Application of ADC Stabilizing Buffers for Long-Term Storage of Antibody-Drug Conjugates

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Abstract: This application note compares various ADCs prepared using CellMosaic's ADC kits and examines the effect of ADC stabilizing buffers on those ADCs. The results show that these stabilizing buffers can be used for long-term storage of ADCs without changing their properties.

Introduction

Antibody-drug conjugates (ADCs) are new types of therapeutics currently being developed by many pharmaceutical and biotech companies. An ADC generally involves linking a very hydrophobic chemical compound to an antibody. An ADC linked through a classical linker tends to aggregate and precipitate out from solution over time. The general guideline at CellMosaic is to prepare ADCs using our conjugation kits immediately before use. Some of the ADCs may be stored at 4°C for a few weeks or months. Freeze storage of ADCs is generally not recommended due to the possibility of accelerated aggregation or precipitation during the freezing process. There is a compelling need to develop a stable formulation for the long-term storage of these ADCs.

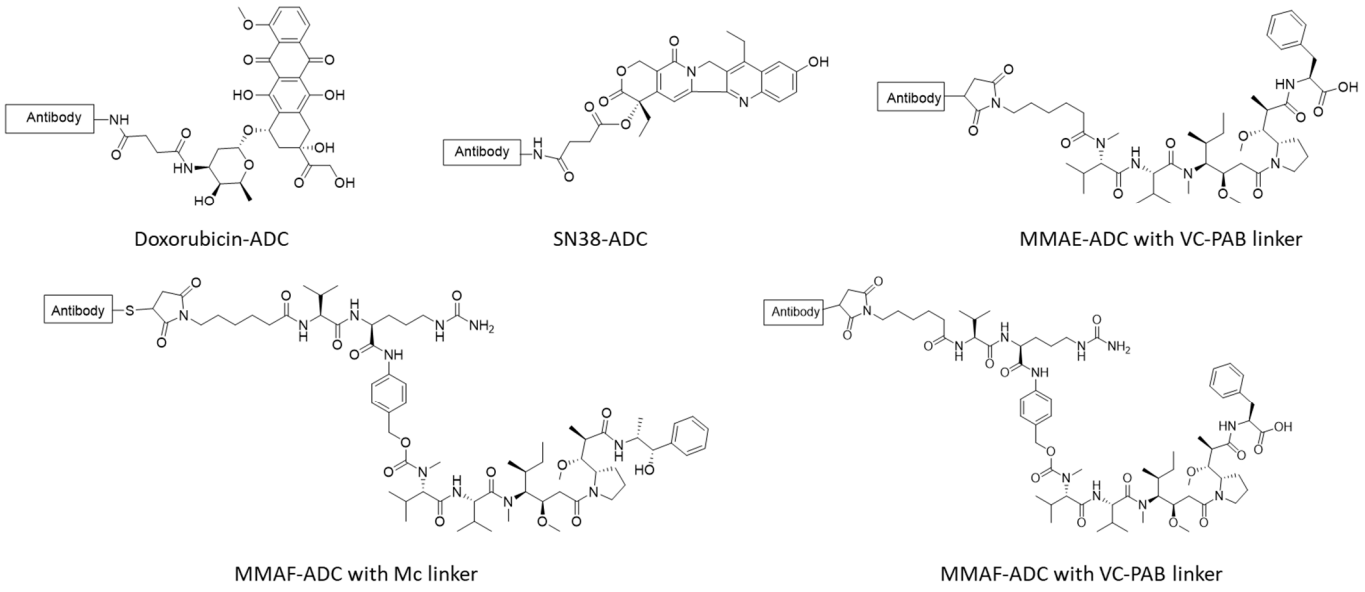

Figure 1. Chemical structures of ADCs prepared using CellMosaic's antibody drug conjugation kits: Doxorubicin-ADC (CM11406), SN38-ADC (CM11408), MMAE-ADC with VC-PAB linker (CM11409), MMAF-ADC with Mc linker (CM11422), MMAF-ADC with VC-PAB linker (CM11425).

