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Authors: Peng Zhang, Priyesh Jain, Caroline Tsao, Kan Wu, and Shaoyi Jiang

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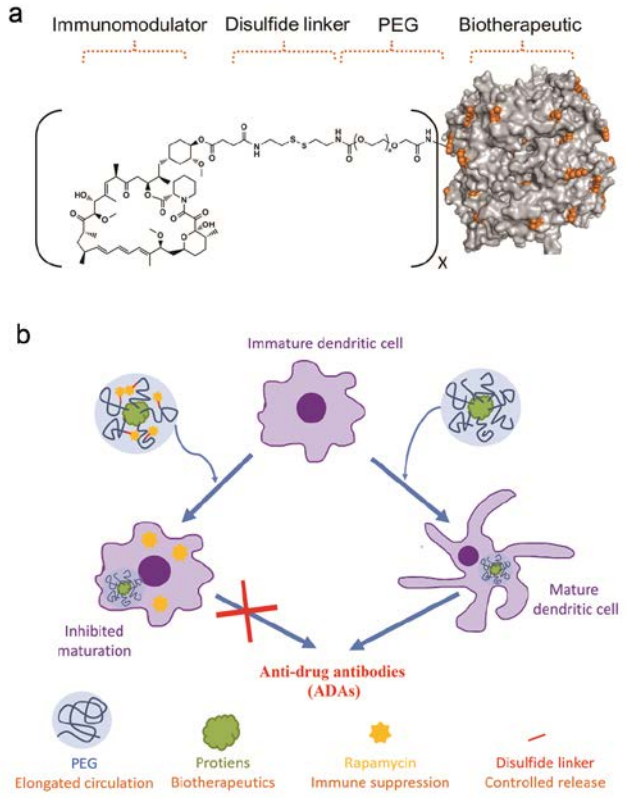
Proactively Reducing Anti-Drug Antibodies via Immunomodulatory Bioconjugation

Peng Zhang, Priyesh Jain, Caroline Tsao, Kan Wu and Shaoyi Jiang*

Abstract: Although PEGylation reduces the immunogenicity of protein drugs to some extent due to its "stealth effect", more and more reports demonstrated its limitations for highly immunogenic biotherapeutics. Here we report a proactive strategy to alleviate the development of anti-drug antibodies (ADAs) against protein drugs by immunomodulatory bioconjugation. An immunomodulator rapamycin was conjugated to a PEGylated protein therapeutic via a cleavable disulfide linker. When circulating in blood, the bioconjugate will play its therapeutic role as a PEGylated drug while the conjugated rapamycin can be released and prevent immune responses once the bioconjugate is uptaken by antigen presenting cells. In vitro and in vivo results showed that the immunomodulatory bioconjugate significantly reduced the titers of ADAs compared with a PEGylated protein. Importantly, the inhibition of immune responses was specific to the conjugated antigen, avoiding systemic immune suppression and the risk of increased susceptibility to infections. The reported immunomodulatory bioconjugation approach breaks the limitations of PEGylation by the proactive prevention of ADAs.

Anti-drug antibodies (ADAs) remain as one of the major obstacles that limit the widespread applications of biologic therapeutics.[1] Although naturally derived biologic macromolecules, including enzymes, antibodies, hormones, cytokines and nucleic acids, comprise a broad reservoir of drug candidates with exceptional medicinal potency and specificity, they carry an inherent risk of eliciting ADAs that can adversely affect the efficacy and safety of the treatment.[1-2] Virtually all biologics can elicit an ADA response, even fully human growth factors and human antibodies.[3] The generated ADAs may negatively affect treatments by neutralizing drug pharmacological activity, expediting blood clearance or causing hypersensitivity reactions (including life-threatening anaphylaxis).[4]

Various approaches have been developed to reduce the immunogenicity of biologic therapeutics.[5] Among which, the conjugation of polyethylene glycol (PEG) (known as PEGylation) has successfully brought a number of PEGylated therapeutics into market.[5c] PEGylation has improved the pharmacokinetics and immunogenicity of biologic macromolecules by sterically shielding their antigenic epitopes from immune system recognition. However, more and more reports demonstrated its limitations for highly immunogenic biotherapeutics.[6] For example, Pegloticase (PEGylated uricase) induces ADAs in ~90% of the refractory chronic gout patients, resulting in a reduction in drug effectiveness and increased risk of infusion reactions.[4b, 4c] Similarly, clinical hypersensitivity occurred in a subset of acute lymphoblastic leukemia patients received Pegaspargase with reported incidence rates of up to 24%.[7] Recently, the US Food and Drug Administration (FDA) has advocated that the biopharmaceutical industry take a proactive risk-based approach to mitigate unwanted immunogenicity of the biologic therapeutics.[8] An effective approach to improve PEGylation to further reduce ADAs is apparently desired.



