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# Mechanism of three-component collision to produce ultrastable pRNA three-way junction of Phi29 DNA-packaging motor by kinetic assessment

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## ABSTRACT

RNA nanotechnology is rapidly emerging. Due to advantageous pharmacokinetics and favorable in vivo biodistribution, RNA nanoparticles have shown promise in targeted delivery of therapeutics. RNA nanotechnology applies bottom-up assembly, thus elucidation of the mechanism of interaction between multiple components is of fundamental importance. The tendency of diminishing concern about RNA instability has accelerated by the finding of the novel thermostable three-way junction (3WJ) motif of the phi29 DNA-packaging motor. The kinetics of these three components, each averaging 18 nucleotides (nt), was investigated to elucidate the mechanism for producing the stable 3WJ. The three fragments coassembled into the 3WJ with extraordinary speed and affinity via a two-step reaction mechanism,  $3WJ_b + 3WJ_c \leftrightarrow 3WJ_{bc} + 3WJ_a \leftrightarrow 3WJ_{abc}$ . The first step of reaction between  $3WJ_b$  and  $3WJ_c$  is highly dynamic since these two fragments only contain 8 nt for complementation. In the second step, the  $3WJ_a$ , which contains 17 nt complementary to the  $3WJ_{bc}$  complex, locks the unstable  $3WJ_{bc}$  complex into a highly stable 3WJ. The resulting pRNA-3WJ is more stable than any of the dimer species as shown in the much more rapid association rates and slowest dissociation rate constant. The second step occurs at a very high association rate that is difficult to quantify, resulting in a rapid formation of a stable 3WJ. Elucidation of the mechanism of three-component collision in producing the ultrastable 3WJ proves a promising platform for bottom-up assembly of RNA nanoparticles as a new class of anion polymers for material science, electronic elements, or therapeutic reagents.

**Keywords:** RNA nanotechnology; nanobiotechnology; bacteriophage; phi29; RNA nanoparticle; viral DNA packaging motor

## INTRODUCTION

The field of RNA nanotechnology has recently undergone rapid expansion mainly due to the fact that RNA has the simplicity characteristic of DNA yet the versatile functionality of proteins (Masquida et al. 1997; Guo et al. 1998; Lilley 2000; Leontis and Westhof 2003; Lescoute and Westhof 2006; Guo 2010; Bindewald et al. 2011; Ye et al. 2012). With the combination of the advantage of these two materials, RNA can easily be designed and constructed through bottom-up assembly and plays diverse roles (Felden et al. 1996; Chworos et al. 2004; Shu et al. 2004; Khaled et al.

2005; Jaeger and Chworos 2006; Afonin et al. 2008). The wide-ranging functionalities of RNA make it a prime candidate for applications in sensing operations, computer parts (Rinaudo et al. 2007; Breaker 2008; Win and Smolke 2008; Benenson 2009; Xie et al. 2010, 2011; Qiu et al. 2013; Shu et al. 2014a), imaging reagents (Calzada et al. 2012; Chen et al. 2012; Paige et al. 2012; Kellenberger et al. 2013), NEM devices (Noy 2011), and therapeutics including the delivery, specific targeting, and treatment of cancer and viral infections (Guo et al. 1998; Shu et al. 2003, 2004, 2011a, 2013; Yingling and Shapiro 2007; Severcan et al. 2009; Afonin et al. 2011; Chang et al. 2012; Delebecque et al. 2012; Haque et al. 2012; Lee et al. 2012, 2015b; Taberner et al. 2013). The

