15x.
1. Ladder
2. Template DNA YEPlac 181+GPl+FLO11
3. PCR control 1 µl DNA
4. 2.5ul DNA V1032N with 4µl 0.1mM primer
5. 2.5ul DNA F1054N with 4µl 0.1mM primer
6. 2.5ul DNA V1137N with 4µl 0.1mM primer
7. Control 5ul DNA
8. 5ul DNA V1032N with 4µl 0.1mM primer
9. 5ul DNA F1054N with 4µl 0.1mM primer
10. 5ul DNA V1137N with 4µl 0.1mM primer

Figure 37. PCR product of site-directed mutagenesis of FLO11. PCR product from site-directed mutagenesis of FLO11 were run on an agarose gel.
Figure 38. Invasion assay with F1054N mutant in the presence of anti-amyloid dyes. Cells were allowed to grow YPAD plates with and without anti-amyloid dyes for two weeks. The cells were then washed off and a section of the agar was cut out. Images were taken of the cross-sections using a Nikon dissecting microscope at 15X total magnification.
Figure 39. **Rhodamine-labelle**d anti-amyloid **peptides** from **Flo11p**. 200μg/ml of peptide was incubated with cells for 30 minutes and then washed three times. Fluorescence microscopy was done with a Nikon Fluorescence Microscope with a 40X objective.
Figure 40. Rhodamine-labelled anti-amyloid peptides from Flo1p. 200µg/ml of peptide was incubated with cells for 30 minutes and then washed three times. Fluorescence microscopy was done with a Nikon Fluorescence Microscope with a 40X objective.
Chapter VII

References


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