Role of polyethylene glycol (PEG) linkers: trends in antibody conjugation and their pharmacokinetics

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ABSTRACT

Polyethylene glycol (PEG) has been the most commonly used polymer for the past few decades in the field of biomedical applications due to its gold standard stealth effect. PEGylation of antibody–drug conjugates, liposomes, peptides, nanoparticles, and proteins is done to improve their pharmaceutical efficacy and pharmacokinetic properties. PEGylation of antibodies with various PEG linkers improves targeting ability by increasing the blood circulation time and thus enhances the biodistribution profiles. It also assists in minimizing the immediate capture by the reticuloendothelial system. In this review, we summarize the effect of PEG linkers in an antibody conjugation and their pharmacokinetics in the field of biomedical imaging.

Key Word: PEG linkers, antibody conjugate, pharmacokinetics, blood circulation

Introduction

Polyethylene glycol (PEG) is a nonionic hydrophilic biocompatible polymer in nature with a gold standard stealth effect. It has been extensively used in human intravenous, oral, nasal, and dermal applications and is also an important ingredient in food and cosmetic industries [1]. PEGylation of molecules with various PEG linkers would be a wise methodology to enhance bioavailability, stability, safety, and efficacy, which aids a wide range of applications in both pharmaceutical development and biomedical imaging. Moreover, the attachment of PEG linkers to any system decreases self-aggregation due to steric stabilization, improves prolonged blood circulation, and avoids blood opsonization. Furthermore, it also enhances

stability over a wide range of pH and temperature changes. Therefore, the insertion of PEG linkers will facilitate an immeasurable significance in both drug development and clinical applications [2]. In recent applications, PEG linkers have been inserted into diverse system such as antibodies (Abs), antibody-drug conjugates, lipid/polymeric nanoparticles (NPs), dendrimers, micelles, and liposomes improving their hydrophilic nature and hydrodynamic size, thereby decreasing immunogenicity, minimizing proteolytic cleavage, and facilitating interactions with blood proteins and mononuclear phagocyte system (MPS) in the cells. In addition to these effects, PEGylation increases the water solubility of hydrophobic anticancer drugs such as doxorubicin, camptothecin, and paclitaxel. Till date, more than 21 PEGylated drugs with different

formulations have been approved by the US FDA, but none of the other synthetic polymer-based drugs have achieved this status [3].

Furthermore, PEGylation has a positive impact in the field of radiopharmaceutical development. Different PEG linkers have been inserted between antibodies and chelators to minimize the immediate capture of radiolabeled antibodies by the reticuloendothelial system (RES) and maximize their delivery to targeted tumors. It has also been reported that the addition of a PEG linker results in increased tumor-to-background ratios that are superior to those of conventional antibody conjugates [4]. In the pretargeting strategy, PEGylated radiolabeled chelates demonstrated an improved tumor uptake compared with non-PEGylated radiolabeled chelates. Moreover, PEG linkers accelerated the rate of bioorthogonal click reaction by inhibiting the cis–trans isomerization of trans-cyclooctene (TCO) in serum [5]. The overall trends justify that insertion of PEG linkers into any type of substrate in turn enhances the following properties: 1) therapeutic or diagnostic property by reducing unintended uptake in normal tissues, 2) decreasing systemic toxicity, and 3) increasing the blood retention time, thereby enhancing tumor accumulation. In this brief review, we outline the PEG linker effect in an antibody conjugation, the in vitro and in vivo behavior, and the biodistribution and pharmacokinetic properties.

Decoding the effect of PEG linkers by optical imaging in antibody conjugation

Recently, PEGylation has emerged as one of the well-recognized methods for maintaining the in vivo stability and pharmacokinetics of biologically important proteins and peptides [6-7]. In addition, adjusting the hydrophobicity by the PEGylation method can help in modifying small molecules and radiopharmaceuticals