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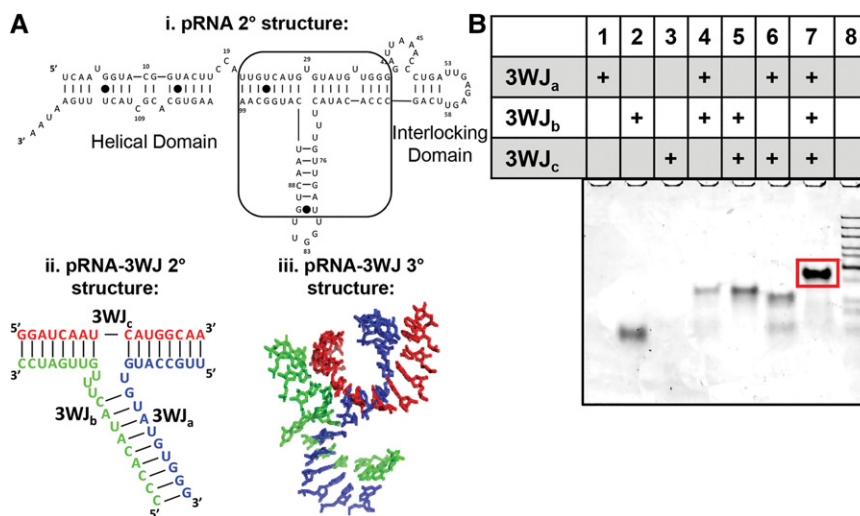
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use of RNA as a delivery vehicle provides several benefits over other systems and technologies. Firstly, RNA nanoparticles can be produced with known stoichiometry and high reproducibility (Guo et al. 1998; Lilley 2000; Shu et al. 2004, 2011a; Afonin et al. 2010; Jasinski et al. 2014; Khisamutdinov et al. 2014a,b), as RNA is composed of simple building blocks with predictable secondary structure. RNA aptamers can be used for specific targeting of cell groups through binding to cell surface receptors much like a protein ligand or antibody (Cerchia et al. 2009; Abdelmawla et al. 2011; Shu et al. 2011b). Furthermore, the replacement of protein-targeting reagents with nucleic acids prevents the induction of antibodies (Abdelmawla et al. 2011; Guo et al. 2012; Shu et al. 2014b), allowing for repeated administration. RNA nanoparticles are typically between 10 and 50 nanometers in size, meaning they are retained in the human body yet are small enough to pass through cell membranes through endocytosis (Maeda 2001; Gao et al. 2005; Jain 2005; Li and Szoka 2007; Maeda et al. 2013; Jasinski et al. 2014; Khisamutdinov et al. 2014b). In *in vivo* testing, RNA nanoparticles have shown favorable pharmacokinetic and biodistribution properties, as they are able to avoid accumulation in healthy organs and tissues with efficient retention in tumors (Shu et al. 2011a; Haque et al. 2012). Additionally, RNA possess unique *in vivo* attributes: (i) transcription, termination, splicing, and processing allow for *in vivo* RNA production; (ii) riboswitches (Mulhbachter et al. 2010) and ribozymes (Sarver et al. 1990; Hoeplich et al. 2003; Lilley 2003; Winkler et al. 2004; Liu et al. 2007) result in *in vivo* processing and possible assembly into nanoparticles in cell for special functions such as intracellular computation (Rinaudo et al. 2007; Breaker 2008; Win and Smolke 2008; Benenson 2009; Xie et al. 2010, 2011; Qiu et al. 2013; Shu et al. 2014a); (iii) RNA can self-assemble without external forces (Shu et al. 2014a); and (iv) RNA is stable in acidic environments allowing for survival in endosome (Guo et al. 2012). Even with these advantages, RNA nanotechnology still has many hurdles that it must overcome in order to be used in therapeutics. Most notably, RNA is known to have stability issues and is prone to degradation by RNase that has since been solved through backbone modifications, such as 2'-fluoro (Pieken et al. 1991; Liu et al. 2011), 2'-O-methyl (Czaderna et al. 2003), or locked nucleotides (Kaur et al. 2006). The dissociation of the self-assembled RNA nanoparticles at low concentrations and elevated temperatures in *in vivo* environments makes it obligatory to address the dynamics and kinetics of RNA nanoparticle assembly (Privalov and Filiminov 1978; Freier et al. 1986; Jaeger et al.

1993; Kawasaki et al. 1993; Lesnik and Freier 1995; Sugimoto et al. 1995; Gyi et al. 1996; Diamond et al. 2001; Brunel et al. 2002).

Recently, a novel ultrastable RNA motif was found in the packaging RNA (pRNA) of the phi29 dsDNA packaging motor (Fig. 1) that has since diminished the concerns of RNA nanoparticles dissociating at low concentrations found *in vivo* because of its unusually high thermostability (Shu et al. 2011a). This motif is a three-way junction (3WJ) that serves as the central core structure of the pRNA and connects the helical domain (Zhang et al. 1994) with the two interlocking looped regions of the pRNA (Reid et al. 1994). It has been determined that the 3WJ serves as the central folding domain and provides the overall high stability seen in pRNA. The motif can be formed from three individual RNA oligos at a high yield even in the absence of metal ions (Shu et al. 2011a). The novelty behind this RNA structure is in the ease of formation, a remarkably low Gibbs free energy ( $\Delta G^\circ$ ) of  $-28$  kcal/mol, and a high melting temperature ( $T_m$ ) of  $59.3^\circ\text{C}$  (Binzel et al. 2014). Furthermore, it has been proven that the pRNA-3WJ allows the inclusion of RNA functional moieties such as receptor-binding aptamer, siRNA, ribozyme, miRNA, or riboswitch, resulting in the production of functional nanoparticles (Shu et al. 2011a; Lee et al. 2015a; Rychahou et al. 2015). The addition of functional groups did not interfere with the proper folding of the 3WJ or the added functional moieties. Previously, attempts have been made to elucidate the mechanism for the kinetic stability of the pRNA-3WJ, yet the 3WJ was found to be too stable to detect its dissociation by the current technology of radioactive chasing (Shu et al. 2011a). However, thermodynamic studies were previously completed on the pRNA-



**FIGURE 1.** Overview of the Phi29 pRNA and the three-way junction (3WJ). (A) (i) Secondary structure of the phi29 pRNA monomer with the pRNA-3WJ outlined by the box, which connects the helical domain to the interlocking procapsid binding domains. (ii) Secondary structure and sequence of the pRNA-3WJ and (iii) the crystal structure of the pRNA-3WJ. (B) Assembly gel of the pRNA-3WJ from the three short RNA oligo strands on 12% native PAGE. The 3WJ (lane 7) shows efficiency of folding and particle homogeneity before purification.

3WJ (Binzel et al. 2014). From the association and melting profiles in the thermodynamic studies, a single transition step was seen, hinting to the pRNA-3WJ folding in a single step of all three strands. Furthermore, there was no evidence of a dimer intermediate in the 3WJ formation.

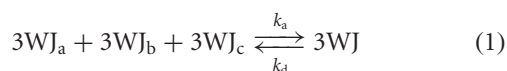
The understanding of the mechanism of assembly of the pRNA-3WJ and the interaction of the RNA strands is of fundamental importance, but the study of three-component collisions has not been well-studied in chemistry, biology, or material sciences. Here, we investigated the kinetic properties of the pRNA-3WJ using surface plasmon resonance (SPR) to study its association and disassociation using bottom-up RNA construction nanotechnics. We provided real-time examination of the three-component collision of the pRNA-3WJ. Through these studies, the pRNA-3WJ proves to be ultrastable and only disassociates at extremely low concentration, which is atypical to other well-studied double-stranded RNA or dimeric macromolecules. Furthermore, the pRNA central motif relies heavily on each of the three strands for rapid folding and slow dissociation rates.

