Template-Dependent (Ir)reversibility of Noncovalent Synthesis Pathways

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Non-covalent synthesis aims at exploiting non-covalent interactions for the controlled hierarchical assembly of nanostructures. Just as the covalent synthesis of molecules is affected by the conditions under which the chemical reaction is carried out, the outcome of a non-covalent synthesis process can be likewise affected by the experimental pathway in case the assembled structures are kinetic, rather than thermodynamic, products. Here, we show how the ATP-templated formation of metallo-amphiphile assemblies results in different kinetically stable structures depending on the self-assembly pathway. Interconversion between the different assembled states is not possible and neither is ATP subject to enzymatic hydrolysis which would convert the system back to the unassembled state. The high stability of the different assembly states is a result of the strong interaction between ATP and the metallo-amphiphile. Indeed, the use of AMP as a weakly interacting template leads to thermodynamically stable assemblies which can be transitioned between different states. The results underline that control over the kinetic processes that regulate self-assembly is a fundamental tool for the hierarchical self-assembly of complex nanostructures and the development of energy-driven synthetic systems.

1. Introduction

Inspired by nature, self-assembly has emerged as a potent tool for the formation of well-defined structures with nanosized dimensions.[1] For most of this period, building blocks for self-assembly processes were designed based on their propensity to assemble in thermodynamically stable structures.[2] Yet, in more recent years the awareness has grown that the kinetics of self-assembly processes may play an essential role in governing the structure and function of the product.[3] In addition, an increasing number of reports have appeared in which the self-assembly process is kinetically regulated by chemical reactions, which paves the way towards out-of-equilibrium self-assembly.[4–6] This new perspective on self-assembly has led recently to a proposition by Vantomme and Meijer to treat self-assembly as the noncovalent equivalent of covalent synthesis.[7–10] The term noncovalent synthesis serves to emphasize the importance of experimental conditions (sample preparation, additives, solvents, stirring …) in determining the outcome of the self-assembly process. It also sets the stage for programming pathways in which subsequent processes are used to gradually assemble systems of increased complexity.[11–12] In this contribution we illustrate how the outcome of a self-assembly process is determined by the sample preparation: different kinetically stable products are formed depending on the timing of the addition of a template. Furthermore, it will be shown that the kinetic stability of the assemblies determines the possibility of assembly interconversion and the possibility to reversibly transition the system between different assembly states.

We previously reported that adenosine triphosphate (ATP) can stabilize vesicular assemblies made of C_{18}TACN–Zn^{2+} amphiphiles through multivalent interactions between the phosphate groups and the charged metalloamphiphile head groups (1,4,7-triazacyclononane (TACN)–Zn^{2+}).[18–20] The addition of an ATPase, e.g. potato apyrase or alkaline phosphatase, was shown to lead to spontaneous dissociation of the assembly, because the waste products of ATP hydrolysis were not able to stabilize the vesicles. Within the context of the results described here, it is of relevance to note that this system responded nearly instantaneously to changes in the ATP concentration. This property marked a strong contrast with the slow kinetics observed for the analogous amphiphile C_{20}TACN–Zn^{2+} containing a C_{18} instead of a C_{16} chain – which we reported recently.[16] It was observed that C_{20}TACN–Zn^{2+} self-assembled into well-defined flat-disk-like structures and that the addition of ATP caused the stacking of these disks leading to 3D-layered structures with dimensions of several hundreds of nanometers. Interestingly, upon the addition of enzyme it was observed that the ATP hydrolysis rate was very slow, spanning a time course of hours, unlike the case of ATP-stabilized vesicles composed of C_{18}TACN–Zn^{2+} whose lifetimes were in the order of minutes. Furthermore, only a minor fraction of ATP was hydrolyzed and the 3D structures remained largely intact. This led to the postulation that ATP was barely accessible to the enzyme because of intercalation between the disks composed of C_{20}TACN–Zn^{2+}. Another important observation was that the waste products, AMP + 2P_i, of ATP hydrolysis were unable to induce aggregate formation of the metalloamphiphile. Although structurally very similar, these two systems represent...
limiting cases in terms of kinetic stability and ATP-mediated reversibility between different states. Here, we discuss the properties of a new amphiphile C_{16Ph}TACN-Zn^{2+} which displays a different behavior. It will be shown that ATP templates the self-assembly of C_{16Ph}TACN-Zn^{2+}, but that the structure of the assemblies depends on the moment ATP is added (Figure 1). Furthermore, the assemblies have such a high kinetic stability that a transition between the assembled states is not possible. Indeed, the system is not susceptible to the enzymatic hydrolysis of ATP. It will be shown that these properties are a result of the strong interaction between ATP and C_{16Ph}TACN-Zn^{2+}; indeed, the weaker templating agent adenosine monophosphate (AMP) provides structures of lower kinetic stability, permitting reversibility between the states and introducing responsiveness to enzymatic hydrolysis (Figure 1).