Scheme 1. a) Schematic showing of the immunomodulatory protein bioconjugate structure. b) When uptaken by dendritic cells, rapamycin released from the bioconjugate inhibits dendritic cell maturation and prevents ADA generation.

Traditional methods, e.g., PEGylation, passively reduce ADAs by physically hiding the underlying protein cargos from immune recognition. However, such passive strategies may suffer from particular limitations, such as material immunogenicity and insufficient protein surface epitope coverage.[6a] A nanogel encapsulation strategy was proposed to overcome the concerns of ADAs by sterically covering the whole protein surface using zwitterionic polymers [5d]. However, as a trade-off, the hydrogel coating strategy can only be applied to enzymes with small substrates. As an alternative, a proactive strategy that endows PEGylated therapeutics with the ability to modulate the immune system is a new effective approach. Rapamycin, a commonly used immunosuppressant to prevent organ transplant rejections, has been shown to inhibit dendritic cell (DC) maturation and induce tolerogenic DCs.[9] Rapamycin encapsulated PLGA nanoparticles have been used to reduce

[*] Dr. P. Zhang, Dr. P. Jain, C. Tsao, K. Wu, Prof. S. Jiang
Department of Chemical Engineering, University of Washington, Seattle, WA, 98195 (USA)
E-mail: sjiang@uw.edu

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ADAs of biotherapeutics, and human clinical trials are currently ongoing.[10] Herein, we report an immunomodulatory bioconjugation chemistry to proactively mitigate ADA responses, by conjugating rapamycin to PEGylated therapeutic proteins. When circulating in blood, the bioconjugate will play its therapeutic role as normal PEGylated drugs; while the conjugated rapamycin can be released and prevent immune responses once the bioconjugate is uptaken by antigen presenting cells. The conjugation chemistry ensures the co-delivery of the immunomodulator and the protein, avoiding potential systemic immune suppression and related complications.

Rapamycin is a macrocyclic polyketide whose biological activities are dependent on the binding of the left-hand portion of the molecule, from C8 to C31, to FKBP12 (FK-506 binding protein) and the subsequent formation of a tertiary complex with mTOR (mammalian target-of-rapamycin) protein.[11] Modification of the 42-OH position has led to a series of derivatives with good activity.[12] Thus, the 42-OH position was selected in our study to introduce the reactive moiety for bioconjugations. Rapamycin 42-hemisuccinate was firstly synthesized via lipase-catalyzed regioselective esterification following a published procedure.[12a] The carboxyl group was then activated and conjugated to PEG molecules (10 kDa) through a disulfide linker, denoted as PEG-ss-rapa. The successful conjugation was verified by both NMR (Fig. S2) and UV-Vis spectra (Fig. 1a). A rapamycin substitution degree of 87% was calculated from the UV absorption intensity. The disulfide bond was introduced as the environmental triggering mechanism to release the immunostimulators intracellularly. As shown in Fig. 1a, when treated with dithiothreitol (DTT), the characteristic absorption peak of rapamycin around 280 nm rapidly disappeared, indicating its release from the conjugate.