## RESULTS AND DISCUSSION

### Determination of kinetic parameters of the pRNA-3WJ

The Bio-Rad ProteOn XPR36 allows for real-time monitoring and analysis of molecule association and dissociation. SPR allows for examination of interactions between molecules on a prism surface. In this case, the ligand RNA strand is bound through a biotin label to the neutravidin-labeled chip surface. As mass accrues or decreases on the chip surface either by ligand labeling or interactions between the ligand and analyte, the angle of light refraction through the chip prism and resonance is changed, thus creating a response that then can be fit with kinetic models. The Bio-Rad ProteOn XPR36 system allows for the observation of 36 interactions at the same time while including controls of ligand and analyte free spots, thus providing a platform for elaborate studies of the association and dissociation of the pRNA-3WJ. Through real-time analysis, we are able to calculate the dissociation constant ( $K_D$ ) by measuring the rates of reactions moving toward 3WJ complex formation and dissociation to single RNA strands.

Through classic definitions of kinetics, all chemical reactions are viewed to reach equilibrium where there is a balance in the production of products and reactants. In the case of the pRNA three-way junction, a single-step chemical reaction is described by Equation 1 below:



and the rate of forming pRNA-3WJ can be written as

$$\frac{d[3WJ]}{dt} = k_a[3WJ_a][3WJ_b][3WJ_c] - k_d[3WJ], \quad (2)$$

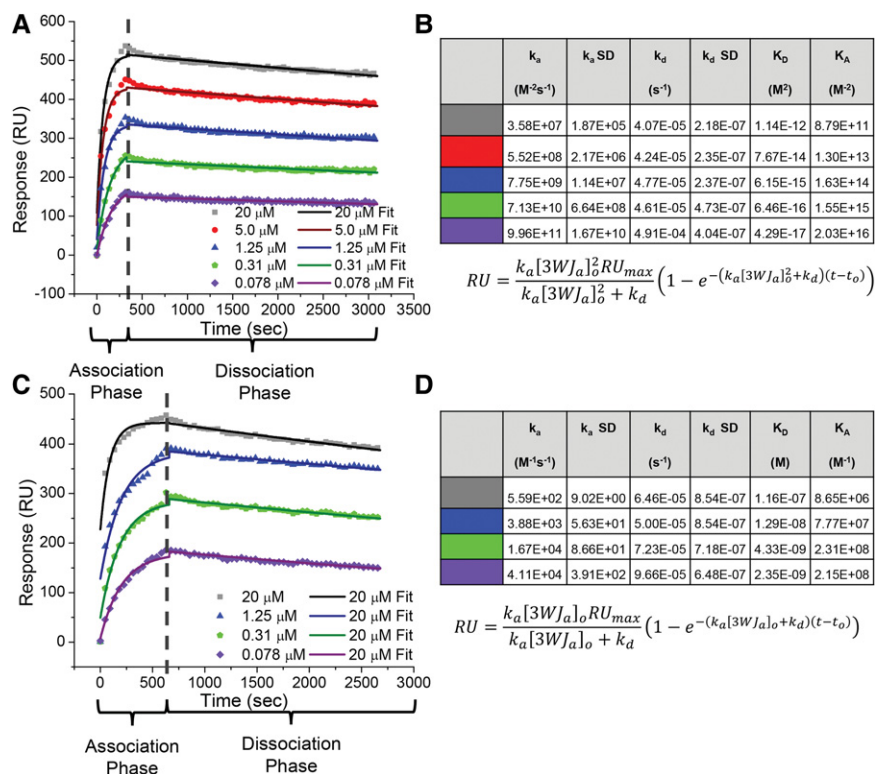
where  $k_a$  is the rate constant of the pRNA-3WJ formation, and  $k_d$  is the rate constant of dissociation into individual RNA strands in units of  $M^{-2} s^{-1}$  and  $s^{-1}$ , respectively. At equilibrium, the rates of forward and backward reactions are equal to each other, creating a balance between formation of the pRNA-3WJ and single-stranded RNA. Together, the two rate constants can be combined to define the disassociation and association constants.

$$K_D = \frac{k_d}{k_a} \quad \text{and} \quad K_A = \frac{k_a}{k_d}. \quad (3)$$

Based on previous experiments (Binzel et al. 2014), it was believed that the pRNA-3WJ was undergoing a single association and dissociation step following what is seen in Equations 1 and 5 (Shu et al. 2011a; Binzel et al. 2014). Using SPR, association and disassociation of the pRNA-3WJ was examined using the  $3WJ_a$  strand immobilized onto the chip surface, hence acting as the ligand, followed by co-injecting  $3WJ_b$  and  $3WJ_c$  strands, acting as analytes. A single transition is seen in the association over a range of concentrations from 20  $\mu M$  to 78 nM. Based on the previous idea that the pRNA-3WJ formed in a single step from the three components, the data were fit to a Langmuir three-component pseudo-first-order model using Equation 5, solving for the reaction rate constants (Fig. 2A,B). In these experiments, a relatively constant dissociation averaged  $4.52 \times 10^{-5} s^{-1}$ ; this value is within expected range for biological samples. However, the association rate constant of the pRNA-3WJ was seen changing on order of magnitudes, meaning a constant association rate across tested concentrations of 20–0.078  $\mu M$ . This clearly negates first-order chemical reactions and indicates error in the assembly kinetic parameters, as one would expect the reaction rate to change with varying concentration leading to a constant  $k_a$ .