2. Results and Discussion

Amphiphile C_{16Ph}TACN-Zn^{2+} contains a phenyl-ring between the C16-chain and the TACN-Zn^{2+} head group and was designed based on the expectation that the presence of the aromatic ring would affect the self-assembly properties and would allow the selective inclusion of small apolar molecules in the hydrophobic part of the assemblies (Figure 1). The metal-free precursor C_{16Ph}TACN was synthesized and characterized as described in the supporting information. DLS measurements of a 50 μM solution in aqueous buffer (HEPES, pH = 7) revealed the presence of small structures with a hydrodynamic radius of around 7 ± 2 nm compatible with the formation of micelles (Figure S10). Apart from some irregular agglomerates, TEM images did not reveal any well-defined assemblies (Figure S11). Slow, but important, structural changes occurred when 1 equivalent of Zn(NO\(_3\))\(_2\) was added to form \textit{in situ} the metalloamphiphile C_{16Ph}TACN-Zn^{2+}. DLS measurements revealed a gradual increase in hydrodynamic radius reaching a constant value of 170 ± 60 nm after around 8 hours (Figure 2b; Figure S12). TEM images taken at regular intervals revealed the gradual formation of disk-like structures I very similar to those obtained previously for C_{16Ph}TACN-Zn^{2+} (Figure 2c–1 + Figure S12). The assembly process could also be monitored by fluorescence spectroscopy exploiting the increase in fluorescent intensity as a result of the solubilisation of the apolar fluorescent dye Nile red (2 μM) in the hydrophobic domain of amphiphile-based assemblies. Immediately after addition of Zn(NO\(_3\))\(_2\) a strong fluorescence signal was measured which gradually decreased over the time course of hours eventually stabilizing at a very low level (Figure 2a). The decrease in FI over time may be ascribed to the dense packing of the apolar C_{16Ph}-chains in the bilayer of the disks, which limits the incorporation of the fluorescent probe inside the assemblies. A similar observation was made previously for C_{16Ph}TACN-Zn^{2+} [16].

In contrast with the ATP-templated stacking of disks observed for C_{16Ph}TACN-Zn^{2+}, addition of ATP (15 μM) to the C_{16Ph}TACN-Zn^{2+} disks (50 μM) did not lead to the formation of well-defined structures. Rather, TEM images taken at different times after the addition of ATP showed that the disks slowly rolled up to form large elongated structures II after 6 hours (Figure 2c–3 + Figures S14 and S15). DLS measurements confirmed the formation of aggregates with dimensions over 1 μm (Figure 2b). Fluorescent spectroscopy revealed a slight increase in intensity immediately after the addition of ATP, after which it remained stable (Figure 2a).

Interestingly, the templating effect of ATP was entirely different when ATP was added to a fresh solution of C_{16Ph}TACN-Zn^{2+} at the same concentration (50 μM), i.e. before C_{16Ph}TACN-Zn^{2+} had self-assembled into disks. DLS measurements revealed the fast formation of large assemblies with a hydrodynamic diameter of around 600 nm, which remained stable in time (Figure 2b). TEM images revealed that spherical assemblies III had formed (Figure 2c–4 + Figure S16). Kinetic FI measurements revealed the persistence of a constant fluorescent signal indicating a very different packing of the C_{16Ph}-chains (Figure 2a).

The entirely different assemblies II and III obtained for mixtures containing an identical molecular composition show how the product of the self-assembly process is determined by the sample preparation: the immediate addition of the templating agent ATP after dilution of the stock solution of C_{16Ph}TACN-Zn^{2+} causes spherical assemblies, whereas the addition of ATP after 12 hours leads to the formation of wrapped elongated structures. Considering the identical mixture composition one – or both – assemblies represent kinetic products of the self-assembly process. To investigate this aspect further, assemblies II and III were exposed to extensive heating and sonication to overcome kinetic barriers. Yet, in both cases
Figure 2. a) Fluorescence intensity (FI) at 636 nm as a function of time following the addition of Zn(NO$_3$)$_2$ (50 μM; red trace) and Nile red (2.5 μM) followed by the addition of ATP (15 μM) after 800 minutes (green trace) and the simultaneous addition of the same amounts of Zn (NO$_3$)$_2$ and ATP (blue trace). The circled numbers in the figure refer to the times at which the DLS measurements were performed and the TEM images in Figure c were taken. b) Hydrodynamic diameter of C$_{16}$PhTACN·Zn$^{2+}$ disks I (1), assemblies 200 minutes after addition of ATP to disks I (2), assemblies 400 minutes after addition of ATP to disks I (3) and assemblies 1200 minutes after the simultaneous addition of ATP and Zn(NO$_3$)$_2$ to C$_{16}$PhTACN (4). c) TEM images of assemblies corresponding to Figure 2a. General experimental conditions: [HEPES] = 5 mM, pH 7.0, T = 25°C.