CellMosaic has developed proprietary stabilizing buffers that can be used for long-term storage of ADCs. The buffers contain stabilizers to prevent the hydrophobic drugs from interacting with one another and, as a result, the ADCs remain in solution (stabilized) when stored below freezing. Stabilizers also help preserve the structure of the ADCs during lyophilization. After lyophilization, ADCs can be dissolved in deionized water without changing their properties. Lyophilized ADCs can also be shipped or stored temporarily at ambient temperature. Furthermore, stabilizing buffers are biocompatible and will not interfere with any *in vitro* and *in vivo* studies. The buffer does not contain preservative such as NaN_3, protease inhibitors, reducing agents, metal chelators such as EDTA, or other carrier proteins. If needed, all buffer components can be removed by dialysis or desalting before use in downstream assays.

This application note reports the performance of the ADC stabilizing buffers on some standard ADCs that were prepared using CellMosaic's ADC kits (**Figure 1**).

Materials and Methods

Materials. Antibody-drug conjugation kits, ADC stabilizing buffers, HIC buffer set, and SEC HPLC protein standard are commercial products from CellMosaic.

Preparation of ADCs. ADCs were prepared using antibody-drug conjugation kits following the protocols in the user manual. A standard human IgG1 antibody was used for all ADC syntheses.

Analysis of ADCS. The extent of aggregation of ADCs was analyzed by size exclusion chromatography (SEC) HPLC using a TSKgel G3000SWxl (7.8 mm x 30 mm) (Tosoh Biosciences). The conjugates were eluted out with an isocratic PBS buffer at a flow rate of 0.75 ml/min or 1 ml/min. SEC separates the conjugates by apparent molecular weight (MW) or size in aqueous solution. The larger the MW of the conjugate, the earlier it elutes. Human IgG1 antibody elutes out at 8.094 minutes at 1 ml/min and 11.066 minutes at 0.75 ml/min. For ADCs prepared via a reduced thiol of the antibody, hydrophobic interaction chromatography (HIC) HPLC was used to calculate the drug-to-antibody ratio (DAR) and the heterogeneity of the ADCs. The conjugates are separated based on hydrophobicity. Antibodies loaded with the same number of drugs (same DAR) will have similar hydrophobicity and be eluted as a single peak. For a typical MMAE ADC, multiple peaks represent various amounts of drug-loaded ADCs. A Waters column (Protein-Pak Hi, Res HIC, 2.5 µm, 4.6x35 mm) was used and the elution method consisted of a linear gradient from 100% buffer A (0.1M sodium phosphate buffer, 1.8M ammonium sulfate, pH 7.0) to 100% buffer B (0.1M sodium phosphate buffer, pH 7.0) in 10 minutes and then held at 100% B for another 2 minutes. The flow rate was set at 0.8 ml/min. Human IgG1 antibody eluted out at 6.188 minutes.

Results

Antibody Doxorubicin Conjugates

Doxorubicin (Dox) is an anticancer drug on the World Health Organization's list of essential medicines. Doxorubicin blocks DNA synthesis by intercalating into the DNA strand and inhibiting DNA topoisomerase II, producing free radicals that trigger apoptosis of cancer cells through DNA damage. Doxorubicin has been linked to an antibody and found to be effective in treating lung cancer [1]. Doxorubicin is very hydrophobic. ADCs with doxorubicin linked through a classical hydrophobic linker tend to aggregate and precipitate out from solution over time. The following example demonstrates the importance of adding ADC-stabilizing PBS buffer (Cat# CM02022).

Dox-ADCs were prepared using the Antibody Doxorubicin Conjugation Kit (Cat#: CM11406) and analyzed by SEC HPLC. The amount of doxorubicin loaded onto the antibody was calculated based on the UV absorbance ratio of the conjugate at 481 nm and 280 nm (see the CM11406 user manual for details). An average DAR of 4.2 was obtained with 26% aggregation. After storing Dox-ADCs in PBS buffer at a