[8-9]. Optical probes with specific targeting ability are important for endoscopic surgical guidance. Several monoclonal antibody-conjugated fluorophores have already been clinically developed based on their unique targeting ability of monoclonal antibodies. Unlike other molecular imaging probes (radionuclides), the important aspect of optical imaging probes is that they are switchable (can be turned off and on as needed). In recent decades, indocyanine green (ICG) has been used as a potential fluorophore with an excellent safety profile for the clinical translation of antibody conjugation due to fluorescence emissions in the NIR region. Generally, the bifunctional ICG derivatives are highly quenchable and the probe can turn on only at the targeted tissue. On the other hand, the signal emanating from always-on probes (IRDye 700 and IRDye 800-labeled probes). Owing to the interaction between ICG and aromatic amino acids on mAbs, ICG conjugation with mAbs results in auto-quenching [10]. Antibody degradation occurs after cellular internalization, and the discharged ICG from mAbs reflects as an effective light emitter. However, even after purification, noncovalently bound ICG is gradually released, which causes more nonspecific background signals, specifically in the liver and abdomen. In 2013, Hisataka Kobayashi et al. demonstrated the incorporation of a short-length PEG linker between a fluorophore and a sulfo-N-succinimidyl residue to minimize the lipophilicity of ICG derivatives (ICG=1.84±0.06, PEG_4-ICG=0.64±0.06 & PEG_8-ICG= -0.03±0.02). Similarly, the dye conjugated antibodies shows decrease dequenching capacity panitumumab-ICG (Pan-ICG=15.1, Pan-PEG_4-ICG=10.2 & Pan-PEG_8-ICG=6.7fold) and increases the covalent binding with the antibody (70-86%) results in high serum stability, in vitro cellular uptake, and enhances tumor recognition. The conjugation ratio of ICG to mAb (>1) was higher than previous studies (0.3 to 0.5). In the in vitro studies demonstrate the fluorescence intensity increases substantially on increasing time. These results indicate that the activation of conjugate occur after internalization and
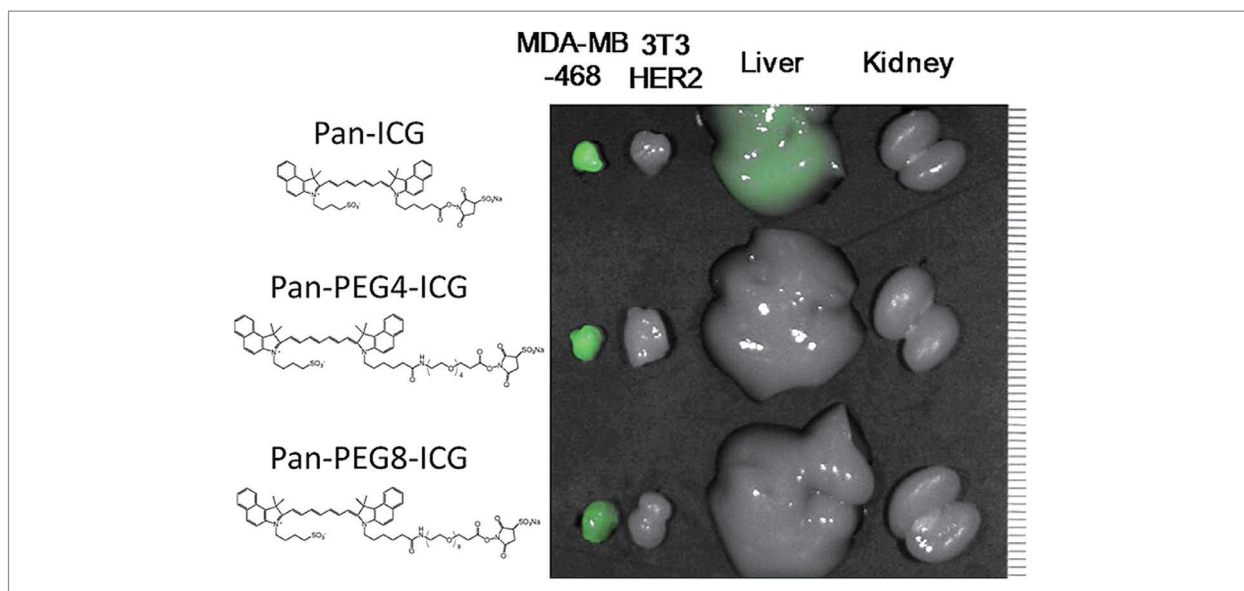
Figure 1. Ex vivo imaging with and without polyethylene glycol (PEG) containing mAb-ICG.

lysosomal processing which leads to complete dequenching by 1 h. In the in vivo fluorescence dynamic imaging study demonstrate Pan-PEG$_4$-ICG were able to detect EGFR-1 (epidermal growth factor receptor 1) with higher tumor-to-background ratios (15.8 for EGFR+ to EGFR- tumor and 6.9 for tumor to-liver ratios at 3 d p.i.) (Figure 1) [11].