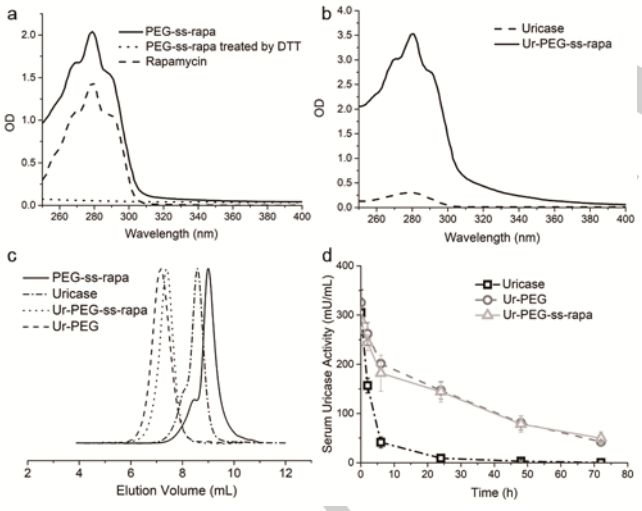

Figure 1. a) UV-Vis spectra of the PEG-ss-rapa polymer before and after DTT treatment (in PBS), in comparison with the spectrum of rapamycin (DMSO solution). b) UV-Vis spectra of the native uricase and bioconjugate, at a protein concentration of 2 mg/ml. c) Gel-permeation chromatography (GPC) traces of uricase and the bioconjugates. d) Circulatory profiles of uricase and bioconjugates (n=6). The serum uricase activity was measured after one intravenous injection in rats.

PEG-ss-rapa was subsequently conjugated to uricase to test its performance both *in vitro* and *in vivo*. Uricase derived from *Candida sp.* was selected as the model protein in this study due to its strong immunogenicity. To perform the protein conjugation, the carboxyl end-group of PEG-ss-rapa was activated as N-hydroxysuccinimide (NHS) ester, and then bioconjugation was completed by reacting with the protein lysine residues. The successful protein conjugation was revealed by UV-Vis spectra (Fig. 1b), as the bioconjugate (denoted as Ur-PEG-ss-rapa) showed an 11-fold stronger absorption at 280 nm compared with the native uricase. Based on the UV absorptions, the conjugation density was calculated to be 18.6 rapamycin molecules with a total of 21.4 PEG polymer chains per uricase tetramer. A PEGylated uricase (Ur-PEG, 22 PEGs per protein tetramer) was also prepared using 10 kDa PEG-NHS for the purpose of comparison. GPC analysis showed that Ur-PEG-ss-rapa possessed a similar hydrodynamic size as the Ur-PEG bioconjugate at a similar polymer conjugation density (Fig. 1c). Both uricase bioconjugate showed residue activities of > 95% with respect to the native enzyme. The *in vivo* circulation behavior of Ur-PEG-ss-rapa was evaluated in a rat model. As shown in Fig. 1d, no significant difference was observed between the serum concentration-time curves of Ur-PEG-ss-rapa and the regular PEGylated uricase, indicating that the conjugation of rapamycin did not sacrifice its circulation half-life.

Rapamycin is known to inhibit dendritic cell maturation, which is considered as the key mechanism for immune tolerance induction against specific antigens.[9c] We incubated the Ur-PEG-ss-rapa bioconjugates with mouse dendritic cells (DC 2.4) to examine its influence on dendritic cell activation. The dendritic cells were firstly incubated with the bioconjugates for 24 h, followed by exposure to lipopolysaccharides (LPS) for another 24 h. The expression of two biomarkers, CD80 and CD86, was examined by flow cytometry. As shown in Fig. 2a, the costimulatory molecules on DCs in the Ur-PEG group were all up-regulated compared with the untreated immature DC control, showing the sign of activation. The Ur-PEG-ss-rapa treated group exhibited lower surface expression of CD86 compared with immature control cells, which is consistent with previous reports regarding rapamycin treated DCs.[9c] Similarly, the up-regulation of CD80 was also reduced compared with Ur-PEG treated cells. Normalization of independent experiments by the conversion of flow data to the percentage of the MFI for the indicated group, relative to the MFI for immature control confirmed that the decreases in CD80 and CD86 on Ur-PEG-ss-rapa treated DCs were statistically significant (Fig. 2b). Collectively, the low expression level of costimulatory molecules CD80 and CD86 in the Ur-PEG-ss-rapa group proved the biological activity of the conjugated rapamycin derivative.