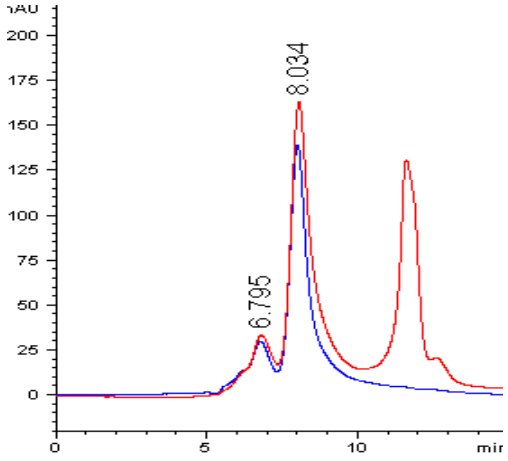

Figure 2. Overlay of the SEC HPLC profiles of Dox-ADCs in ADC-stabilizing PBS buffer (red line) and in PBS (blue line). Flow rate: 1 ml/min. Unaggregated ADC elutes at 8.034 minutes. Peaks between 11–13 minutes are for small molecules such as stabilizing buffer.

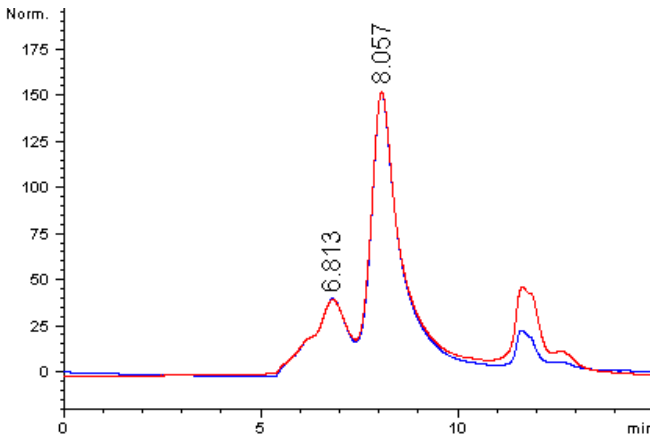

Figure 3. Overlay of the SEC HPLC analysis of Dox-ADCs before (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer. Flow rate: 1 ml/min. Unaggregated ADC elutes at 8.057 minutes. Peaks between 11–13 minutes are for small molecules such as stabilizing buffer.

concentration of 1 mg/ml at ambient temperature for 48 h, we observed some precipitation. The Dox-ADCs were filtered and analyzed again by HPLC.

Approximately 80% of the ADC remained in solution based on the total amount of ADC detected by UV at 220 nm (20% precipitation). The Dox-ADCs were further diluted at a concentration of 0.63 mg/ml and stored separately in PBS buffer and ADC-stabilizing PBS buffer at 25°C for 65 h (**Figure 2**). At this concentration, no precipitation was observed for Dox-ADCs stored in the ADC-stabilizing PBS buffer, whereas roughly 5% precipitation occurred in PBS buffer. Dox-ADCs were also lyophilized in the ADC-stabilizing PBS buffer. After lyophilization, the product was dissolved in deionized water and re-analyzed by SEC HPLC (**Figure 3**). The profile is essentially the same as the one before lyophilization. This result confirms that lyophilization in ADC-stabilizing buffer will not induce aggregation or change the configuration of ADCs.

Antibody SN38 Conjugates

7-Ethyl-10-hydroxycamptothecin, known as SN38, is another very hydrophobic drug that can be used for antibody-drug conjugation. SN38 is an inhibitor of topoisomerase I that eventually leads to inhibition of both DNA replication and transcription. Recently, the FDA has granted a priority review designation to a BLA for sacituzumab govitecan (Immunomedics, NJ), an ADC that consists of SN-38 linked with a humanized IgG antibody targeted against TROP-2, for the treatment of patients with metastatic triple-negative breast cancer (mTNBC) following at least two prior therapies for metastatic disease. TROP-2 is a cell-surface glycoprotein expressed in more than 90% of TNBCs. SN38-ADC with a releasable ester linkage was prepared using the Antibody SN38 Conjugation Kit (Cat# CM11408). An average DAR of 3.3 was obtained with 13.6% aggregation. SN38-ADCs are relatively more stable than Dox-ADCs. After storing the ADCs at RT for 7 days, there is a slight increase in aggregation (15%), but no precipitation. No SN38 is released. We tested two ADC-stabilizing buffers for SN38-ADCs. No difference was found before and after lyophilization in either ADC-stabilizing PBS buffer (**Figure 4**) or ADC-stabilizing general buffer (Cat# CM02024) (**Figure 5**).