Generally, compare to the larger size IgG, smaller minibody exhibits rapid blood clearance and improve the target-to-background ratio in the in vivo imaging. Fab and F(ab)´2 fragments, diabodies, and minibodies were produced by enzymatic and genetic modification of mAbs [12]. In 2014, the same group Hisataka Kobayashi et al extended their work by incorporating the short PEG linker between the minibody and indocyanine green (ICG) against PSMA-MB (prostate-specific membarane antigen) resulted in better tumor-specific signal. In this work, the following dequenching results were observed by adding 1% SDS to dye-conjugated (PSMA-MBs) PSMA-MB-ICG=36.3, PSMA-MB-PEG$_4$-ICG=16.9, PSMA-MB-PEG$_8$-ICG=6.2 and PSMA-MB-IR700=1.4 fold. The covalent binding ICG to PSMA-MB were PSMA-MB-ICG=16.8%, PSMA-MB-PEG$_4$-ICG=67.6%, PSMA-MB-PEG$_8$-ICG=67.8% and PSMA-MB-IR700=73.4%

respectively. The conjugated ratio of dyes to PSMA-MB was PSMA-MB-ICG=3.5, PSMA-MB-PEG$_4$-ICG=3.0, PSMA-MB-PEG$_8$-ICG=2.6 and PSMA-MB-IR700=4.1 respectively. The fluorescence activated cell sorting flow cytometry and in vivo imaging showed PSMA-MB-(PEG$_{4\ \&\ 8}$)-ICG exhibited minimal signal in PSMA-negative cells, whereas considerably high response for PSMA-positive cells observed (Figure 2) [13]. However, PSMA-MB-IR700 (always-on probe) shows high background signal. Nevertheless, in the in vivo biodistribution the radioiodinated ^{125}I-PSMA-MB-PEG$_8$-ICG and ^{125}I-PSMA-MB displayed identical distribution profiles. Both mainly distributed to the kidney, lung, heart, liver and spleen at 1 h post injection and the radioactivity gradually decrease thereafter. The activity was decreased in the other organs over time as well as uptake in the stomach showed transient increase at 6 h. High TBR was observed PSMA-MB in PSMA-positive tumor upto 3 d, while the radioactivity in PSMA -negative tumors declined much more rapidly. After intravenous injection into a xenograft mouse model, an anti-PSMA mAb-ICG conjugate required 1-10 d to detect PSMA-positive tumor. For clinical translations, this delay is not appropriate. To resolve this issue, they used
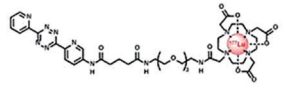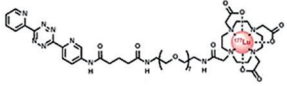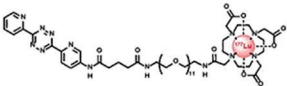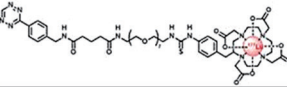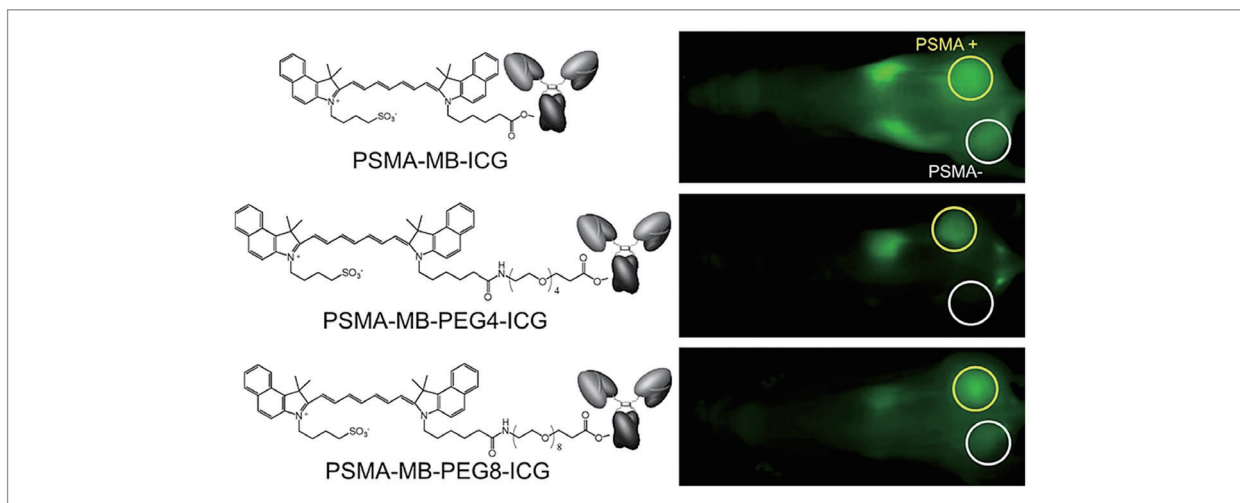
Figure 2. In vivo images of PSMA+ and PSMA− tumors of mice injected with PSMA-MB-PEG$_0$-ICG, PSMA-MB-PEG$_4$-ICG, and PSMA-MB-PEG$_8$-ICG.

