ABSTRACT: Virus-like particles (VLPs) are stable protein cages derived from virus coats. They have been used extensively as biomolecular platforms, e.g., nanocarriers or vaccines, but a convenient in situ technique is lacking for tracking functional status. Here, we present a simple way to monitor disassembly of 19F-labeled VLPs derived from bacteriophage Qβ by 19F NMR. Analysis of resonances, under a range of conditions, allowed determination not only of the particle as fully assembled but also as disassembled, as well as detection of a degraded state upon digestion by cells. This in turn allowed mutational redesign of disassembly and testing in both bacterial and mammalian systems as a strategy for the creation of putative, targeted-VLP delivery systems.

Virus-like particles (VLPs) are icosahedral protein cages made up of hundreds of capsid protein subunits from different viruses. They have well-defined structures and can be strikingly stable under extremes of temperature,1,2 pH,2 and in different solvents.3 These render them potentially suitable for encapsulating materials such as proteins,4–7 synthetic polymers,6,7 oligonucleotides,6,7 and smaller molecules.5,11 Furthermore, their protein surface can be used to append different functional groups, ligands or antigens for targeting,12–14 imaging,15 vaccination,16,17 and other biomedical purposes. Cellular internalization of VLP has been predominantly determined by fluorescence microscopy12,20 and/or transmission electron microscopy (TEM).21,22 Although these techniques provide robust data on the position of particles relative to target cells, they do not provide much information on, e.g., disassembly status. The latter can be particularly important when VLPs are applied as carriers, as cargo release is greatly dependent on breakdown. Thus, it would be useful to develop a convenient way for monitoring the multimeric state of VLPs to aid their design and use.

19F-protein labeling can prove invaluable because 19F generally has a low background in biological samples. It is NMR-active with a wide chemical shift range making it sensitive to the local environment, and has a high sensitivity, making it easy to detect;23 both useful for monitoring structural and interaction changes. Thus, “background-free” virus tracking in vivo could use 19F-NMR without obscurity from the complex mixture of biomacromolecules in the cell. We envisaged that labeling VLPs with a 19F-containing unnatural amino acid (uAA) would allow us to monitor structural change of particles at a molecular level via 19F-NMR.

The VLP derived from the bacteriophage Qβ is formed from 180 copies of a 132 amino acid subunit and was chosen as a model for the introduction. Qβ-VLP is considered to be more stable compared with other VLPs, such as MS2, due to intersubunit disulfide linkages.24 One approach to introducing uAAs involves the commandeering of “sense” codons for amino acids such as methionine (Met) to incorporate Met analogs;25–28 trifluoroMet (Tfm) was chosen for close structural similarity, relatively high F content (and so NMR sensitivity), and F magnetic equivalence (and so simpler, stronger signal). Tfm has been used to probe enzyme mechanism27,29 and suggested as a residue that allows creation of unusual physicochemical properties.30 Wild-type (WT) Qβ contains no Met sites, thus conversion of the Lys16 codon in the Qβ gene to Met codon would allow site-specific incorporation. Site 16 is one of the most exposed on the particle,31 and we reasoned would also provide an excellent probe site (Figure 1a).

Expression of this gene in Escherichia coli Met auxotroph B834(DE3) in the presence of Tfm (~1.7 mM) under optimized conditions (see SI) allowed the production of Qβ-Lys16Tfm (“Qβ-F”) with ~85% F-incorporation (Figure 1b), a level consistent with prior levels in other proteins;32 Met competes well with Tfm for the methionyl-tRNA synthetase (MetRS)32 and even after exhaustive Met depletion 15% is incorporated. The integrity of VLPs formed from the self-assembly of expressed Qβ-F was confirmed by both dynamic light scattering (DLS) (Figure 1c) and TEM (Figure 1d). Their measured radius (15.3 ± 0.6 nm) was found to be identical to WT within experimental error (Figure S2).

When these intact Qβ-F VLPs were analyzed by 19F-NMR, a broad resonance (full width at half height, FWHH = ~240 Hz) was observed with a shift of −40.68 ppm, and a remarkably large R2 value of 760 s−1 (Figure 2a and Figure S16). Controlled disassembly of the particles was achieved through titrated addition of denaturant and reductant33 and monitored by 19F-NMR. Denaturant SDS (0.2 M) gave a single sharp peak (FWHH = 12.6 Hz) with a shift of −40.90 ppm, and an R2 value of 8 s−1 (Figure S16); the significant change of chemical shift and peak width suggested clear change of particle structure. Reducant DTT alone had negligible effect. This was confirmed by native-PAGE (dissemination of corresponding bands); when further
analyzed under denaturing conditions by SDS-PAGE, hexamers and pentamers were observed (Figure 2c), consistent with persistent disulfide bonds in subassemblies. Further treating the same sample with reductive dithiothreitol (DTT) led to formation of monomers (Figure 2c, right), characterized by similar chemical shift and peak width to the 5/6-mers ($\delta_F = -40.90 \text{ ppm}$; FWHH $\approx 7 \text{ Hz}$, Figure 2a). Taken together, these results reveal that we can monitor the assembly state of the VLPs using $^{19}$F NMR.