### $K_D$ calculation of pRNA-3WJ dimers

In order to confirm RNA/RNA interactions on the SPR chip were in fact the formation of the 3WJ, dimers of the pRNA-3WJ were tested and assembled (Table 1; Fig. 3). In each of the dimer interactions ( $3WJ_{ab}$ ,  $3WJ_{ac}$ , and  $3WJ_{bc}$ ), association and dissociation periods were produced. Furthermore, once the data were fit with a pseudo-first-order Langmuir model due to the expected 1:1 interaction between ligand and analyte, it was seen that each of the calculated  $k_a$ s and  $k_d$ s produced were constant and concentration independent as expected by kinetics. This means the pRNA-3WJ dimers were forming properly on the chip surface. Interestingly, in comparing the association rate constants of each of the dimer species, the  $3WJ_{bc}$  dimer produced the most rapid “on” rate with a  $k_a$  of  $1.37 \times 10^5 M^{-1} s^{-1}$ ; this is nearly an order of magnitude higher than either the  $3WJ_{ab}$  or the  $3WJ_{ac}$  dimers. Here the association rate constant changes units to  $M^{-1} s^{-1}$  as only two components are interacted, whereas before three components were fused, resulting in the  $M^{-2} s^{-1}$  unit. This



**FIGURE 2.** SPR of the pRNA-3WJ. (A) Assembly of the 3WJ of three-component strands. 5'-Biotin-labeled 3WJ<sub>a</sub> was first immobilized to the SPR chip surface. 3WJ<sub>b</sub> and 3WJ<sub>c</sub> strands were mixed at equal, varying concentrations, ranging from 20  $\mu$ M to 78 nM, and injected across the chip surface for 330 sec (association phase). Following the interaction of the 3WJ strands, blank TMS buffer was injected across the chip surface for 2700 sec (dissociation phase). The association and dissociation curves were fit using a three-component pseudo-first-order model. (B) Calculated kinetic parameters from SPR curve following a three-component pseudo-first-order kinetic model. (C) SPR assay for pRNA-3WJ under the assumption of previous dimer formation data. 5'-Biotin-labeled 3WJ<sub>a</sub> was first bound to the SPR chip surface. 3WJ<sub>b</sub> and 3WJ<sub>c</sub> strands were mixed at equal, varying concentrations, ranging from 20  $\mu$ M to 78 nM, and were injected across the chip surface for 660 sec followed by blank TMS buffer for 2700 sec. Association and dissociation curves were fit using a two-component pseudo-first-order model. (D) Kinetic parameters from SPR curves following a two-component pseudo-first-order kinetic model.

increase in association rate may be due to less internal structuring of the 3WJ<sub>c</sub> strand, as we see no ethidium bromide staining of the single strand, thus giving a better diffusion to the 3WJ<sub>b</sub> compared to the 3WJ<sub>a</sub> forming the 3WJ<sub>ab</sub> dimer (Fig. 1A). In comparing the dissociation rate constants ( $k_d$ ) of the dimers, the 3WJ<sub>ab</sub> dimer is seen to be the most stable ( $6.78 \times 10^{-5} s^{-1}$ ), followed by 3WJ<sub>ac</sub> with a  $k_d$  of  $6.34 \times 10^{-4} s^{-1}$ , and finally the weakest dimer being the 3WJ<sub>bc</sub> ( $3.95 \times 10^{-3} s^{-1}$ ). This indicates that the 3WJ<sub>bc</sub> dimer not only would form the quickest but also dissociates the most

rapidly. Additionally, out of concern that the biotin end label on the ligand RNA interfered or altered the binding of RNA complexes on the SPR chip surface, duplicate studies of dimer interactions were completed by switching the ligand strand to analyte and analyte to ligand through changing the biotin label to the second strand. Through these interactions no obvious change in association profiles or  $k_a$  values were seen indicating the biotin label allows for the binding of the ligand strand to the chip surface, showing no significant effect on RNA folding.

