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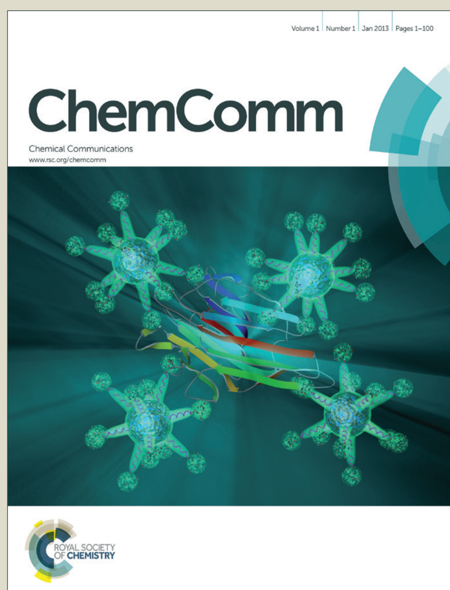

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# Transiently responsive protein-polymer conjugates via a grafting-from RAFT approach: for intracellular co-delivery of proteins and immune-modulators

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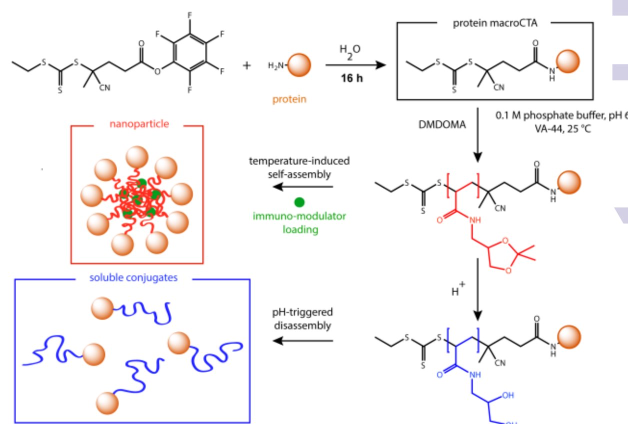
**We report on transiently responsive protein-polymer conjugates that temporarily change protein conformation between soluble and particle-like. 'Grafting-from' RAFT polymerization of a dioxolane-containing acrylamide with a protein macroCTA is used to design polymer-protein conjugates that self-assemble into nanoparticles at physiological temperature and pH. Acidic triggered hydrolysis of the dioxolane units into diol moeities rendered the conjugates fully water soluble irrespective of temperature.**

Efficient polymer-protein conjugation is a crucial step in the design of many therapeutic protein formulations including vaccine nano-formulations, antibody-drug conjugates and to prolong the *in vivo* circulation times of therapeutic proteins.[1] Covalent modification with stimuli-responsive polymers is particularly of interest to confer the responsive properties of these polymers to the attached protein molecules. For example, temperature-responsive polymers conjugated to proteins can self-assemble into nanoparticles above the cloud point temperature ($T_{cp}$), due to their amphiphilic character.[2] This controlled and reversible aggregation of proteins could be exploited for vaccine delivery. Indeed, formulating protein antigens as nanoparticles has proven to be a promising strategy to modulate and increase the adaptive antigen-specific CD8+ T-cell response. This occurs through the stimulation of the cross-presentation pathway and can further be improved by co-encapsulation of immune-modulators that shape the direction and strength of the adaptive immune response.[3] In addition, ultra-small nanoparticles and albumin-binding amphiphiles, are efficiently transported via the interstitial flow and lymphatic capillaries to the draining lymph nodes where they are taken up by dendritic cells (DCs).[4]




**Scheme 1.** Molecular structure and schematic representation of the synthesis, self-assembly and disassembly of transiently responsive protein-polymer conjugates via 'grafting from' RAFT polymerization.

We are particularly interested in designing transiently responsive protein-polymer conjugates. We define these as conjugates that can change from soluble to aggregated state in response to temperature, but become fully soluble, irrespective of temperature, by acid triggered hydrolysis. These conjugates would thus be responsive to the acidic endosomal milieu where nanoparticles are usually stored upon phagocytosis.[5] These features are essential for clearance of the polymeric carrier from the body to avoid long-term accumulation.
Kizhakkedathu and co-workers described the synthesis and polymerization of [(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide (DMDOMA), yielding temperature-responsive polymers with acid-labile dioxolane side groups.[6] Gradual hydrolysis of these dioxolane groups into diol moieties increases the $T_{cp}$ of the polymers from below room temperature upwards until they become fully water soluble, irrespective of temperature. Such transiently responsive homopolymers are also expected to possess a better predictable behavior than combining multiple co-monomers yielding temperature- and pH-responsive polymers, as recently reported by our laboratories.[7]