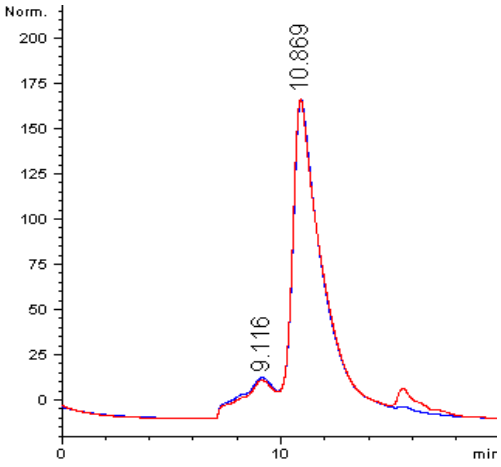

Figure 4. Overlay of the SEC HPLC profiles of SN38-ADCs before (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer. Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 10.869 minutes. Peaks between 15–18 minutes are for small molecules such as stabilizing buffer.

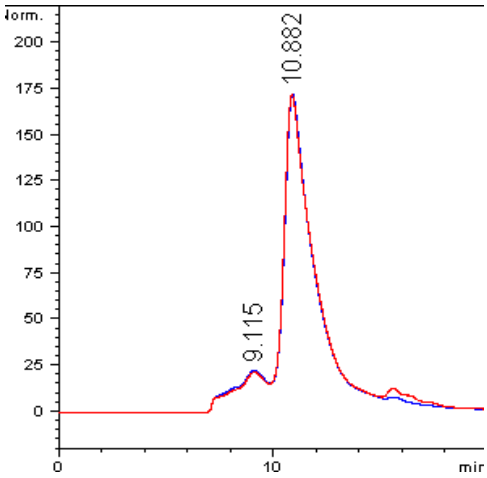

Figure 5. Overlay of the SEC HPLC profiles of SN38-ADCs before (red trace) and after lyophilization (blue trace) in ADC-stabilizing general buffer. Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 10.882 minutes. Peaks between 15–18 minutes are for small molecules such as stabilizing buffer.

Antibody MMAE Conjugates

Monomethyl auristatin E (MMAE) is one of the most frequently used toxins for antibody-drug conjugation [2]. MMAE inhibits cell division by blocking the polymerization of tubulin. It is a peptide-based toxin but very hydrophobic. MMAE is generally modified with maleimidocaproyl (Mc) valine-citruline p-aminobenzylcarbamate (VC-PAB) linker. VC-PAB is stable in extracellular fluid but cleaved by cathepsin B inside the tumor cell, activating the antimitotic mechanism. VC-PAB further increases the overall hydrophobicity of the toxin.

MMAE-ADC with a VC-PAB linkage was prepared using CellMosaic's conjugation kit (Cat# CM11409). An average DAR of 3.3 was obtained with 3.9% aggregation. Purified conjugates were stored in PBS buffer at 0.62 mg/ml and analyzed by SEC HPLC over time. **Figure 6** shows that with time, aggregates slowly built up (9.687 minutes, from 3.9% to 19.2% after 6 days at RT). Storing the conjugates in ADC-stabilizing PBS buffer will slow down the aggregation process (18% after 6 days at RT). However, if we lyophilize the ADC in ADC-stabilizing PBS buffer immediately and then re-dissolve in deionized water, the profile is essentially the same as before (**Figure 7**). No aggregation built up during the lyophilization.