anti-PSMA minibody showing higher contrast compare to the always on (PSMA-MB-IR700) probe at 6 h post injection and extended up to 5 d p.i. To summarize, the incorporation of a short PEG linker increased the covalent binding percentage of ICG to the minibody, and increasing tumor to background ratio by reducing the release of ICG monomers. Hence, minibody–ICG was demonstrated as an alternative optical probe over full antibody–ICG in the field of optical imaging.

Evaluation of PEGylation on antibody by bioorthogonal pretargeting strategy

Pretargeting strategy is a promising approach in the field of tumor diagnosis and therapy. As a result of pretargeting by the bioorthogonal inverse electron demand Diels–Alder reaction (IEDDA) by tetrazine (Tz) and trans-cyclooctene (TCO), it represents a multistep process. The TCO-modified targeting agent is initially injected, and then, after a required period of time, the radiolabeled probe-bearing Tz is injected, forming in vivo radioimmunoconjugate. Hence, reducing the circulation time of the radiation and

avoids the radiation uptake in nontargeted organs [14]. In the attempts of pretargeted radioimmunotherapy (PRIT), to our knowledge, Françoise Degoul et al. were the first to report in 2019 about the bioorthogonal click chemistry in peritoneal carcinomatosis, orthotopic tumor models, using 35A7 monoclonal antibody (mAb) by targeting carcinoembryonic antigen [15]. In this study, TCO was conjugated with mAb and ^{177}Lu labeled DOTA was conjugated to Tz with various lengths of PEG linkers (PEG$_3$, PEG$_7$ or PEG$_{11}$) and denoted as Tz-1 for PEG$_3$, Tz-2 for PEG$_7$ and Tz-3 for PEG$_{11}$. The influence of PEG was clearly determined in collective experiments, primarily in the clearance study. While the radiolabeled probe with short PEG Tz-1 was excreted through liver, intestine and kidney, while Tz-2 and Tz-3 were excreted via kidney. Studies on pretargeted biodistribution have shown that Tz-2 actively targets tumors with 6.29 %ID/g at 144 h with longer blood circulation time than other Tz probes, and hence Tz-2 was further used for PRIT that significantly reduced tumor growth. The probe Tz-2 demonstrated a similar profile compared with the established Tz-4 probe, which had the same PEG length, except for the acid groups on DOTA. However, both probes exhibited a hydrophilic nature, whereas Tz-1 exhibited a lipophilic nature, resulting in high background nonspecific signals in the liver and intestine (Figure 3).

Figure 3. Structures and radiochemical properties of radioligands with varying lengths of PEG linker.

In addition, in another attempt, the same research group reported about pretargeting strategy by optimizing factors such as the number of PEG linkers (PEG_0, PEG_4, PEG_{12}) between mAbs and TCO along with modification of mAbs with different equivalents (eq.) of TCO [16]. These modifications were conducted on two different non internalizing mAbs, Ts29.2 and 35A7, with different grafting numbers of TCO (1.3 to 16.0) by changing the number of eq. of TCO in the reaction which was confirmed by MALDI-TOF MS (Figure 4). However, in the in vitro analysis immunofluorescence intensity was increased with the increasing number of PEG, and this trend was observed in both mAbs, and the maximum intensity was found in PEG_4 with 10 eq. of TCO. Similarly, with fluorescent Tz probes the specific interaction of TCO and Tz was confirmed. In vivo assessment of $PEG_{0, 4, 12}$ by direct targeting in the HT29 xenograft model revealed no significant difference between PEGylated and non-PEGylated Ts29.2 mAbs. The pretargeting strategy in same tumor model revealed, the region of interest (ROI) average radiance was slightly higher in PEG_0 than in PEG_{4} $_{\& 12}$ but two-fold lower than direct targeting strategy. In pretargeting study for HT29 xenografts, significant signal from lymph node was obtained due to extravasations in lymphatic system of Tz probe and was reduced over a

period of time. Similarly, in peritoneal carcinomatosis A431 xenografts, the in vivo fluorescence imaging demonstrated that higher fluorescence signal was emitted in PEG_0 than the $PEG_{4, 12}$ in the peritoneal cavity of mice by pretargeting with 35A7 TCO modified mAb at 24 h, 48 h, and 72 h injection before Tz probe. Overall, TCO grafting was more efficient when PEG linkers were involved, and the longest $PEG_{4, 12}$ in both antibodies grafted more TCO moieties than PEG_0 without altering the ability of mAb to recognize cell membrane antigen. However increased PEG number reduces the reactivity of TCO while PEG_0 representing the strong contestant for PRIT.