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Amongst the different controlled radical polymerization techniques,[8] reversible addition-fragmentation chain transfer (RAFT) polymerization in particular, has shown to be tolerant to many chemical groups, solvent media and offers a straightforward route to synthesize polymers with a protein-reactive end-group via the use of a functional chain transfer agent (CTA).[2b, 9] Previously we reported on a head-to-head evaluation of several protein-reactive RAFT CTAs for protein-polymer conjugation via 'grafting-to'.[10] The advantage of 'grafting-to' is the use of pre-synthesized polymer, which allows for thorough characterization of the polymer and avoiding exposure of the protein to potentially denaturing polymerization conditions. However, we found that a large molar excess (up to 20-40 fold) of protein-reactive polymer is required to obtain full protein conjugation.

An alternative approach is 'grafting-from', where the polymer chain is grown directly from a protein that is modified with a CTA.[11] The major advantage of this approach is that the prepared conjugates only need to be purified from low molecular weight compounds (i.e. unreacted monomer, initiator, etc…). This can easily be done by dialysis, unlike the removal of unreacted polymer, involved in 'grafting-to', which is often tedious and requires preparative gel filtration chromatography. However, possible steric hindrance during polymerization can lead to a substantial amount of unreacted protein-bound CTA.

In this paper, we design transiently soluble polymer-protein conjugates via a grafting-from RAFT approach (**Scheme 1**) and demonstrate the use of these conjugates for simultaneous delivery of protein and a hydrophobic immune-modulator to dendritic cells. Bovine serum albumin (BSA), used as model protein, was reacted with a 20-fold molar excess of a pentafluorophenyl (PFP) functionalized trithiocarbonate RAFT CTA. The choice for PFP as activated ester moiety to modify lysine residues is based on our previous work where we showed that PFP outperformed the more widespread NHS esters in terms of protein-conjugation efficiency, likely due to increased hydrolytic stability.[10]

UV/VIS analysis of the obtained protein macroCTA ($\lambda_{max}$ of the PFP-CTA is at 306 nm) revealed an average of 5 CTA molecules grafted per BSA molecule (**Figure S1**, Supporting information). Polymerization of DMDOMA at a targeted DP (degree of polymerization) of respectively 100 and 200 was conducted at 25°C in phosphate buffer pH 6 using the water-soluble azo initiator VA-044 as radical source.[11b] Overnight reaction yielded a turbid mixture, indicating self-assembly to occur during the polymerization reaction. Such phenomenon has been termed by several groups as polymerization-induced self-assembly (PISA).[12] SDS-PAGE analysis clearly proved that polymers were grown from the protein backbone, as the protein bands had shifted to higher molecular weights (**Figure 1A**). As expected, the protein-polymer band for the targeted DP of 200 was visible at higher molecular weights than the one for DP 100. Integration by ImageJ software, indicated that approximately 30% of the protein remained unmodified. This can likely be attributed to uneven distribution of the CTA units over the different BSA molecules, and/or BSA molecules with too sterically hindered CTA moieties that do not allow for 'grafting-from' polymerization.

DLS analysis of the reaction mixture before ($T_0$) and after ($T_e$) polymerization clearly proofs the formation of temperature-responsive conjugates (**Figure 1B**). Before the onset of polymerization, there is no significant difference in size upon increase in temperature. After polymerization, DLS analysis indicates the presence of temperature-responsive behavior, with a $T_{cp}$ of 27°C and 19°C for BSA-pDMDOMA$^{DP100}$ and BSA-pDMDOMA$^{DP200}$ respectively (**Figure S2**, Supporting information). This difference in $T_{cp}$ could be expected as a shorter pDMDOMA chain length will yield less hydrophobic polymer moieties on the conjugates.