The ADA generation against Ur-PEG-ss-rapa bioconjugate was revealed by measuring both protein and bioconjugate specific antibodies after three weekly intravenous (I.V.) injections in a rat model. An uricase-PEG-rapamycin (denoted as Ur-PEG-rapa) bioconjugate without cleavable disulfide bond was included for comparison. As shown in Fig. 3a, the native uricase stimulated a robust antibody response due to its strong immunogenicity, while regular PEGylation reduced anti-uricase by about 6-fold. By contrast, Ur-PEG-ss-rapa bioconjugate

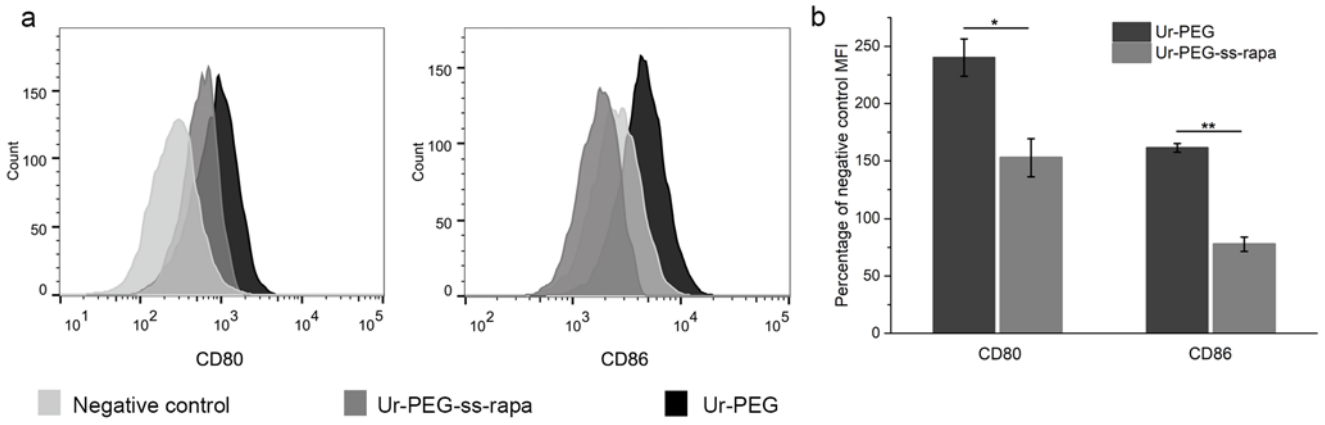

Figure 2. Ur-PEG-ss-rapa inhibited dendritic cell maturation. DC 2.4 cells were treated by the bioconjugates for 24 h and stimulated by LPS. The control cells were neither treated by bioconjugates nor the LPS. The upregulation of CD80 and CD 86 was analyzed by flowcytometry. a) Histograms and b) averaged mean fluorescence intensity (MFI) of the samples (n=3). Student's t test was chosen to compare two small sets of quantitative data, **p*<0.05, ***p*<0.01.

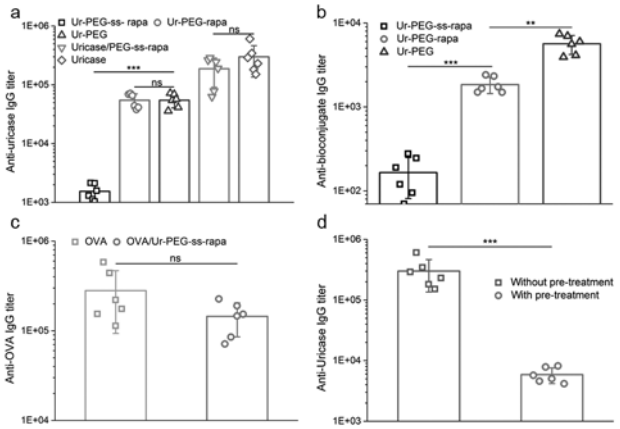

Figure 3. Antibody responses measured by enzyme-linked immunosorbent assay (ELISA). a) Anti-uricase and b) anti-bioconjugate antibodies after three weekly injections. c) Anti-OVA antibodies of the groups received three weekly OVA or OVA/Ur-PEG-ss-rapa co-injections. d) The pre-treatment group firstly received three weekly administrations of the immunomodulatory bioconjugates. After two weeks, three weekly administrations of native uricase were then given. The antibody responses were compared with the group received solely three injections of the native uricase. All statistical analyses were performed using a one-way ANOVA with a Bonferroni posttest, n=6, **p*<0.05, ***p*<0.01, ****p*<0.001.