Reducing the hepatic radiation uptake via PEGylation (^{111}In-DTPA-PEG-C225)

The epidermal growth factor receptor (EGFR), a transmembrane glycoprotein with an intracellular tyrosine kinase domain. It has been considered as a targeting receptor for both antitumor diagnosis and therapy due to its higher expression level on a variety of cancerous solid tumor cells such as breast, renal cell, colon, squamous cell,
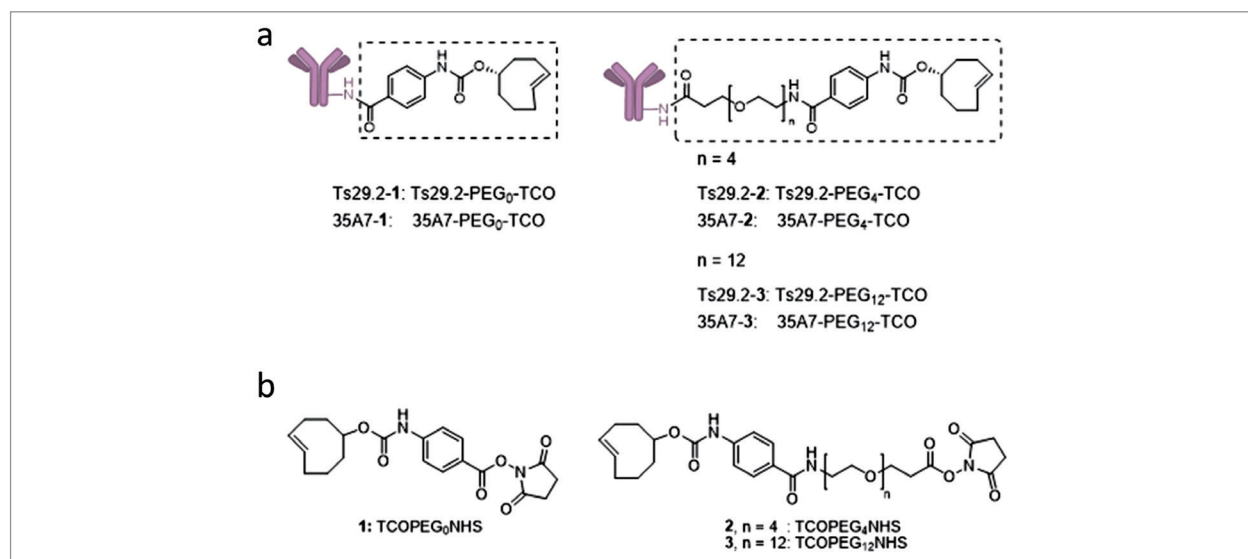
Figure 4. Structures depicting general modifications in Ts29.2 and 35A7 mAb conjugates with varying PEG linkers (a). Structure of TCO-1 with PEG_0 and TCO-2 and TCO-3 with PEG_4 and PEG_{12}, respectively (b).

ovarian, and prostate cancers [17, 18]. C225 is an EGFR-targeting human-mouse chimeric monoclonal antibody. It has also been reported that C225 antibody prevents cancer cell proliferation by inhibiting tyrosine kinase activation [19]. In this context, few C225–drug conjugates have been developed for treating different types of cancers, which are currently in phase II clinical trials [20]. The major obstacle in radioimmuno detection using radiolabeled antibodies is the liver uptake, which consequently hampers their clinical application. To address this issue, Chun Li et al. reported that PEGylated radioimmunoconjugate (^{111}In-DTPA-PEG-C225) enhances the imaging of anti-EGFR compared to the unmodified conjugate (^{111}In-C225-DTPA) by reducing the liver uptake [21]. DTPA-PEG-C225 with different ratios of PEG-DTPA (1:10 and 1:30) and C225-DTPA were synthesized according to reported protocols. Then, the radioimmunoconjugates (^{111}In-DTPA-PEG-C225, 1:10 and 1:30) were injected into EGFR-rich A431 cancer xenografts and successfully imaged. As anticipated, there was clear reduction in the liver uptake for ^{111}In-DTPA-PEG-C225 [27.0% ± 3.0% ID/g (1:10); 25.5% ± 2.0% ID/g (1:30) in comparison with 46.9% ± 2.5% ID/g for DTPA-C225 at 48 h p.i. and the changes in reduction uptake were purely dependent on the increased