Figure 3. a) Fluorescence intensity (FI) at 636 nm as a function of time following the addition of Zn(NO$_3$)$_2$ (50 μM) to a solution of C$_{16}$PhTACN·Zn$^{2+}$ (50 μM; red trace) and Nile red (2.5 μM) followed by the addition of AMP (50 μM) after 800 minutes (green trace) and the simultaneous addition of the same amounts of Zn(NO$_3$)$_2$ and AMP (blue trace). The circled numbers in the figure refer to the times at which the DLS measurements were performed and the TEM images in Figure c were taken. b) Hydrodynamic diameter of C$_{16}$PhTACN·Zn$^{2+}$ disks I (1), assemblies 50 minutes after addition of AMP to disks I (2), assemblies 600 minutes after addition of AMP to disks I (3) and assemblies 1500 minutes after the simultaneous addition of AMP and Zn(NO$_3$)$_2$ to C$_{16}$PhTACN (4). c) TEM images of assemblies corresponding to Figure 3a. General experimental conditions: [HEPES] = 5 mM, pH 7.0, T = 25°C.
the treatment led to precipitation, suggesting that both assemblies II and III are kinetically trapped structures.

Currently, there is a growing interest in the development of assemblies that are maintained in a dynamic kinetic state through the concomitant occurrence of an anabolic and catabolic reaction in the system. In such systems a fuel-driven anabolic reaction activates the building blocks for self-assembly, whereas the waste-producing catabolic reaction deactivates the building blocks causing disassembly. The key characteristic is that the installment of a dynamic kinetic state requires a continuous supply of energy. This is highly attractive as it allows the chemical functions of the assembled state to be regulated. The exploitation of energy to drive self-assembly processes also brings us closer to the development of life-like systems that operate out-of-equilibrium.

Previously, we have shown that the dissipative conditions installed by the presence of an ATPase during ATP-templated self-assembly of vesicles leads to the transient formation of vesicles with a lifetime that depends on the amount of added ATP. We were very interested to observe whether for the current system enzyme-mediated transitions between assembly states were possible. Therefore, the enzyme potato apyrase (PA), an enzyme that hydrolyses ATP into adenosine 5'-monophosphate (AMP) and two molecules of orthophosphate $P_i$, was added to assemblies II and III (3.0 U ml$^{-1}$, [CaCl$_2$] = 0.5 mM, 25°C) and the fluorescence intensity was monitored as a function of time (Figure S17). In both cases only minimal changes in the FI were observed indicating that in neither assembly ATP is accessible as a substrate for the enzyme. This corroborates with the results previously obtained for the ATP-templated disk stacks made up of the amphiphile $C_{20}$TACN · Zn$^{2+}$, which were also inert to the presence of enzyme. This implies that the structural stability of the assemblies is too high to induce transientness, implying that the ATP-templated system cannot operate under dissipative conditions.

Altogether, these results indicate that assemblies II and III formed from $C_{16}$PhTACN · Zn$^{2+}$ and ATP have a too high kinetic stability which precludes the formation of the thermodynamic assembly product. Moreover – and of importance for fueled transient self-assembly processes – the reversible transition between different assembly states in the presence of enzyme appears to be blocked by the strong interactions between $C_{16}$PhTACN · Zn$^{2+}$ and ATP in the assembled state.

