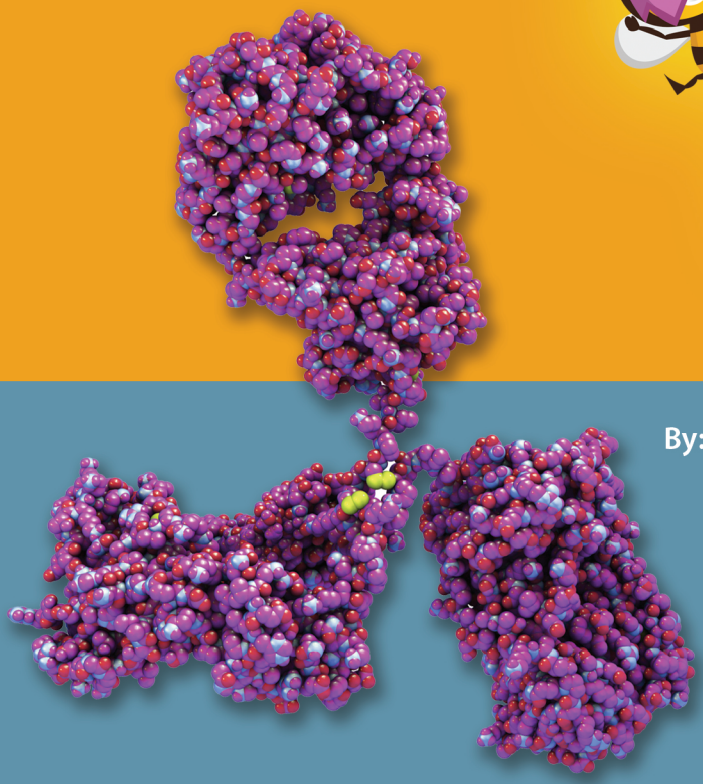
PBD-Dimer Payloads for Antibody Drug Conjugates

A Robust Approach to cGMP Production



By: **Bertrand Cottineau**
R&D Manager HPAPI & Bioconjugation

Rachel De Luca
Site Director, Le Mans

Mélanie Derde
Analytical Manager, Bioconjugation

Francois D'Hooge
Scientific Director, Bioconjugation

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From the 1960s when monomeric pyrrolobenzodiazepines (PBDs) such as anthramycin were isolated and characterized, to the dramatic increase of potency induced by dimerization in the 2000s, PBD dimers have evolved as one of the leading classes of antibody drug conjugate (ADC) payloads (Figure 1). Currently, 17 ADCs are in clinical trials involving PBD-dimers — second only to auristatins (23 ADCs), and more than those with maytansines (16 ADCs). Four different PBD-dimer ADC payloads are in clinical trials: Talirine (SGD-1910) from Seattle Genetics; Tesirine (SG3249) from Spirogen, MedImmune, ADC Therapeutics, and Abbvie; and DGN462 and DGN549 from Immunogen. With the most advanced PBD-ADC program, Abbvie-StemCentrx' Rovalpituzumab-tesirine (Rova-T) closing to commercial launch, we summarize herein the production challenges and solutions presented by the PBD-dimer platform.

Several other companies also are involved in preclinical development of PBD-dimers ADC payloads, either through a license from Spirogen (Genentech, Mitsubishi Tanabe Pharma, Regeneron, and Gamamabs Pharma) or through proprietary internal payload development programs (Sanofi, Bristol-Myers Squibb, Cellerant Therapeutics, and Abzena).

PBD-dimers are DNA crosslinkers or alkylators. Upon ADC internalization and lysosomal processing, PBD-dimer payloads are released in the cytosol, then diffuse through the nuclear membrane and bind in the minor groove of DNA. After binding, the imine then can alkylate or crosslink the two DNA strands and block DNA replication. As shown on the computer-generated graphic (Figure 2), PBD-dimer minor groove binding does not change DNA geometry, which makes this modification hard to identify and repair. PBD-dimer geometry is induced by the two H11a stereochemistries, so stereochemical control is absolutely necessary for biological activity.