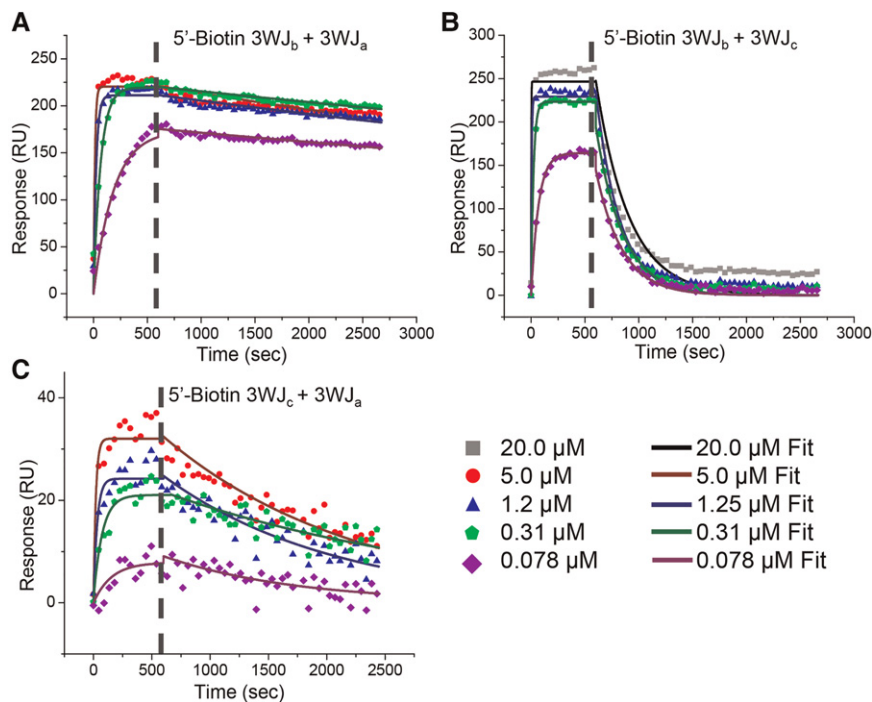
Next, in hopes of gaining insight into the assembly mechanism of the pRNA-3WJ, the association rates of the dimer species were compared with the association rate of the tested three-component one-step reaction of the pRNA-3WJ. This was completed by calculating concentration-independent association rates ( $k_a'$ ) by taking the fitted rate constants and multiplying by the concentration of the studied interaction resulting in an  $s^{-1}$  unit (Table 2). It is important to note that the pRNA-3WJ was multiplied by a concentration squared value while dimers only by concentration, due to the difference in association reaction orders. Surprisingly, it is seen that the pRNA-3WJ produces a rate of reaction on average an order of magnitude below the 3WJ<sub>bc</sub> dimer. This indicates that the formation of the dimer species is occurring before the formation of the pRNA-3WJ, thus creating an intermediate step that was previously not seen in thermodynamic studies (Binzel et al. 2014).

### Determination of time constants of each pRNA-3WJ strand

To further gain insight into the formation of a dimer species, time-based strand displacement was completed on each of the individual pRNA-3WJ strands. Using a time-based strand replacement electrophoretic mobility shift assay (EMSA),

**TABLE 1.** Kinetic parameters of pRNA-3WJ dimer species

Ligand	Analyte	$k_a$ ( $M^{-1} s^{-1}$ )	$k_a$ error	$k_d$ ( $s^{-1}$ )	$k_d$ error	$K_D$ (M)	$K_A$ ( $M^{-1}$ )
3WJ <sub>b</sub>	3WJ <sub>a</sub>	$4.18 \times 10^4$	$2.55 \times 10^2$	$6.78 \times 10^{-5}$	$2.71 \times 10^{-7}$	$2.30 \times 10^{-9}$	$6.73 \times 10^8$
3WJ <sub>c</sub>	3WJ <sub>a</sub>	$3.59 \times 10^4$	$1.37 \times 10^3$	$6.34 \times 10^{-4}$	$8.86 \times 10^{-6}$	$2.71 \times 10^{-8}$	$6.17 \times 10^7$
3WJ <sub>b</sub>	3WJ <sub>c</sub>	$1.37 \times 10^5$	$6.31 \times 10^3$	$3.95 \times 10^{-3}$	$1.95 \times 10^{-5}$	$2.88 \times 10^{-8}$	$3.49 \times 10^7$



**FIGURE 3.** SPR assay for pRNA-3WJ dimers. Dimer species that make up the pRNA-3WJ were examined through SPR at varying concentrations ranging from 20  $\mu\text{M}$  to 78 nM. The injected RNA strand was injected for 660 sec (association phase) followed by blank TMS buffer for 2700 sec (dissociation phase). The histograms were then fit using a two-component pseudo-first-order Langmuir model for bound 5'-biotin 3WJ<sub>b</sub> and 3WJ<sub>a</sub> (A), bound 5'-biotin 3WJ<sub>b</sub> and 3WJ<sub>c</sub> (B), bound 5'-biotin 3WJ<sub>c</sub> and 3WJ<sub>a</sub> (C).

time constants were calculated for each of the RNA strands in order to examine which, if any, of the strands is responsible for the fast folding that has been seen in the pRNA-3WJ and the stability at high and low concentrations. The results show that the 3WJ<sub>a</sub> and 3WJ<sub>b</sub> strands produce a similar  $\tau$  of 38.89 min and 35.93 min, respectively, while the 3WJ<sub>c</sub> showed a  $\tau$  of only 11.81 min (Fig. 4). The longer times for the 50% strand replacement of the 3WJ<sub>a</sub> and 3WJ<sub>b</sub> strands suggest that the structure and property of the 3WJ<sub>c</sub> strand is special and unique. Using ethidium bromide or SYBR-green staining within gels, the 3WJ<sub>c</sub> is undetectable, whereas both 3WJ<sub>a</sub> and 3WJ<sub>b</sub> are detected, indicating a lack in internal structuring of the 3WJ<sub>c</sub> strand (Shu et al. 2011b). From these studies, it was determined that 3WJ<sub>a</sub> and 3WJ<sub>b</sub> strands are es-

sential for the strong stability of the pRNA-3WJ motif; however, the high reactivity of the 3WJ<sub>c</sub> strand allows for the rapid association seen in SPR studies and previous thermodynamic studies (Binzel et al. 2014). These results further confirm and support the SPR dimer studies showing a rapid rate of reaction from the 3WJ<sub>c</sub>-containing dimers with a strong stability or dissociation of the 3WJ<sub>ab</sub> dimer species.