only generated a negligible anti-uricase response, which was 35-fold lower than the regular PEGylated uricase. Simply mixing and co-injection of uricase with PEG-ss-rapa free polymers did not significantly reduce the anti-uricase response compared with the native uricase. Other studies also suggested that co-injection of free rapamycin with protein antigens cannot induce antigen specific tolerance,[10a,10b] indicating that rapamycin needs to be co-delivered with the antigen. It should be noted that the Ur-PEG-rapa bioconjugate showed a similar anti-uricase response with regular PEGylated uricase, demonstrating that rapamycin needs to be released as the free form to function as an immunomodulator. The anti-bioconjugate antibody responses showed a similar trend as that of the anti-uricase antibody (Fig. 3b). The non-releasing Ur-PEG-rapa bioconjugate showed a 3-fold less anti-bioconjugate titer compared with regular PEGylated uricase, while the cleavable Ur-PEG-ss-rapa reduced the titer by 34-fold to a negligible level. As a classic passive strategy, PEGylation reduced ADAs generation by the "stealth" effect; nonetheless, the effectiveness is limited by the achievable protein conjugation density. In addition, the immunogenic protein carrier amplified the immunogenicity of the haptenic PEG molecules, which results in the generation of anti-bioconjugate ADAs.[6a] The combination of the proactive mechanism with traditional PEGylation breaks the limitation of the passive strategy, as the released drug actively modulates the immune activity. In order to inhibit ADAs, the protein/antigen and rapamycin need to be delivered into the same dendritic cell. Compared with the linked protein/antigen in the bioconjugate, co-injected free antigens have far less opportunity to be uptaken by a dendritic cell that also uptakes rapamycin. As a result, only the immune responses against the conjugated proteins are suppressed.

The usage of immunosuppressive drug may cause non-selective immunosuppressive effect, which could result in increased susceptibility to infections.[13] To examine the selectivity of the antibody generation inhibition, we co-injected Ur-PEG-ss-rapa together with ovalbumin, and compared the anti-ovalbumin antibody titers with the group received ovalbumin alone. As shown in Fig. 3c, both groups had strong responses against ovalbumin, with no significant differences in antibody titers. The similar antibody levels demonstrated that the conjugated rapamycin suppresses the immune responses in an antigen-specific manner. As showed in the above *in vitro* tests, the conjugated rapamycin inhibits DC maturation, and the immature DCs are known to induce immune tolerance.[14] To further test whether the antibody inhibition is a result of antigen-specific tolerance, we conducted another study by measuring the antibody response of the native uricase administration post a pre-treatment regimen of the Ur-PEG-ss-rapa. In this test, the animals firstly received three dosages of Ur-PEG-ss-rapa as the pretreatment. Two weeks later, three weekly administrations of native uricase were given and the antibody titers were examined

two weeks post the final injection. As shown in Fig. 3d, the pretreatment resulted in a 51-fold lower anti-uricase IgG titer compared with the control group, demonstrating a persistent antigen-specific tolerance. As a benefit, the rapamycin-antigen conjugation strategy may also have the potential to be used as a treatment for autoimmune disease in addition to the reduction of ADAs.

In conclusion, we report a proactive strategy to reduce ADAs of the PEGylated biologic drugs via immunomodulatory bioconjugation chemistry. The rapamycin derivative was conjugated to PEGylated proteins via a cleavable disulfide linker. *In vitro* tests showed that the conjugated rapamycin is capable of inhibiting the maturation of dendritic cells. *In vivo* studies demonstrated that rapamycin conjugated via the cleavable linker effectively mitigates ADAs against PEGylated proteins. Further studies proved that the inhibition of ADAs was a result of the antigen-specific immune tolerance induced to the conjugated protein, which avoided systemic immune suppression and the risk of increased susceptibility to infections. All of these results indicate that rapamycin bioconjugation offers a promising approach to minimize the issues of ADAs, which are associated with adverse hypersensitivity reactions and the loss in the efficacy of otherwise effective drugs.

Acknowledgements

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Keywords: bioconjugation • immune response • PEGylation • protein therapeutic • rapamycin

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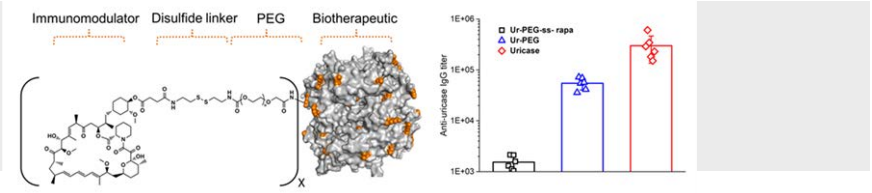

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