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pH Responsive Janus-like Supramolecular Fusion Proteins for Functional Protein Delivery

Seah Ling Kuan,[‖,†] David Y. W. Ng,[‖,†,‡] Yuzhou Wu,[†,‡] Christina Förtsch,[§] Holger Barth,[§] Mikheil Doroshenko,[‡] Kaloian Koynov,[‡] Christoph Meier,[†] and Tanja Weil*[,†]

[†]Institute of Organic Chemistry III, Macromolecular Chemistry, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany

[‡]Max Planck Institute for Polymer Research Ackermannweg 10, D-55128 Mainz, Germany

[§]Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Albert-Einstein-Allee 11, D-89081 Ulm, Germany

Supporting Information

ABSTRACT: A facile, noncovalent solid-phase immobilization platform is described to assemble Janus-like supramolecular fusion proteins that are responsive to external stimuli. A chemically postmodified transporter protein, DHSA, is fused with (imino)biotinylated cargo proteins via an avidin adaptor with a high degree of spatial control. Notably, the derived heterofusion proteins are able to cross cellular membranes, dissociate at acidic pH due to the iminobiotin linker and preserve the enzymatic activity of the cargo proteins β-galactosidase and the enzymatic subunit of *Clostridium botulinum* C2 toxin. The mix-and-match strategy described herein opens unique opportunities to access macromolecular architectures of high structural definition and biological activity, thus complementing protein ligation and recombinant protein expression techniques.

The development of a versatile approach to derive stimuli responsive, Janus-like fusion proteins holds great promise in the realization of precise multifunctional platforms that utilize the tumor microenvironment as a trigger, e.g., acidic[1] or reducing[2] milieus, for the delivery and controlled release of functional proteins. Genetic fusion is to date the major route to access these chimeras as exemplified by antibody-enzyme conjugates.[3,4] However, controlled degradation of the recombinant fusion protein proceeds mainly via enzymatic cleavage, and it is not possible to introduce pH or light sensitive break points via genetic engineering. Chemical conjugation strategies are valuable alternatives to engineer fusion proteins, since they allow the combination of native and chemically postmodified proteins, thereby expanding the repertoire of proteins nature offers.[5−7] Furthermore, chemical approaches allow small molecules to be programmed into the protein chimeras[8] for controlled disintegration of the fusion proteins. In particular, noncovalent approaches are of emerging interest, since mild conditions can be applied to retain bioactivity of the fusion construct to impart in vivo functionality.[8−11] Avidin−biotin technology has been long since employed as a molecular adaptor for heterofunctional bioconjugates with extensive use in cancer pretargeting.[12,13] Nevertheless, precise control of the resultant stoichiometry and distinct orientation of the conjugate is limited. Despite attempts to improve stoichiometric control through the production of monovalent or divalent streptavidin proteins via genetic engineering,[14,15] the applications are still hampered by lower binding constants[15] and a lack of spatial control. A noncovalent strategy to rapidly self-assemble the protein components with a high degree of control over composition and spatial arrangement is therefore highly attractive for the systematic construction of asymmetric protein motifs in a "built-to-order" fashion.

Iminobiotin, an imine analogue of biotin, interacts noncovalently with avidin, and its resultant binding affinity is dependent on the extent of protonation of the imine group (Figure 1a, basic pH, K_d∼10^{-11} M; acidic pH, K_d ∼10^{-3} M).[16]

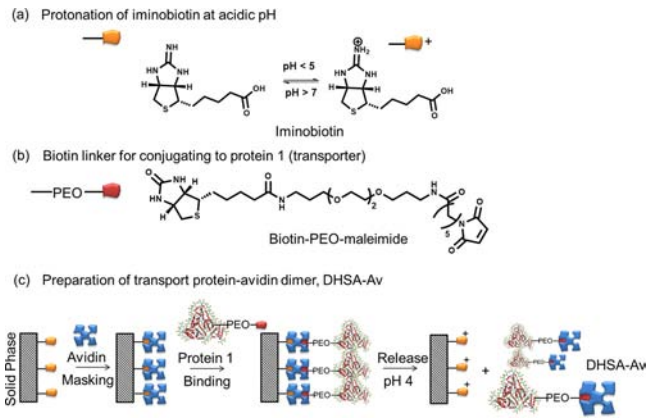

Figure 1. Synthesis of the DHSA-Av dimer. (a) Protonation of the imine group in iminobiotin at acidic pH. (b) Biotin-PEO-maleimide linker that is conjugated to the SH-group of protein 1 (DHSA). (c) Schematic overview for the toposelective solid phase immobilization to assemble DHSA-Av dimers.