### Association mechanism of the pRNA-3WJ

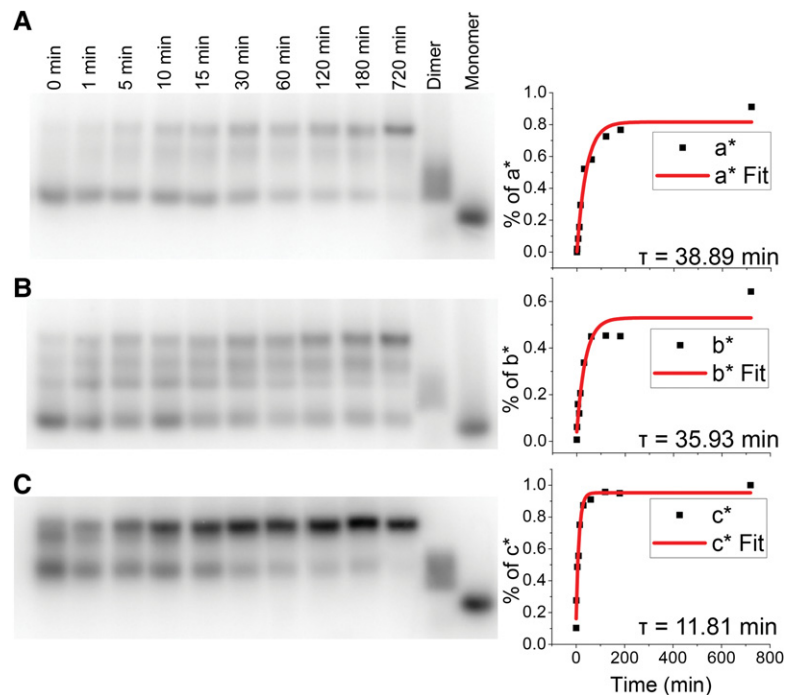
The previous thermodynamic studies on the pRNA-3WJ showed that the three RNA fragments associated very rapidly and the formation of a dimer intermediate was undetected. While a one-step association between the three strands seemed unlikely, it was believed that the pRNA-3WJ formed through the initiation of a pseudo-dimer with a very rapid addition of the third RNA strand. Here we have found that in fact the 3WJ<sub>b</sub> and 3WJ<sub>c</sub> strands form a dimer species at a rapid rate and faster than the modeled association as three strands together. Thus,

the two analytes (3WJ<sub>b</sub> and 3WJ<sub>c</sub>) were forming into a dimer complex before reaching the chip surface and being able to interact with the 3WJ<sub>a</sub> ligand strand on the chip surface. With this idea the pRNA-3WJ data were reanalyzed to follow a pseudo-first-order reaction that would result in an assembly mechanism of  $3WJ_b + 3WJ_c \leftrightarrow 3WJ_{bc} + 3WJ_a \leftrightarrow 3WJ_{abc}$  (Fig. 2C,D). Here the same dissociation rates were observed as in the three-component, one-step system. Additionally, the rates of association were independent of concentrations tested, leading to varying rate constants. This again negates first-order reaction laws; however, the varying rate of association constants can be attributed to using a range of working concentrations and thus was observed  $k_a$ s several orders of magnitude above the real association rate constant. This

**TABLE 2.** Association and dissociation rates of pRNA-3WJ and its components

	Ligand Analyte	$k_a'$ ( $\text{s}^{-1}$ ) <sup>a</sup>				$k_d$ ( $\text{s}^{-1}$ ) ( $\times 10^{-3}$ )			
		3WJ <sub>b</sub> 3WJ <sub>a</sub>	3WJ <sub>c</sub> 3WJ <sub>a</sub>	3WJ <sub>b</sub> 3WJ <sub>c</sub>	3WJ <sub>a</sub> 3WJ <sub>b</sub> + 3WJ <sub>c</sub>	3WJ <sub>b</sub> 3WJ <sub>a</sub>	3WJ <sub>c</sub> 3WJ <sub>a</sub>	3WJ <sub>b</sub> 3WJ <sub>c</sub>	3WJ <sub>a</sub> 3WJ <sub>b</sub> + 3WJ <sub>c</sub>
Concentration	$5.00 \times 10^{-6}$	0.0846	0.0502	0.650	0.0138	0.0850	0.601	3.25	0.0424
	$1.25 \times 10^{-6}$	0.0415	0.0312	0.185	0.0121	0.0715	0.679	4.45	0.0477
	$3.10 \times 10^{-7}$	0.0152	0.0129	0.0414	0.00685	0.0550	0.378	3.99	0.0461
	$7.80 \times 10^{-8}$	0.00533	0.00567	0.0105	0.00606	0.0598	0.879	4.11	0.0491

<sup>a</sup>Concentration-independent association rates were calculated by multiplying fit association rate constants ( $k_a$ ) by their concentrations.



**FIGURE 4.** Time constants of 3WJ strands by electrophoretic mobility shift assays (EMSA). The half-life of each 3WJ strand in the pRNA-3WJ was calculated by EMSA using  $^{32}\text{P}$ -labeled free strands, replacing unlabeled strands on assembled pRNA-3WJ. The transition of monomer to 3WJ was then plotted, and the data were fitted to calculate  $\tau$ . (A) 3WJ<sub>a</sub>, (B) 3WJ<sub>b</sub>, and (C) 3WJ<sub>c</sub>.

means that the rate of the reaction for the  $3\text{WJ}_{bc} + 3\text{WJ}_a \leftrightarrow 3\text{WJ}_{abc}$  is occurring much quicker than the observable level on SPR and the required concentrations needed to slow the reaction to an observable level would be too low to be sensed on the SPR chip surface. This situation differs from the previous changing association rate constants seen in the one-step association fitting due to the fact that here we know from the timing scale and the dimer studies that the 3WJ<sub>bc</sub> dimer is forming before the two strands are able to interact with the 3WJ<sub>a</sub> ligand bound to the chip. This leads to a simple 1:1 interaction of the 3WJ<sub>a</sub> ligand with the 3WJ<sub>bc</sub> dimer flowing across the chip. Additionally, through electrophoresis studies, a 1:1:1 interaction is always seen further supporting the Langmuir model used in the SPR studies.