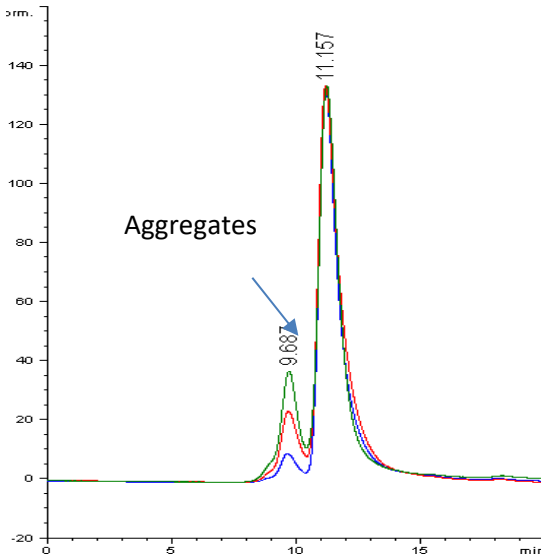

Figure 6. SEC HPLC spectra of MMAE-ADCs in PBS buffer (blue: 0 h; red:1 day at RT; green: 6 days at RT). Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 11.157 minutes.

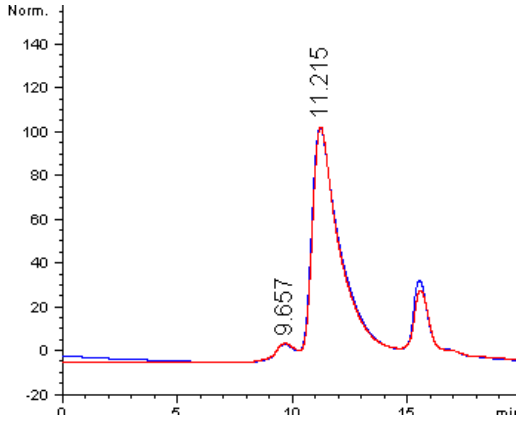

Figure 7. Overlay of the SEC HPLC profiles of MMAE-ADCs before (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer. Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 11.215 minutes. Peaks between 15–18 minutes are for small molecules such as stabilizing buffer.

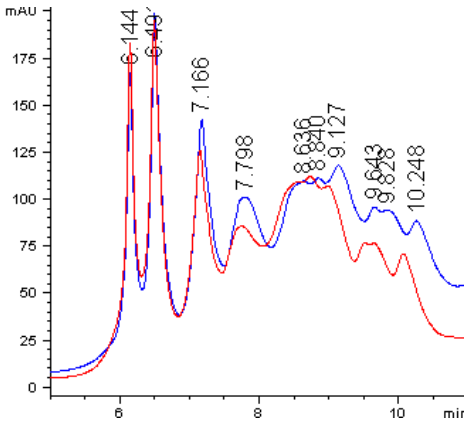

Figure 8. Overlay of the HIC HPLC profiles of MMAE-ADCs in PBS and stored overnight at RT (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer.

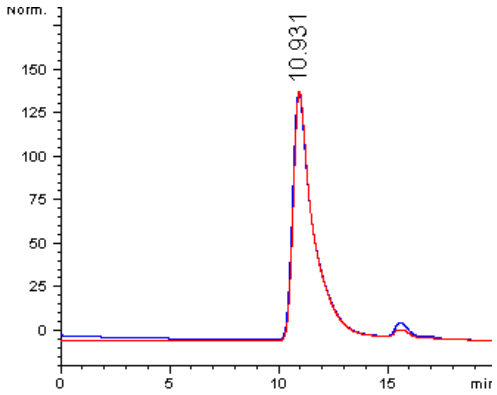

Figure 9. Overlay of the SEC HPLC profiles of MMAF-ADCs with Mc linker before (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer. Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 10.931 minutes. Peaks between 15–18 minutes are for small molecules such as stabilizing buffer.

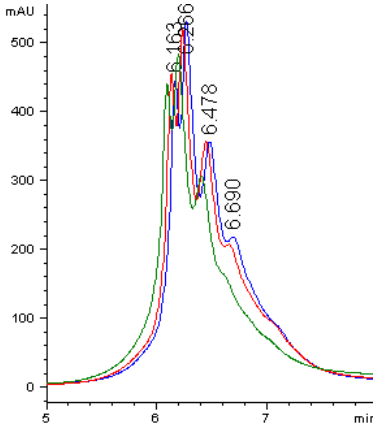

Figure 10. Overlay of the HIC HPLC profiles of MMAF-ADCs with Mc linker in PBS buffer and stored at RT overnight (green trace), in ADC-stabilizing PBS buffer and stored at RT overnight (red trace), and after lyophilization (blue trace) in ADC-stabilizing PBS buffer.