Below the $T_{cp}$, a slight increase in size (from 7.25 nm to 8.79 nm and from 6.93 nm to 13.18 nm for BSA-pDMDOMA$^{DP100}$ and BSA-pDMDOMA$^{DP200}$ respectively) is observed. This can be attributed to the presence of grafted polymer chains on the protein which increase the hydrodynamic radius. Above the $T_{cp}$, the protein-polymer conjugates form particles of approximately 48 and 187 nm for BSA-pDMDOMA$^{DP100}$ and BSA-pDMDOMA$^{DP200}$ respectively. This temperature-reversible transition between globules and fully soluble unimers suggests that no significant crosslinking occurred during polymerization. The critical micellar concentration (CMC) was similar for both conjugates: 48 µg/mL and 51 µg/mL for BSA-pDMDOMA$^{DP100}$ and BSA-pDMDOMA$^{DP200}$ respectively (**Figure S3**, Supporting information).

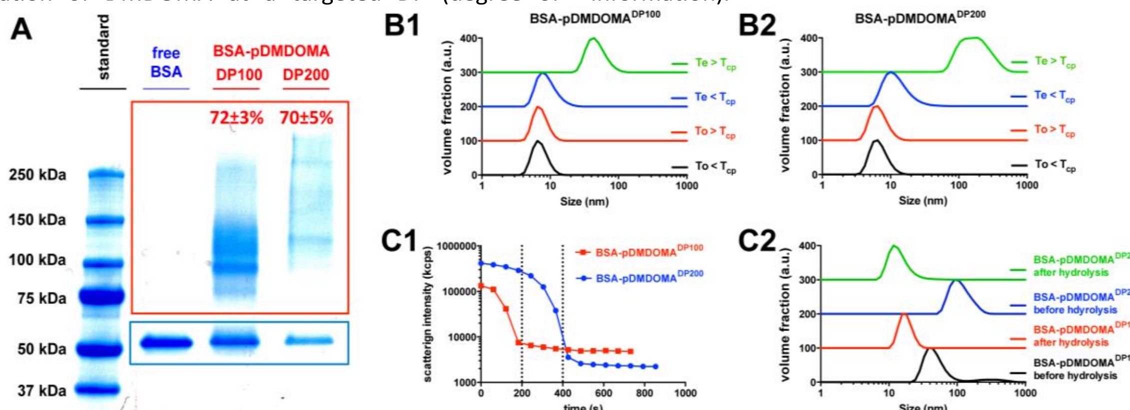


**Figure 1.** (A) SDS-PAGE analysis of BSA-pDMDOMA conjugates. (B) Size distribution measured by DLS of the BSA-pDMDOMA reaction mixtures before ($T_0$) and after ($T_e$) polymerization. (C) Light scattering intensity (C1) measured by DLS as function of time during the hydrolysis of the BSA-pDMDOMA conjugates at 37 °C and (C2) corresponding size distribution curves measured at 37 °C.

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To investigate whether the dioxolane moieties allow the polymer-protein conjugates to undergo pH-triggered transition from amphiphilic structures into fully water soluble structures, we exposed the conjugates to acidic medium (50 mM HCl as proof of concept) at 37 °C and monitored the evolution of particle size and light scattering intensity by DLS. As shown in **Figure 1C1**, the light scattering intensity drops as function of time, indicating the hydrolysis of the hydrophobic dioxolane moieties into hydrophilic diol moieties resulted in a gradual dissolution of the self-assembled conjugates. In addition, **Figure 1C2** confirms that the protein-polymer conjugates lose their self-assembly capacities after hydrolysis, as their size at 37 °C returns to that of the non-hydrolyzed conjugates below their $T_{cp}$. SDS-PAGE (**Figure S4**) and $^1$H-NMR (**Figure S5**) analysis of BSA, pDMDOMA or BSA-pDMDOMA suggests that hydrolysis of the ketal moieties and not protein hydrolysis is responsible for the pH-triggered transition from globules to unimers of the BSA-pDMDOMA conjugates.

In the second part of this work we aimed at establishing an *in vitro* proof-of-concept showing that pDMDOMA-conjugation can be used for intracellular co-delivery of proteins and small hydrophobic molecules loaded into the hydrophobic domains of the pDMDOMA above its $T_{cp}$. For this purpose we used dendritic cells (DCs). These are the most potent class of antigen presenting cells of the immune system and a key target cell population for vaccination and immune-therapy.[13]

First, the conjugates were fluorescently labeled with Cy5-NHS, that can bind to the lysine residues in the BSA backbone. Next the Cy5-BSA-pDMDOMA particles were loaded via solvent displacement from ethanol with the fluorescent dye Cy3-alkyne, as a model hydrophobic molecule. Non-encapsulated Cy3-alkyne (i.e. precipitated particulates) was removed by filtration. As a control, the same procedure was repeated for PBS buffer and soluble BSA macroCTA.