The pH responsive iminobiotin−avidin interaction has been employed mostly for protein affinity purification[17] and, more recently, in the synthesis of layer-by-layer nanoparticles that exhibit a pH-sensitive outer stealth layer.[18] We hypothesize that adopting the iminobiotin−avidin technology will allow us to fulfill two criteria: (1) The combination with solid phase synthesis will lead to the construction of heterofusion proteins with high degree of spatial control. (2) Programming the pH

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trigger, iminobiotin, into the cargo protein will allow controlled release and preservation of bioactivity in vivo, which opens access to stimuli responsive release of the protein cargo inside acidic cancer cells,[1,19] thereby serving as an attractive option to existing release strategies.[20] Herein, the first examples of pH responsive Janus-like supramolecular fusion proteins based on the iminobiotin−avidin linkage are reported. Five different asymmetric supramolecular fusion proteins with opposite "faces" are assembled, i.e., a biotinylated chemically post-modified transporter protein at one site and a biotinylated or pH responsive iminobiotinylated protein cargo at the opposite site. The release and preservation of enzymatic activity of the assembled supramolecular fusion proteins are also investigated.

Dendronized human serum albumin (DHSA)[21] is selected as a chemically postmodified transporter protein because of its enhanced cellular uptake and low cytotoxicity and its potential to deliver drug molecules into cells.[21] As the first step to construct the fusion protein, biotin is tethered to the single unpaired sulfhydryl of the Cys34 residue on HSA via a PEO-maleimide linker (Figure 1b, Chart S1, Supporting Information (SI)). Dendronization of HSA is accomplished by conjugation of ethynyl-polyamidoamine dendrons to azido-functionalized HSA via the Huisgen 1,3-dipolar cycloaddition as reported previously (Figure S1 (SI)).[21] Avidin (Av) is first immobilized onto iminobiotin beads to mask one hemisphere of Av, followed by assembly of biotinylated DHSA onto the toposelective Av (Figure 1c). The DHSA-Av heterodimer is isolated by subsequent cleavage from the beads at acidic pH (Figure 1c) and finally purified by cation exchange chromatography. The resultant DHSA-Av construct possesses a vacant site at the opposite hemisphere, formerly occupied by the solid phase, for the conjugation of the "cargo" protein entity (Figure 2). The purified DHSA-Av (Figure S3 (SI)) is characterized by

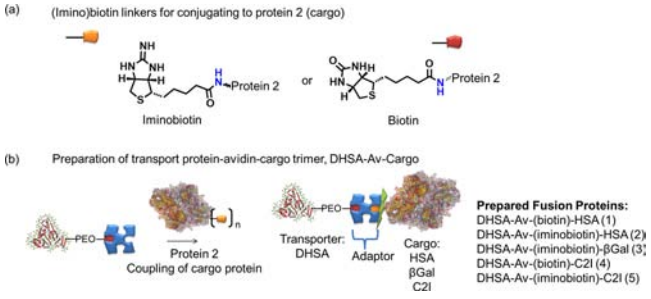

Figure 2. Assembly of DHSA-Av-Cargo complexes. (a) Iminobiotin (pH-cleavable) and biotin (non-pH-cleavable) NHS esters conjugated to amine groups of the cargo proteins HSA, βGal, and C2I. (b) Assembly of DHSA-Av with the (imino)biotinylated cargo proteins HSA, βGal, or C2I.

the SDS-PAGE, MALDI-ToF and fluorescence polarization (Figures 3a, S4, S5 (SI)), which corroborates with the formation of the fusion construct. Further characterization is carried out using atomic force microscopy (AFM) and fluorescence correlation spectroscopy (FCS) to determine the size and the homogeneity of the resultant construct. The mean hydrodynamic radius of DHSA-Av determined by FCS is 7.4 nm (Figures 3b, S6 (SI)) supports the formation of the fusion construct, which is consistent with AFM data (8 nm, Figure S8 (SI)). AFM allows not only the estimation of the complex sizes but also provides information about sample homogeneity and the physical arrangement of the constituent components in the observed multiprotein complexes.[22,23] Estimation of the