Next, we exploited this ability to determine particle-state as a guide to particle design. As a proof of concept, we set out to develop a more “flimsy” VLP system that might allow more ready or rapid cargo release. Our $^{19}$F-NMR studies on Qβ-F had suggested a role for reducible cross-linking. We therefore generated a triple mutant (Qβ-K16M-C74S-C80S) in which the Cys74 and Cys80 that contribute to intersubunit disulfide covalent cross-linking were converted to isosteric but nonreactive Ser. Tfm was incorporated into protein as for Qβ-F to generate named Qβ-CS, Figure S4). Pleasingly, Qβ-CS still formed discrete particles, even without disulfide bonds at C74 and C80 (Figure S2), consistent with prior studies that have identified the dominant contribution of noncovalent interactions between viral subunits.34 The relative robustness of these Qβ-CS VLPs was probed with $^{19}$F-NMR: in contrast to Qβ-F, Qβ-CS VLPs disassembled directly into monomers under denaturing conditions when treated only with SDS (Figure 2c), consistent with our intended design. The chemical shifts of Qβ-CS VLPs in the absence and presence of SDS were highly similar ($-40.63 \text{ ppm}$, FWHH $\approx 200 \text{ Hz}$; $-40.88 \text{ ppm}$, FWHH $\approx 6 \text{ Hz}$, respectively) to those observed for Qβ (Figure 2b). Reductant DTT alone had negligible effect.

We further characterized multimer and monomers using $^{19}$F diffusion NMR (Figure 3 and Figure S5).35 Despite their similar chemical shifts in 1D $^{19}$F-NMR spectra, their diffusion coefficients were different. Notably, adding DTT in addition to SDS to the solution resulted in an increase in diffusion coefficient, consistent with destabilizing subassemblies, and the value from [Qβ-F]+SDS+DTT was essentially identical to that from [Qβ-CS]+SDS, as expected, as well as Qβ-F monomer prevented from disulfide formation by Cys-alkylation (Table S1). Diffusion coefficients for the full particle were challenging to measure owing to the very large $R_2$ of the resonance (Figure 2a and Figure S5). The values obtained were significantly smaller than measured for degraded assemblies (in the presence of SDS and DTT) but larger than expected for full size particle, suggesting that they stem from high order multimers only observed in the presence of intact particles, thereby allowing us to monitor assembly state of the VLPs.
Importantly, this validation of particle disassembly states also allowed us to determine the extent of particle dissociation and to test putative disassembly pathways. Thus, gradual titration of Qβ-F with SDS led to a gradual diminution in the intensity of the broad particle peak (δF ≈ 40.7 ppm) with simultaneous emergence in intensity of the peak ascribed to disassembled species (Figure S6). Interestingly, this suggests that the mechanism of disassembly in the presence of denaturant is not a cooperative catastrophic collapse but a gradual drift in population in response to environmental stress.

Next, we tested this ability to monitor particle state in a biological context (Figure 4a). Qβ-F VLPs were introduced to a range of representative mammalian cell types: A549 epithelial cells, THP-1 monocytes, and THP-1-derived macrophages. Flow cytometry (Figure S9) and confocal microscopy (Figure S10), following chemical, fluorescent, surface-labeling (Figures S7–S9), revealed that only THP-1-derived macrophages readily engulfed VLPs (Figure 4b,c). Confocal fluorescence microscopy confirmed internalization (Figure 4d and Figure S11); observations after 20 min suggested entry via an endosomal pathway (Figure S12).

To assess the more detailed behavior of VLPs in the milieu of the cell interior, we incubated VLPs with cell lysates and probed their behavior by 19F-NMR. In addition to the internalization by THP-1-derived macrophages discovered here, E. coli is the natural host cell of Qβ virus; lysates from both “host” cell types were therefore chosen (Figure 4a). In the viscous cellular environment, it is expected that all 19F-NMR resonances would be broadened due to macromolecular crowding. Nonetheless, we were still able to observe the broad resonance thereby allowing monitoring of intact virus assemblies in both lysates, owing to the particular suitability of the pairing of 19F-NMR and our system (Figure 5a and S13). Upon prolonged incubation at 37 °C, a sharp peak with a shift of −40.95 ppm (FWHH = 9.9 Hz) appeared, with increasing intensity over time. NMR measurements determined the diffusion coefficient to be 3.0 × 10−10 m2 s−1 (Table S2), suggesting an even smaller species than those found previously. This was confirmed upon additional chemical denaturation (added SDS+DTT): the resonance from this smaller species was readily discernible from the disassembled species observed previously (Figure 5a and Figure S13). Together, these data suggest: (i) the formation of smaller (monomeric or peptidic) Tfm-containing fragments of Qβ derived from lysate (likely mediated by proteolysis) and (ii), importantly, that our developed system can distinguish between intact particles, intervening multimeric states and a further stage of disassembly (or degradation) generated within complex milieu such as cell lysates.

In turn, this system, vitally, allowed us to analyze our designed “flimsy” VLP system Qβ-CS. Comparison of the degradation processes of Qβ-F and Qβ-CS revealed a significantly shorter half-life for Qβ-CS in both lysate systems (Figure 5b), consistent with in vitro data and with the designed removal of disulfide bonds mediated by Cys74 and Cys80. Interestingly, whereas both Qβ-F and Qβ-CS were disassembled by E. coli, only the more stable Qβ-F was not significantly disassembled by the macrophage cells. Consistent with these observations, VLP-derived peptides were directly identified by MS/MS; time course analyses revealed formation (including Tfm-containing peptides) and further digestion into fragments. Notably, more peptides were observed from “weakened” Qβ-CS in both E. coli and macrophage (Figures S17–S19).

Finally, to illustrate the potential of these VLP platforms for further elaboration, we tuned their cellular uptake. Chemical-modification with putative ligands for cell-surface receptors as “cell-targeting” groups allowed a proof-of-principle of such
further adaptation. Thus, we extended the tropism of Qβ-F VLPs from *E. coli* and macrophages toward other cells through surface chemical-attachment of D-mannosyl residues; these alterations increased uptake of VLPs by THP-1 monocytes (Figures S14 and S15).

In summary, we have established a system for monitoring the disassembly of VLPs using 19F-NMR (see Figure S16 for further discussion) that is applicable even in complex biological milieu. Successful application to design, tested here in such discussion) that is applicable even in complex biological milieu.

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