The HIC HPLC profile of the MMAE-ADC is also similar for ADCs before and after lyophilization (**Figure 8**).

Antibody MMAF Conjugates

Monomethyl auristatin F (MMAF) is a new auristatin derivative with a charged *C*-terminal phenylalanine residue. CellMosaic has commercialized two antibody MMAF conjugation kits: one uses maleimidocaproyl (Mc) MMAF, and the other uses MMAF with Mc-VC-PAB linker. Both MMAF ADCs have been prepared using CellMosaic's conjugation kits (Cat# CM11422 and

CM11425) but with 80% of the drugs added during the reaction compared to a standard preparation. The effect of ADC-stabilizing PBS buffer on the ADCs was also tested. For Mc-MMAF labeling, an average DAR of 2 was obtained with no aggregation (**Figure 9**); 4.1% aggregation occurred after 6 days in PBS buffer at RT and 2.3% in ADC-stabilizing PBS buffer. For Mc-VC-PAB MMAF labeling, an average DAR of 3 was obtained with 3.4% aggregation (**Figure 11**); 8.8% aggregation occurred after 1 week in PBS buffer and 7.5% in ADC-stabilizing PBS buffer. No aggregates formed after lyophilization. Mc-MMAF is less hydrophobic than Mc-VC-PAB-MMAF. The HIC profile of Mc-VC-PAB-MMAF ADC shows more separate conjugates than Mc-MMAF ADC (**Figure 10** and **12**). There was no difference in the profiles for both ADCs after lyophilization.

Lyophilizaiton of ADCs in PBS buffer

Table 1 summarizes the results we obtained from various ADCs using ADC-stabilizing buffers. For comparison, various ADCs were dissolved in PBS buffer and then lyophilized directly in PBS buffer. Their SEC HPLC profiles prior and after lyophilization were compared. Table 2 summarizes the data. All of the ADCs tested had slightly increased aggregation (1-5% on average) after the lyophilization and a 10-15% decrease in the amount due to precipitation during lyophilization.

Conclusion

In conclusion, ADC-stabilizing buffers can slow down the aggregation or precipitation process during storage for antibodies labeled with very hydrophobic drugs. Furthermore, ADC-stabilizing buffers allow the long-term storage of ADCs in solution at less than -20°C or as a lyophilized powder.

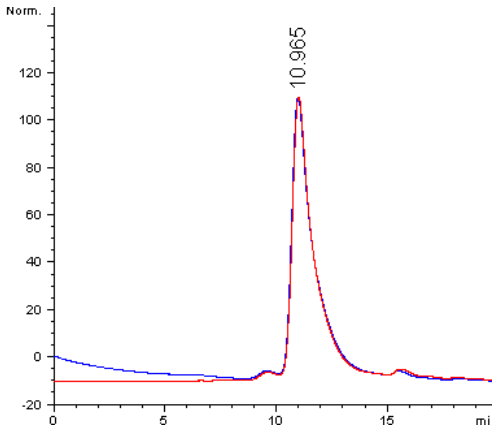

Figure 11. Overlay of the SEC HPLC profiles of MMAF-ADCs with VC-PAB linker before (red trace) and after lyophilization (blue trace) in ADC-stabilizing PBS buffer. Flow rate: 0.75 ml/min. Unaggregated ADC elutes at 10.965 minutes. Peaks between 15–18 minutes are for small molecules such as stabilizing buffer.