PEG ratios (1:10 and 1:30) (Figure 5). In contrast, there was no sufficient change in the tumor uptake with these conjugates compared with ^{111}In-DTPA-C225; however, clear tumor visualization was observed. Based on these observations, the unchanged tumor uptake for ^{111}In-DTPA-PEG-C225/^{111}In-DTPA-C225 implies that the binding mechanism for EGFR is similar and it was not affected by PEG modification. To further confirm the mechanism, blocking studies were conducted using native C225 (100-fold excess) that was injected 20 h before the injection of ^{111}In-DTPA-PEG-C225 to block the overexpressed EGFR. The ɤ–images and ex vivo analysis at 48 h p.i. were well-matched with each other, and the tumor-to-blood ratios (2.5–2.7-fold) and tumor-to-liver ratios were also dramatically reduced after pretreatment with C225. Finally, the binding mechanism was evaluated using EGFR-rich (MDA-MB-468 and A431 EGFR-positive) and EGFR-deficient (MD-MBA-435) tumor models. The EGFR-rich xenografts were clearly visualized with ^{111}In-DTPA-PEG-C225, whereas such visualization was not observed in MD-MBA-435-negative xenografts at 24 h p.i. To summarize, Insertion of PEG linker between C225 antibody and radioligand reduces the liver uptake dramatically by restoring its receptor-mediated binding

affinity toward EGFR. Such an observation was not noticed with non-PEGylated radioimmunoconjugate. Further studies are required to explore whether PEG modification can cause any change in the immunogenicity of C225 before its clinical application.

Impact of linkers and conjugation chemistry on antibody–drug conjugates

Chemotherapy is one of the most common treatment for various cancers, which involves delivering anticancer drugs to kill cancerous cells; however, the major obstacle is the off-target delivery of cytotoxic pay loads [22]. To overcome this challenge, antibody–drug conjugates (ADCs) were developed, wherein a monoclonal/humanized antibody is linked to a cytotoxic payload through different chemical linkers. This novel strategy is used to deliver small cytotoxic drugs site-specifically (cancer cells), thus improving the treatment efficacy by reducing the off-target delivery of payloads,

Figure 5. Quantification of tumor-to-liver ratios from sequential γ-images with/ without PEG-modified C225 conjugates, presented as mean ± SEM (n = 3).