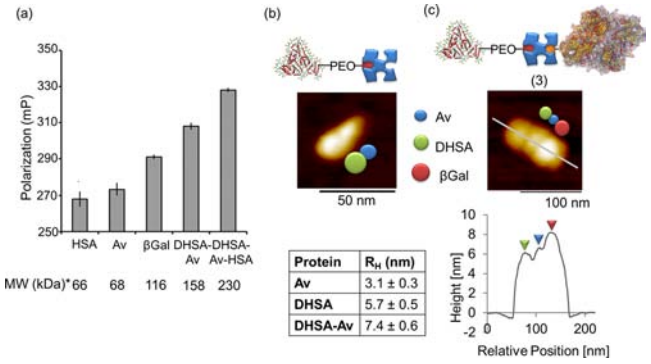

Figure 3. Characterization of the supramolecular fusion proteins. (a) Fluorescence polarization of DHSA-Av and DHSA-Av-HSA against protein standards. *Molecular weights of the protein standards as reported or calculated from MALDI-ToF (b) AFM image (top) showing DHSA-Av heterodimers (H = 8 nm) on mica surface and hydrodynamic radii of DHSA-Av determined by FCS measurements (bottom). (c) AFM image (top) and height profile (bottom) of DHSA-Av-(iminobiotin)-βGal (3) trimer on mica surface. The respective structures of the constructs are depicted above the AFM images. Shapes that are expected for the projections of the heterofusion proteins cross section are indicated in the AFM images.

homogeneity of the resultant fusion construct and the statistical analysis of the height profile and aspect ratios of the macromolecules indicate a population composing of 89% dimers with one Av per DHSA molecule (Figures 3b, S8 (SI)). Noteworthy, only one protein is coupled to each site for steric reasons, and therefore, no higher order assemblies with three or four proteins are detected. These findings indicate the efficiency of the proposed method for the construction of defined protein conjugates in a controlled fashion. The membrane translocation of Av is shown to be mediated by the appended DHSA, with a 6-fold increase in uptake (Figure S11 (SI)).

Three different types of cargo proteins, namely, HSA, the enzymatic subunit of *Clostridium botulinum* C2 toxin (C2I) and β-galactosidase (βGal) have been applied to construct the supramolecular fusion proteins. All three cargo proteins, HSA, C2I and βGal, have been statistically labeled with biotin or iminobiotin by reaction with the respective NHS esters (Figure 2a, Chart S1b (SI)).

Thereafter, they are assembled with DHSA-Av by incubation (Figure 2b) to yield the resultant DHSA-Av-Cargo complexes (1−5), which are used for subsequent characterization and biological evaluation. The formation of the fusion DHSA-Av-Cargo complexes is determined by fluorescence polarization or AFM. The formation of the protein trimers DHSA-Av-(biotin)-HSA (1) is apparent from the size increase (MW ∼ 230 kDa) detected in fluorescence polarization (Figure 3a). AFM characterization is applied to the DHSA-Av-(iminobiotin)-βGal (3), since it allows better differentiation of the protein entities due to the greater size difference of βGal to the protein components in DHSA-Av. The AFM image shows the formation of a heterotrimer (Figure 3c). The heights of DHSA, Av and βGal in the heterotrimeric structure are determined to be 6, 6.5, and 8 nm, respectively. The heights of DHSA and βGal are consistent with the sizes of the individual entities. Av has a higher than expected height of 6.5 nm, as it is supported by the two larger proteins (Figure 3c).

The pH responsiveness of the avidin-iminobiotin interaction is verified by a competitive binding assay using 4′-hydroxyazobenzene-2-carboxylic acid, which has a lower

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binding affinity for Av than iminobiotin (Figure 4a).[1] In the following evaluation, pH-induced release of the cargo protein