Therefore, it is proposed that the pRNA-3WJ undergoes a two-step reaction, in which the second reaction takes place immediately following the formation of the dimer intermediate at a rate that is too fast to currently quantify on SPR (Fig. 5). This high reaction rate is due to the fact that the initiation of 3WJ<sub>bc</sub> formation increases the number of bases for binding from eight or nine bases from the single strand to 17 bases on the duplex for interaction with the 3WJ<sub>a</sub> strand (Fig. 5, middle panel). This increase in bases results in a higher probability of reaction per collision between the RNA dimer and the third strand, giving a significantly increased rate. Furthermore, this increase in base-pairing from 8 to 17 nucleotides (nt) significantly lowers the Gibbs free energy of formation ( $\Delta G^\circ$ ), indicating the reaction will occur more spontane-

ously. With the increase of nucleotides upon formation of the 3WJ<sub>bc</sub>, the 3WJ<sub>a</sub> is recruited to form the completed 3WJ with a much higher affinity. Thus, this second high-speed reaction relies on the formation of the 3WJ<sub>bc</sub> dimer that is the rate-limiting factor for 3WJ assembly. Additionally, due to the rapid reaction of the second step, only a low concentration of the dimer intermediate is needed to drive the 3WJ assembly. Thus, to the naked eye the assembly of the pRNA-3WJ is seemingly a singular step reaction without the evidence of an intermediate step.

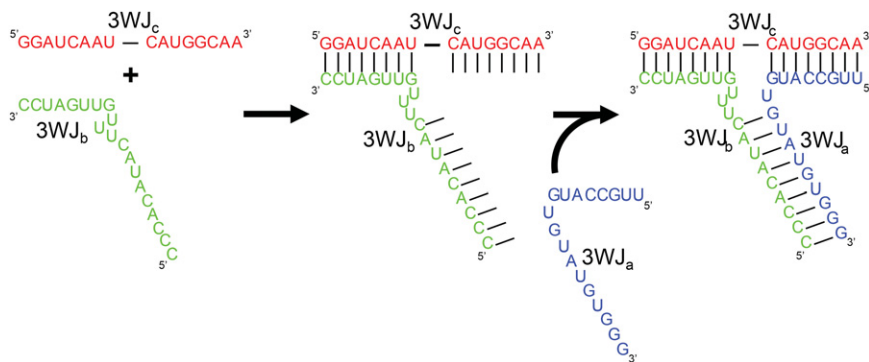
Furthermore, each strand and helical region of the pRNA-3WJ plays an important role in rapid formation and high stability. As discussed earlier, from the  $\tau$  values we know that the 3WJ<sub>c</sub> strand is highly reactive; this results in the highest  $k_a$  of the 3WJ<sub>bc</sub> dimer. Although the dimer between the 3WJ<sub>b</sub> and the 3WJ<sub>c</sub> strands forms the quickest, it also displays the shortest dissociation rate, showing that the pRNA-3WJ stability is provided through the other helical domains. The 3WJ<sub>a</sub> and 3WJ<sub>c</sub> strands

provide the high stability of the 3WJ, as shown in their long half-lives and the slow  $k_d$  seen in SPR studies of the 3WJ<sub>ab</sub>. However, although the pRNA-3WJ relies heavily on the 3WJ<sub>ab</sub> helix region for its stability, the pRNA-3WJ itself is the most stable motif, as shown in having the slowest “off” rate ( $k_d$ ) (Table 2). This can be attributed to the fact that if one helical region of the pRNA-3WJ breaks, the local concentration of the strand is very high compared to what would be seen in solution, thus initiating an immediate refolding of that helical region as the second half of the strand is still attached to the pRNA-3WJ. As a result of these kinetic studies, the high association rate and slow dissociation rate of the pRNA-3WJ result from contributions of each of the RNA strands, leading to a three-component collision.

## Conclusions

The pRNA-3WJ formation follows a two-step assembly mechanism of  $3\text{WJ}_b + 3\text{WJ}_c \leftrightarrow 3\text{WJ}_{bc} + 3\text{WJ}_a \leftrightarrow 3\text{WJ}_{abc}$ . The 3WJ<sub>bc</sub> dimer formation is the first step, occurring at a slower and less dynamic rate, while the formation of the pRNA-3WJ occurs at a much faster rate since each of the two-component pairs only contain 8 nt for complementation. While assembly of each dimer resulted in an uninterrupted 17 nt sequence, the third complementary component locks the unstable 3WJ<sub>bc</sub> complex into a highly stable 3WJ<sub>abc</sub>. It is suggestive that dimeric intermediates might produce the 3WJ<sub>abc</sub> at a fast rate, and formation of any pair of dimers can





**FIGURE 5.** Assembly mechanism of pRNA-3WJ. The pRNA-3WJ assembles through a two-step association mechanism in which the  $3WJ_{bc}$  dimer first forms, as shown in the first panel. This association step greatly increases the attraction of the  $3WJ_a$  strand through the doubling of nucleotides presented for binding resulting in a dropped  $\Delta G^\circ$ . This second reaction occurs at an unobservable rate and results in forming a stable nanostructure, in which each of the three strands is locked into the structure by two areas of base-pairing.

promote a fast assembly with the third components to produce the  $3WJ_{abc}$  when all three strands are incubated in the solution. Additionally, this mechanism relies on having a balanced concentration of the pRNA-3WJ strands. Thus the pRNA-3WJ shows extraordinary stability and ease of formation, resulting in a branched motif that can be used for the construction of stable RNA nanoparticles.