pharmacokinetics/pharmacodynamics (PK/PD), and biodistribution compared with traditional chemotherapies [23]. In this context, the choice of the linker plays a vital role in linking the antibody and cytotoxic payload to determine the effectiveness of ADC therapy. Linkers that are used in ADCs must be stable in blood serum to avoid premature release of payloads, be less immunogenic, and possess efficiency in delivering the cytotoxic payloads from ADCs site-selectively [24]. In general, heterobifunctional linkers are favorable for preparing ADCs as they consist of reactive functional groups at both ends, which in turn are easily available for coupling with both antibody or drug molecules according to the choice. Overall, the choice of linkers is most important in the development of ADCs. To understand the effect of linkers on ADCs, William M. Atkins et al. reported the impact of linkers and conjugation chemistry on antibody–drug conjugates [25]. To explore the strategy two model idiotype monoclonal antibodies (IgG1) like as H10 and aHIS has been chosen. These are most common type of antibodies used in ADCs platform. The IgG1 contains 16 disulfide bonds (2 heavy-heavy and 2 heavy weight), 12 disulfide bonds at intrachain along with many lysine residues available for different conjugations. Here, in this study, three different linkers with different length and hydrophobicity were used to attach the model drug biotin on each IgG1 (thiol, amine, and oxidized carbohydrates) with different payload ratios. First, the thermal stability of each conjugate was evaluated using DSC analysis. The functionality of both antibodies with amine and carbohydrate linkers had minimal effects on thermostability (T_m) compared with thiol conjugates. However, the thiol linkers had a greater impact on thermostability for H10 than aHIS ADCs with comparable biotin payload. The thermostability of reduced antibodies (thiol reduction with TCEP) was tested, and results demonstrated that thermostability is purely dependent on IgG, and not on thiol reduction. Next, the antibody binding affinities toward antigen were determined for each of the linker conjugates and compared with unmodified
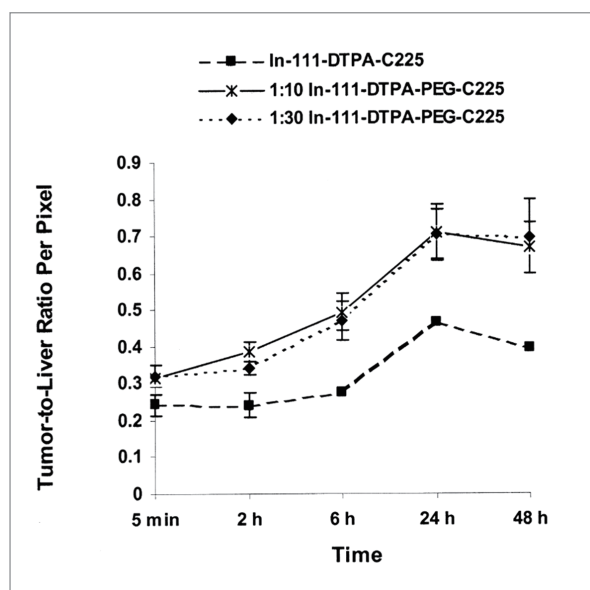
antibody. None of the ADCs showed a significant impact on antigen binding; the amine conjugate $H10_NPEG_4$ (anti-hen lysozyme coupling to amines with PEG_4) showed some adverse effects on antigen binding, most probably due to the high payload of biotin at the lysine residue. Finally, the effects of all three conjugate linkers on Fc receptor (CD32b) binding interactions along with protein G and protein A (A known as inhibitors of FcR binding) binding assays were investigated using the SAR sensor surface method. Amine-coupled $H10_NPEG_{4H}$ exhibited some negative effect on FcR binding due to high payload ratios of biotin and not due to the linker and conjugation chemistry. This type of reduction in FcR binding is common for PEGylated antibodies [26]. However, none of the other conjugates displayed any impact on FcR binding, except $aHiS_tPEG_8$ (anti-6xHis coupling to thiols with PEG_8) and $aHiS_NLC$ (anti-6xHis coupling to amines with 6-aminohexanoic acid) conjugates (minimum effect).

Conclusion

We have overviewed the effects of PEGylation on antibodies and its drug conjugates based on multimodality approaches, varying from optical imaging to nuclear imaging. Biocompatible PEG derivatization is performed to improve hydrophilicity, which results in prolonged blood circulation, low immunogenicity, and elevation of tumor uptake and avoids the premature release of drugs in the bloodstream. Furthermore, in the field of nuclear imaging, PEGylation reduces the nonspecific interaction of radiation in vital organs, according to the above-described pretargeting strategies that allow the use of high doses of radiation as well as short-lived radioisotopes. However, further comprehensive studies are required to explore or understand the complete role of the diversity of PEG linkers in the field of modern-day radiopharmaceuticals, biomedical diagnostics, and therapeutic applications.

Acknowledgements

References

1. Knop K, Hoogenboom R, Fischer D, Schubert SU. Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew Chem Int Ed* 2010;49(36):6288–6308.

2. Cheng T, Chuang K, Chen B, Roffler RS. Analytical Measurement of PEGylated Molecules. *Bioconjugate Chem* 2012;23(5):881−899.

3. Kozmaab TG, Shimizu T, Ishida T, Szebeni J. Anti-PEG antibodies: Properties, formation, testing and role in adverse immune reactions to PEGylated nano-biopharmaceuticals. *Adv Drug Delivery Rev* (DOI:10.1016/j.addr.2020.07.024).

4. Kwon YL, Scollard AD, Reilly MR. ^{64}Cu-Labeled Trastuzumab Fab-PEG$_{24}$-EGF Radioimmunoconjugates Bispecific for HER2 and EGFR: Pharmacokinetics, Biodistribution, and Tumor Imaging by PET in Comparison to Monospecific Agents. *Mol Pharmaceutics* 2017;14(2):492−501.

5. Rahim KM, Kota R, Haun BJ. Enhancing Reactivity for Bioorthogonal Pretargeting by Unmasking Antibody Conjugated trans-Cyclooctenes. *Bioconj Chem* 2015;26(2): 352–360.

6. Harris MJ, Chess BR. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discovery* 2003;2(3):214-221.

7. Roberts JM, Bentley DM, Harris MJ. Chemistry for peptide and protein PEGylation. *Adv Drug Delivery Rev* 2002;54(4):459−476.