To investigate how the dynamics of the assembly process is affected by the strength of interaction between $C_{16}$PhTACN · Zn$^{2+}$ and ATP, we studied the templating effect of adenosine monophosphate (AMP). Previous studies had shown that AMP has a much weaker affinity for multivalent surfaces composed of TACN-Zn$^{2+}$ owing to the lower charge. Similar to the ATP

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Figure 4. a) Schematic representation of the reversible transition between $C_{16}$PhTACN · Zn$^{2+}$ disks I and AMP-templated assemblies IV in the presence of alkaline phosphatase. b) Fluorescence intensity (FI) at 636 nm as a function of time following the addition of alkaline phosphatase (AP) (3 U ml$^{-1}$) to AMP-templated assemblies IV (turquoise trace). c) Hydrodynamic diameter of disks I (1), assemblies 1500 minutes after addition of AMP to disks I (2), assemblies 60 minutes after addition of alkaline phosphatase to assemblies IV (3) and assemblies 24 hours after addition of alkaline phosphatase to assemblies IV (4). d) TEM images of assemblies corresponding to Figure 4a. General experimental conditions: [HEPES] = 5 mM, pH 7.0, $T$ = 25°C.
studies, we added AMP to a solution of C_{16}Ph-TACN·Zn^{2+} before and after the formation of the disk-like structures. The kinetics of the self-assembly process was followed by fluorescence measurements, DLS and TEM analysis (Figure 3). It was observed that the immediate addition of AMP (50 μM) to an amphiphile solution (50 μM), i.e. before the formation of disks, led to the formation of small spherical assemblies IV with a diameter of around 30 ± 15 nm according to DLS and TEM (Figure 3b and 3c – Figure S18), which contrasts strongly with the large spheres observed when ATP was added. Kinetic fluorescence measurements revealed a rather slow kinetics of this process in the order of hours (Figure 3a). Importantly, addition of AMP to the C_{16}Ph-TACN·Zn^{2+}-disks led to a spontaneous reorganization of the system to give assemblies with the identical characteristics of assembly IV (Figure 3c–4). TEM images obtained shortly after addition of AMP to the disks clearly showed how the spherical assemblies formed on the surface of the disks (Figure 3c–2 + Figure S19). In fact, DLS measurements revealed a gradual decrease in size of the assemblies to reach a hydrodynamic diameter identical to that observed for assemblies IV (Figure 3b). Finally, the fluorescence intensity increased to reach the same value over the time course of hours (Figure 3a). These combined data clearly show that assembly IV is the thermodynamic product of the AMP-templated self-assembly process. It also clearly indicates that the high kinetic barriers operated when ATP was used are a result of the (too) strong interactions between ATP and the multivalent surfaces. The lower kinetic stability of the AMP-templated assemblies suggested that, contrary to ATP, an enzyme-mediated transition between the different assembly states should be possible. To that end, alkaline phosphatase (AP) was used instead of potato apyrase, because it can dephosphorylate AMP to adenosine (A) and P. The addition of AP (3 U mL⁻¹) to assemblies IV caused a gradual conversion of the AMP templated aggregates back into the disk-like assemblies I over a period of almost 24 hours (Figure 4a). DLS measurements revealed a gradual increase in size of the assemblies until a constant value of hydrodynamic radius was obtained corresponding to those of the disks (170 ± 60 nm) (Figure 4c). Finally, TEM images regularly taken over a period of time after addition of AP to the AMP templated assembly IV showed how the small spherical aggregates rearranged back into the disk-like structures (Figure 4d – Figures S21–S23). Moreover, the outcome remained the same regardless of the pathway followed, because the addition of enzyme to the system when AMP is added to a solution of fresh C_{16}Ph-TACN·Zn^{2+} before disks had formed led to the same thermodynamic end state. These results show that the AMP-templated assemblies can be transitioned between different states. Nonetheless, it should be noted that refueling of the system in assembly state I with AMP does not lead to reformation of assembly IV, because the enzymatic hydrolysis of AMP is faster than the (slow) rate of the formation of assembly IV.

3. Conclusion

In conclusion, we have shown how a multistep noncovalent pathway can be used to form hierarchically ordered structures. It is demonstrated that the order and timing in which the building blocks are combined determine the outcome of the self-assembly process. Depending on the synthetic pathway, the same building blocks can be assembled in two distinct kinetically stable states which do not interconvert. It is further shown that the kinetic stability of the assemblies can be tuned by reducing the strength of interaction between the building blocks. As a result of the lower kinetic stability of the assemblies, reversible transitions between different states become possible. Overall, the results show that the transition from thermodynamically controlled self-assembly to self-assembly under kinetic control adds new layers of complexity to self-assembly processes and significantly widens its scope for the formation of (transient) nanostructures.

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To be or not to be (reversible): The interaction strength between building blocks determines whether self-assembly occurs under kinetic or thermodynamic control. The self-assembly process under thermodynamic control permits reversible transitions between the different assembly states.