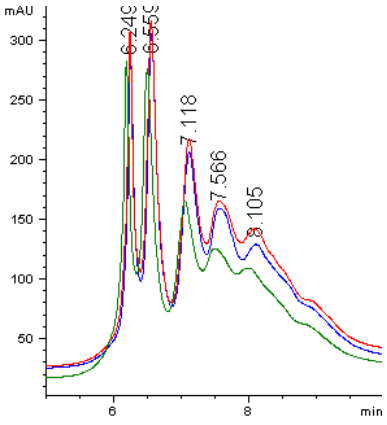

Figure 12. Overlay of the HIC HPLC profiles of MMAF-ADCs with VC-PAB linker in PBS buffer and stored at RT overnight (green trace), in ADC-stabilizing PBS buffer and stored at RT overnight (red trace), and after lyophilization (blue trace) in ADC-stabilizing PBS buffer.

Table 1: Summary of ADC preparation and aggregation (Aggr.) data (Prec.: precipitation; d: days)

ADC Type	Reaction scale	DAR	Recovery (%)	Aggr. in PBS buffer (Initial)	Aggr./Prec. in PBS at RT (Conc. time)	Aggr. in ADC-stabilizing PBS buffer at RT	Changes after lyophilization
Dox	3 mg	4.2	67%	26%	20% Prec. (1 mg/ml, 48 h). Additional 5% prec. (0.63 mg/ml, 65 h).	0% Prec. (0.63 mg/ml, 65 h)	No
SN38	3 mg	3.3	73.6%	13.6%	15.6% Aggr. (1.4 mg/ml, 7 d)	15.9% Aggr. (1.4 mg/ml, 7 d)	No
Mc-VC-PAB-MMAE	1 mg	3.3	64.5%	3.9%	19.2% Aggr. (0.69 mg/ml, 6 d)	18% Aggr. (0.69 mg/ml, 6 d)	No
Mc-MMAF	1 mg	2	76%	0	4.1% Aggr. (0.8 mg/ml, 6 d)	2.3% Aggr. (0.8 mg/ml, 6 d)	No
Mc-VC-PAB-MMAF-	1 mg	3	72%	3.4%	8.8% Aggr. (0.77 mg/ml, 6 d)	7.5% Aggr. (0.77 mg/ml, 6 d)	No

Table 2: Summary of ADC SEC HPLC analysis prior and after lyophilization in PBS buffer

ADC Type	Reaction scale	DAR	Aggr. in PBS buffer (Initial)	Aggr. After lyophilization	Small molecule falls off	% of ADCs in solution after lyophilization
SN38	3 mg	3.4	15.5%	20%	0.4%	89%
SN38	3 mg	2.3	14.7%	14.9%	0.6%	91.8%
Mc-VC-PAB-MMAE	1 mg	3.3	17.6%	18.6%	1.6%	88%
Mc-MMAF	1 mg	3.0	7.4%	12%	None	86.4%
Mc-VC-PAB-MMAF-	1 mg	3.4	11.5%	12%	0.1%	83.8%

References:

1. Trail *et al*. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. 1993, *Science*, 261, 212-215
2. Sun *et al.* Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides. 2005, *Bioconjugate Chem*., 16, 1282-1290

Ordering information for ADC stabilizing buffers

Product Number	Name	Qty	Usage
CM02022	ADC Stabilizing PBS Buffer (5x)	5 mL	Containing 5x PBS buffer and other stabilizers. Use for ADCs in PBS buffer.
CM02023	ADC Stabilizing Citrate Buffer (5x)	5 mL	Containing 5x Citrate buffer and other stabilizers. pH 6.5
CM02024	ADC Stabilizing General Buffer (5x)	5 mL	Containing only stabilizers

Ordering information for ADC prepration kits

Product Number	Name
CM11409	Antibody MMAE Conjugation Kit (with VC-PAB Linkage)
CM11410	Antibody Mertansine (DM1) Conjugation Kit
CM11422	Antibody Mc-MMAF Conjugation Kit
CM11425	Antibody MMAF Conjugation Kit (with VC-PAB Linkage)
CM11406	Antibody Doxorubicin Conjugation Kit
CM11408	Antibody SN38 Conjugation Kit

Ordering information for ADC Analysis Reagents

Product Number	Name
CM92002	SEC (Gel filtration) HPLC Protein Standard
CM02025	Hydrophobic Interaction Chromatography Buffer Set

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