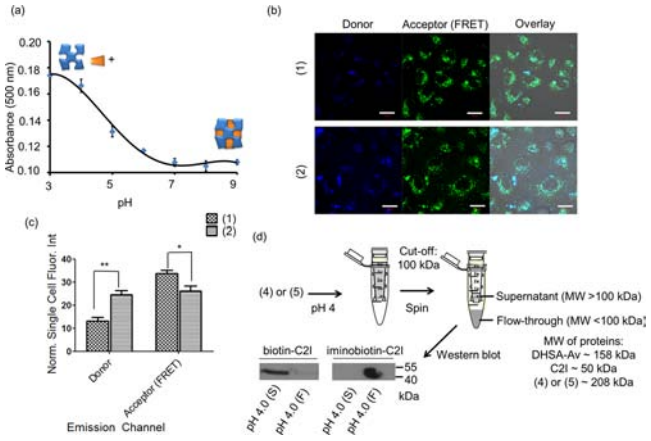

Figure 4. pH-induced disassembly of DHSA-Av-Cargo complexes. (a) Extracellular competitive binding assay with 4′-hydroxyazobenzene-2-carboxylic acid against iminobiotin-Av showing pH responsiveness. (b) Confocal images for the intracellular pH release study of DHSA-Av containing biotin-HSA (1) and iminobiotin-HSA (2). Scale bar is 20 μm. (c) The normalized single cell fluorescence intensity shows enhanced quenching in the donor emission channel due to energy transfer in (1). Data are expressed as mean ± SEM, $N = 5$ (SI). Statistical significance is calculated using student's t test (*$p < 0.05$, **$p < 0.005$). (d) Schematic overview for the pH release experiment of DHSA-Av containing biotin-C2I (4) and iminobiotin-C2I (5) and the corresponding Western blot analysis. The supernatant (S) and flowthrough (F) fractions after incubation of (4) and (5) at pH 4 and subsequent ultrafiltration (100 kDa cutoff) are collected and analyzed by Western blotting with an antibody against C2I.

HSA from the complexes (1) and (2) has been investigated by Förster resonance energy transfer (FRET). FRET allows detecting the proximity between two fluorophores up to 10 nm distances.[24] It is thus possible to evaluate the release of the HSA cargo by FCS either at the single molecule level in vitro or in living cells directly by fluorescence imaging. The DHSA-Av carrier is labeled with Atto-425 as FRET donor and biotin- or iminobiotin-HSA cargos are labeled with Atto-520 as FRET acceptor. FCS studies in the emission channel of the donor (Figure S7 (SI)) reveal similar extent of quenching for the biotin-HSA containing complex (1) and iminobiotin-HSA containing complex (2) at pH 9. However, at pH 4, (2) exhibits significantly diminished quenching (about 3-fold less than (1)) indicating cargo release. In addition, the fusion constructs, (1) and (2), have been evaluated for HSA release in living cells by fluorescence imaging.

Confocal microscopy studies reveal that both complexes (1) and (2) are taken up by A549 cells after incubation overnight regardless of the different linkers used (Figure S12 (SI)). The fluorescence images and intensities of A549 cancer cells incubated with (1) and (2) are recorded with excitation at 458 nm and emission at two channels: 463−509 nm (donor) and 520−658 nm (acceptor), (Figures 4b,c, S13 (SI)). The confocal studies reveal enhanced quenching in the donor emission channel due to energy transfer in (1) compared with (2), further substantiating the release of the iminobiotinylated HSA but not the biotinylated HSA in A549 cells.

The retention of activity is a key concern in protein delivery and the enzymatic activity provides a stringent test. Thus, the pH-induced disassembly of the enzymatic subunit of the *Clostridium botulinum* C2 toxin, C2I, is evaluated in vitro. C2I contributes to the cytotoxic activity of the toxin,[25,26] and it has been selected to investigate the pH-triggered release of bacterial protein toxins from the fusion DHSA-Av carrier. In addition, the preservation of the cytotoxicity after release is of interest for the induction of apoptotic cell deaths.[27] The cytotoxic activity of C2I has been assessed by the observation of toxin-specific cell-rounding due to C2I-catalyzed ADP-ribosylation of actin in the cytosol.[28] Both biotin-C2I and iminobiotin-C2I cargo proteins are conjugated to DHSA-Av at basic pH, and unbound C2I is removed by ultrafiltration with a cutoff of 100 kDa. Thereafter, the thus-assembled DHSA-Av-(biotin)-C2I (4) and DHSA-Av-(iminobiotin)-C2I (5) are incubated for 30 min at pH 4 to release the C2I cargo. DHSA-Av (~158 kDa) and the released cargo (~50 kDa) are then separated by ultrafiltration with a 100 kDa cutoff (Figure 4d). The flow-through and supernatant fractions obtained are subsequently analyzed by Western blotting (Figure 4d). Only C2I conjugated via the iminobiotin linker is detected in the flow-through fraction by a specific C2I-antibody in the Western blot analysis (Figure 4d). For (4), no C2I is detected in the flow-through. The flow-through fractions of carrier-released iminobiotin-C2I is then analyzed for the presence and the preservation of the toxin activity (Figure 5a).