Dendritic cells (immortalized DC2.4 cell line) were pulsed with the conjugates, and after overnight incubation analyzed with flow cytometry. **Figure 2A** shows that particulate Cy5-BSA-pDMDOMA is taken up more efficiently than soluble Cy5-BSA, as the mean fluorescence of the Cy5 channel is significantly higher for the Cy5-BSA-pDMDOMA conjugates.

Also the mean fluorescence in the Cy3 channel clearly indicates that formulation of the hydrophobic Cy3-alkyne dye with Cy5-BSA-pDMDOMA conjugates results in higher cell uptake. To confirm whether the conjugates are internalized by the cells, rather than sticking to the cell surface, confocal microscopy imaging was performed. **Figure 2B** shows that the Cy5-BSA-pDMDOMA particles loaded with the Cy3-alkyne are indeed internalized by the dendritic cells. Additionally, a strong co-localization of the Cy3 and Cy5 channel is observed,

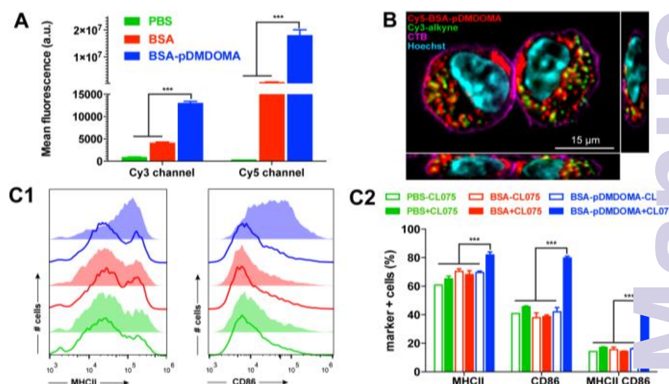


**Figure 2.** (A) Mean cell fluorescence measured by flow cytometry of DC2.4 cells pulsed with Cy3-alkyne formulated in PBS, Cy5-BSA or Cy5-BSA-pDMDOMA. (B) Corresponding confocal microscopy image of DC2.4 cells pulsed with Cy3-alkyne formulated in Cy5-BSA-pDMDOMA. (C) DC maturation ((C1) flow cytometry histograms and (C2) quantification) induced by CL075 formulated in PBS, BSA and BSA-pDMDOMA and the corresponding negative controls. (***; p<0,001)

suggesting that the BSA-pDMDOMA conjugates act as delivery carrier for the hydrophobic Cy3-alkyne dye.

Next, we aimed at utilizing the BSA-pDMDOMA system to deliver the hydrophobic immune-stimulatory molecule CL075.[14] CL075 triggers the Toll-like receptor 7, present on endosomes of DCs, and boosts cellular immune responses against tumors and intracellular pathogens.[15] CL075 is water insoluble and precipitates from solution upon solvent displacement from ethanol into water. Formulation was performed similarly as for the model compound Cy3-alkyne. Samples were added to mouse bone marrow derived dendritic cells (BM-DCs) followed by analysis of the induction of the cell surface maturation markers MHCII and CD86 by flow cytometry. Control samples included PBS buffer and BSA macroCTA with and without CL075, as well as unloaded BSA-pDMDOMA conjugates. Although the basal expression level of MHCII was already high for PBS treated DCs, only DC's treated with CL075 formulated in BSA-pDMDOMA nanoparticles exhibited a significant further increase in MHCII expression. Similar, and even more outspoken, was the effect of CL075 formulated in BSA-pDMDOMA nanoparticles on CD86 expression and on the co-expression of both MHCII and CD86. (**Figure 2C**). These findings indicate that relative to PBS and native BSA, the BSA-pDMDOMA conjugates possess sufficient amphiphilicity to encapsulate the hydrophobic CL075, while still allowing it to exert its biological activity on DCs.

In conclusion, we have demonstrated that transiently soluble and acid-degradable polymer-protein conjugates could be synthesized via a grafting-from RAFT approach. These conjugates form self-assembled nanoparticles that can be used for intracellular delivery of proteins and hydrophobic molecules. The latter was demonstrated by delivering an immune-modulating compound that holds potential for vaccine

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delivery. Our ongoing efforts concentrate on further exploring ketal chemistry to accelerate acidic hydrolysis at endosomal pH and applying this technology for clinically relevant vaccine antigens.

## Notes and references

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