## MATERIALS AND METHODS

### Oligonucleotides and assembly of 3WJs

The pRNA-3WJ was constructed from three RNA oligo fragments. RNA strands were produced by Integrated DNA Technologies (IDT). Assembly of the pRNA-3WJ was completed by mixing each of the three RNA strands ( $3WJ_a$ ,  $3WJ_b$ ,  $3WJ_c$ ) at equal molar concentrations at room temperature in TMS buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10 mM  $MgCl_2$ ). Samples were then run on a 12% native polyacrylamide gel at 100 V for 2 h at 4°C in TBM running buffer (89 mM Tris, 200 mM borate acid, and 5 mM  $MgCl_2$ ) to ensure pRNA-3WJ size and folding.

### SPR studies of the pRNA-3WJ

The Bio-Rad ProteOn XPR36 was used to complete real-time interaction studies of the pRNA-3WJ motif. A neutravidin-coated HLC chip (Bio-Rad) was used to immobilize one of the three 3WJ strands with a 5' biotin label. Several studies were completed by immobilizing the  $3WJ_a$  and  $3WJ_c$  strands to the chip surface, followed by injection and interaction of the remaining two 3WJ strands. All studies were conducted at room temperature and in TMS buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM  $MgCl_2$ ) with added 0.1% Tween. RNA strands were injected at a concentration ranging from 20.0  $\mu M$  to 78.0 nM at a constant flow rate of 25  $\mu L/min$  for a total of 660 sec. This assures the analyte RNAs are in high excess in the association phase followed by injection of blank TMS buffer for 2700 sec at 25  $\mu L/min$  in the dissociation phase. Real-time association and dissociation was observed and displayed

in a histogram of total response (relationship on mass on chip surface) and time.

### SPR studies of the pRNA-3WJ dimers

Utilizing a similar approach as the pRNA-3WJ, the formation and dissociation of dimers from the pRNA-3WJ were analyzed. In order to remain consistent with the studies, the same concentration gradients were used to examine the formation of two of the three strands coming together within the three-way junction.

### Kinetic modeling of surface plasmon data

Data were extracted from the Bio-Rad ProteOn XPR36 and modeled using Igor Pro 6.37. The association periods of the SPR plots were modeled using a Langmuir pseudo-first-order model consisting of two and three components using Equations 4 and 5, respectively. These models were selected due to the nature of SPR studies, in that we are observing a 1:1 interaction (thus Langmuir) in which the interacting strand is in large excess to the bound strand, creating a pseudo-first-order interaction. The dissociation of all RNA motifs was modeled using a Langmuir zero-order reaction as shown in Equation 6.

$$R = \frac{k_a[3WJ_a]_0 R_{\max}}{k_a[3WJ_a]_0 + k_d} (1 - e^{-(k_a[3WJ_a]_0 + k_d)(t-t_0)}), \quad (4)$$

$$R = \frac{k_a[3WJ_a]_0^2 R_{\max}}{k_a[3WJ_a]_0^2 + k_d} (1 - e^{-(k_a[3WJ_a]_0^2 + k_d)(t-t_0)}), \quad (5)$$

$$R = R_0 e^{-k_d(t-t_0)}, \quad (6)$$

where  $R$  is the SPR response in terms of  $t$ , time in seconds. These kinetic models are derived in the [Supplemental Material](#). In fitting the data, the global fitting package was used in linking the association and dissociation of each sample through the  $k_d$  and  $R_{\max}$  and  $R_0$ .

### Calculation of time constant ( $\tau$ ) of pRNA-3WJ<sub>a</sub>, 3WJ<sub>b</sub>, and 3WJ<sub>c</sub>

The time constant ( $\tau$ ) of the pRNA-3WJ core strand was determined through electrophoresis mobility shift assays (EMSA). A single strand of the pRNA-3WJ fragments was radiolabeled at the 5'-terminus using  $\gamma$ - $^{32}P$  ATP (PerkinElmer) and denoted with an asterisk, as previously described (Binzel et al. 2014). The radiolabeled oligo was held at a constant concentration of 10 nM and incubated over varying time points ranging from 0 to 720 min with completed pRNA-3WJ structure at 100 nM concentrations. Samples were snap frozen on dry ice and then an electrophoresis 12% native polyacrylamide gel at 100 V for 2 h at 4°C in TBM running buffer was run. The gel was then imaged by transferring the radio signal to a phosphor screen for 12 h at  $-80^\circ C$  and visualized using a Typhoon 7000 (GE). Band quantification was completed using ImageJ. The ratio of single strand to 3WJ was then calculated and plotted against time in OriginPro 8.5. The curve was fit using Equation 7 below:

$$[3WJ]_x = [3WJ]_0 e^{-t/\tau}, \quad (7)$$

where  $t$  is the time in seconds, and  $\tau$  is the time constant at which 50% of the labeled 3WJ strand was in single-strand state and 50% had replaced unlabeled strand in the 3WJ complex (Novikova et al. 2010). These studies were completed as a way to compare the strand displacement between each of the pRNA-3WJ strands.

## SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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