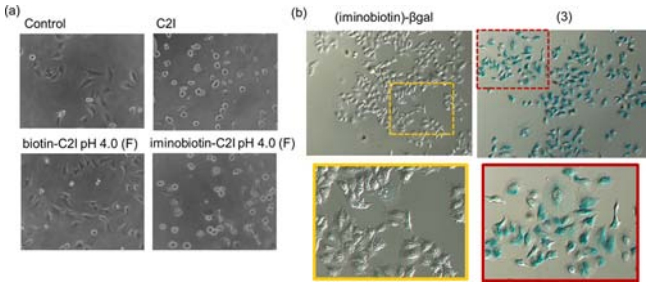

Figure 5. Protein activity assays. (a) The presence of catalytically active C2I protein in the flow-through fractions (F) has been analyzed in the cell rounding assay. HeLa cells are incubated for 24 h at 37 °C with the flow-through fractions of carrier-released iminobiotin-C2I or biotin-C2I. The transport component of C2 toxin, C2IIa, has been added to mediate the transport of C2I into the cytosol of the cells. For control cells are left untreated or treated with C2IIa and C2I for positive control. (b) Microscopic images of A549 cells treated with 100 nM of (left) iminobiotin-βGal and (right) DHSA-Av-(iminobiotin)-βGal (3), followed by treatment with the substrate X-gal.

The transport component of C2 toxin, C2IIa, is then added together with the flow-through fractions into cultured cells to mediate the transport of C2I into the cytosol of the cells to assess whether it still displays functional activity. Notably, cell rounding is observed for the flow-through fractions of carrier-released iminobiotin-C2I but not that of biotin-C2I (negative control). Taken together, the results show that only iminobiotin-C2I, but not biotin-C2I, is released from the DHSA-Av carrier under acidic conditions. Moreover, the carrier-released iminobiotin-C2I retains activity to induce cell rounding in HeLa cells.

Thereafter, cellular studies have been accomplished to assess the enzymatic activity of cargo proteins inside cells. βGal is a tetrameric membrane-impermeable protein that catalyzes the hydrolysis of β-glycosidic bonds. DHSA-Av nanocarrier platform is applied for the delivery of βGal into A549 cancer cells to directly study the retention of enzymatic activity of the cargo

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after cell uptake. The enzymatic activity of βGal can be detected by a colorimetric assay through the incubation of the treated cells for 24 h with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a substrate for βGal. Cellular delivery of βGal is accomplished by assembling iminobiotinylated βGal (iminobiotin-βGal) and DHSA-Av. Confocal microscopy shows that (3) is internalized into A549 cells (Figure S14 (SI)). The enzymatic activity of βGal after membrane transduction is investigated by incubating A549 cells that are pretreated with (3) for a further 24 h with X-gal. Upon enzymatic hydrolysis of the substrate X-gal to galactose and 5-bromo-4-chloro-3-hydroxyindole by βGal, the color changes from colorless to blue, thus demonstrating the successful intracellular delivery of the protein. No color change is observed when cells were treated with βGal alone (Figure 5b). Our findings clearly indicate that the catalytic activity of βGal is preserved upon internalization.

In summary, we have demonstrated a versatile, supramolecular approach to derive defined heteroassembly Janus-like fusion proteins. A high degree of spatial control is achieved using solid phase immobilization based on the pH responsive iminobiotin−avidin interaction. Following this approach, five precision Janus-like fusion conjugates have been prepared: Two noncleavable, biotin-containing cargoes and three pH-cleavable, iminobiotin-containing cargoes, which are the first examples of pH responsive Janus-like fusion proteins based on the iminobiotin−avidin interaction. Our strategy allows the noncovalent conjugation of both native and chemically postmodified proteins, which grants access to greater diversity and expands the toolbox nature offers, as exemplified by the dendronized transporter protein DHSA. Notably, we have demonstrated (a) through FRET studies and in vitro assays that the iminobiotin moiety can serve as a pH trigger for the release of the cargo proteins, iminobiotin-HSA and -C2I, (b) that the DHSA-Av carrier can transport the cargo proteins into cells and the cargo proteins are released in the acidic tumor environment, as exemplified by the HSA derivatives, and (c) that the catalytic activity of the carrier-released iminobiotin-C2I and the iminobiotin-βGal that is internalized into cells is preserved. One could envision programming fusion proteins combining cell- or tissue-selectivity and intracellular release at acidic pH found in the tumor microenvironment, which is highly attractive to achieve targeted delivery and controlled release of functional proteins, e.g., catalytic component of toxins. The mix-and-match strategy described opens unique opportunities to access macromolecular biohybrid architectures of high structural definition and biological activity and in this way complements protein ligation and recombinant protein expression techniques.

■ ASSOCIATED CONTENT

Supporting Information

Supporting figures (Scheme 1, Chart S1, Figures S1−15) and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

tanja.weil@uni-ulm.de

Author Contributions

‖S.L. Kuan and D.Y.W. Ng contributed equally.

Notes

The authors declare no competing financial interest.

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