8. Stephenson AK, Chandra R, Zhuang ZP, Hou C, Oya S, Kung MP, Kung FH. Fluoro-pegylated (FPEG) imaging agents targeting Abeta aggregates. *Bioconjugate Chem* 2007;18(1):238−246.

9. Ono M, Watanabe R, Kawashima H, Cheng Y, Kimura H, Watanabe H, Haratake M, Saji H, Nakayama M. Fluoropegylated chalcones as positron emission tomography probes for in vivo imaging of beta-amyloid plaques in Alzheimer's disease. *J Med Chem* 2009;52(20):6394−6401.

10. Ogawa M, Kosaka N, Choyke LP, Kobayashi H. In vivo molecular imaging of cancer with a quenching near-infrared fluorescent probe using conjugates of monoclonal antibodies and indocyanine green. *Cancer Res* 2009;69(4):1268−1272.

11. Sano K, Nakajima T, Miyazaki K, Ohuchi Y, Ikegami T, Choyke LP, Kobayashi H. Short PEG-linkers improve the performance of targeted, activatable monoclonal antibody-indocyanine green optical imaging probes. *Bioconjugate Chem* 2013;24(5):811−816.

12. Wu MA, Senter DP. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol* 2005;23(9):1137−1146.

13. Watanabe R, Sato K, Hanaoka H, Harada T, Nakajima T, Kim I, Paik HC, Wu MA, Choyke LP, Kobayashi H. Minibody-indocyanine green based activatable optical imaging probes: The role of short polyethylene glycol linkers. *ACS Med Chem Lett* 2014;5(4):411–415.

14. Altai1 M, Membreno R, Cook B, Tolmachev V, Zeglis MB. Pretargeted Imaging and Therapy. *J Nucl Med* 2017;58(10):1553–1559.

15. Rondon A, Schmitt S, Briat A, Ty N, Maigne L, Quintana M, Membreno R, Zeglis MB, Navarro-Teulon I, Pouget JP, Chezal JM, Miot-Noirault E, Moreau E, Degoul F. Pretargeted radioimmunotherapy and SPECT imaging of peritoneal carcinomatosis using bioorthogonal click chemistry: probe selection and first proof-of-concept. *Theranostics* 2019;9(22):6706–6718.

16. Rondon A, Ty N, Bequignat JB, Quintana M, Briat A, Witkowski T, Bouchon B, Boucheix C, Miot-Noirault E, Pouget JP, Chezal JM, Navarro-Teulon I, Moreau E, Degoul F. Antibody PEGylation in bioorthogonal pretargeting with trans-cyclooctene/tetrazine cycloaddition: in vitro and in vivo evaluation in colorectal cancer models. *Scientific Reports* 2017;7(14918):1–11.

17. Modjahedi H, Dean C. The receptor for EGF and its ligands: expression, prognostic value and target for therapy in cancer. *Int J Oncol* 1994;4(2):277–296.

18. Ethier PS. Growth factor synthesis and human breast cancer progression. *J Natl Cancer Inst* 1995;87(13):964–973.

19. Mendelsohn J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin Cancer Res* 1997;3(12):2703–2707.

20. Baselga J, Pfister D, Cooper RM, Cohen R, Burtness B, Bos M, D'Andrea G, Seidman A, Norton L, Gunnett K, Falcey J, Anderson V, Waksal H, Mendelsohn J. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. *J Clin Oncol* 2000;18(4):904–914.

21. Wen X, Wu Q, Ke S, Ellis L, Charnsangavej C, Delpassand SA, Wallace S, Li C. Conjugation with ^{111}In-DTPA-Poly(Ethylene Glycol) Improves Imaging of Anti-EGF Receptor Antibody C225. *J Nucl Med* 2001;42(10):1530–1537.

22. DeVita TV, Chu E. A history of cancer chemotherapy. *Cancer Res* 2008;68(21):8643–8653.

23. Tsuchikama K, An Z. Antibody-drug conjugates:

recent advances in conjugation and linker chemistries. *Protein Cell* 2018;9(1):33–46.

24. Ojima I, Geng X, Wu X, Qu C, Borella PC, Xie H, Wilhelm DS, Leece AB, Bartle ML, Goldmacher SV, Chari JVR. Tumor-specific novel taxoid-monoclonal antibody conjugates. *J Med Chem* 2002;45(26):5620–3.

25. Acchione M, Kwon H, Jochheim MC, Atkins MW. Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates. *MAbs* 2012;4(3):362–372.

26. Anderson WL, Tomasi TB. Polymer modification of antibody to eliminate immune complex and Fc binding. *J Immunol Methods* 1988;109(1):37–42.