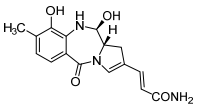
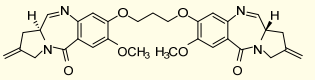
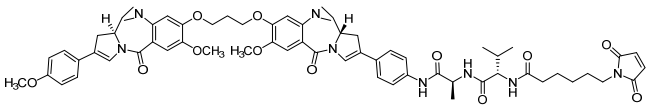
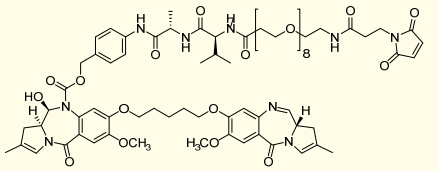
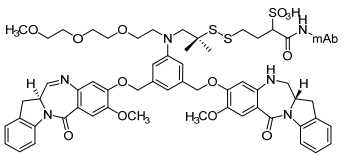
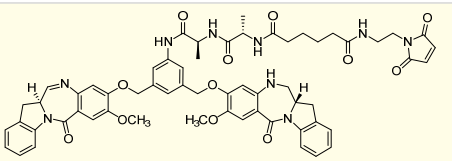
Specific Challenges of PBD Synthesis and Purification

PBD-dimer payloads are ultrapotent molecules that require very high containment (occupational exposure limits below 30 ng/m^3/8 h). Novasep's chemical and bioconjugation laboratories and production suites offer a consistent set of equipment fitted with the best available barrier technology allowing for ultracontained handling and transfer of PBD samples and bulk product. Combined with essential know-how of specially trained staff, exposure can be as low as a few ng/m^3, as demonstrated by periodical sampling at the workstations.

From a process standpoint, the principal challenge is that PBD-dimers are highly unstable in acidic and basic media as well as under nucleophilic conditions. To produce a robust process and highly pure PBD-dimers, experience in PBD chemistry and expertise in process development are essential. For such compounds, Novasep's approach is to perform a detailed process characterization on key steps of the process using design of experiment (DoE) tools. The main impurities to control include diastereomers, aromatized impurities, and maleimide and conjugable impurities.

Diastereomers: As described by Tiberghien, the possible epimerization of H11a can occur under

Figure 1: History of PBD-dimer ADC payload

	Structure		Year
Pyrrolobenzodiazepine (PBDs)		Anthramycin	1965
		Spirogen SJG136 / SG2000	2001
		Seattle Genetics SGD-1910 / Talirine	2010
		Spirogen SG3249 / Tesirine	2013
Indolinobenzodiazepines (IBDs)		Immunogen DGN462	2014
		Immunogen DGN549	2016

basic conditions, for example using TBAF deprotection of the TBS group (**2**). It can be controlled by using TBAF buffered with acetic acid.

Aromatized Impurities: On highly aromatic compounds such as SGD-1910, the H11a position is very sensitive to elimination, leading to a flat pi-conjugated system in which DNA binding potency is widely reduced. This elimination needs to be under control for the whole process.

Maleimide and Conjugable Impurities: The last steps of the synthesis of SG3249 or SGD-1910 (Figure 3) involve introduction of a maleimide moiety for the antibody-cysteine conjugation. Extra care must be taken to ensure that no other maleimide-bearing impurity is present. Expertise in high-pressure chromatography is key to developing a robust process, guaranteeing high and consistent purity of PBD-dimer payload when the process is transferred to the manufacturing facilities.

Conjugation of PBD-Dimer Payloads

Four commercial ADCs are available currently: Pfizer's Mylotarg (gemtuzumab ozogamicin) and Besponsa (inotuzumab ozogamicin), Seattle Genetics' Adcetris (brentuximab vedotin), and Roche/Genentech's Kadcyla (ado-trastuzumab emtansine). Figure 4 shows that they are produced using two different conjugation technologies: stochastic lysine conjugation and stochastic cysteine conjugation (Figure 4A, 4B). For PBD-dimer payloads, the same conjugation technologies were used, along with a more advanced site-specific cysteine conjugation for Seattle Generics' vadastuximab talirine, where an antibody is engineered with extra cysteines at specific site for conjugation purpose only (Figure 4c).

Drug-to-Antibody Ratio (DAR) and Conjugation Process

For each of the three processes illustrated in Figure 5, the main critical quality attributes are drug-to-antibody ratio (DAR) and monomeric

Figure 2: Mode of action (**1**)

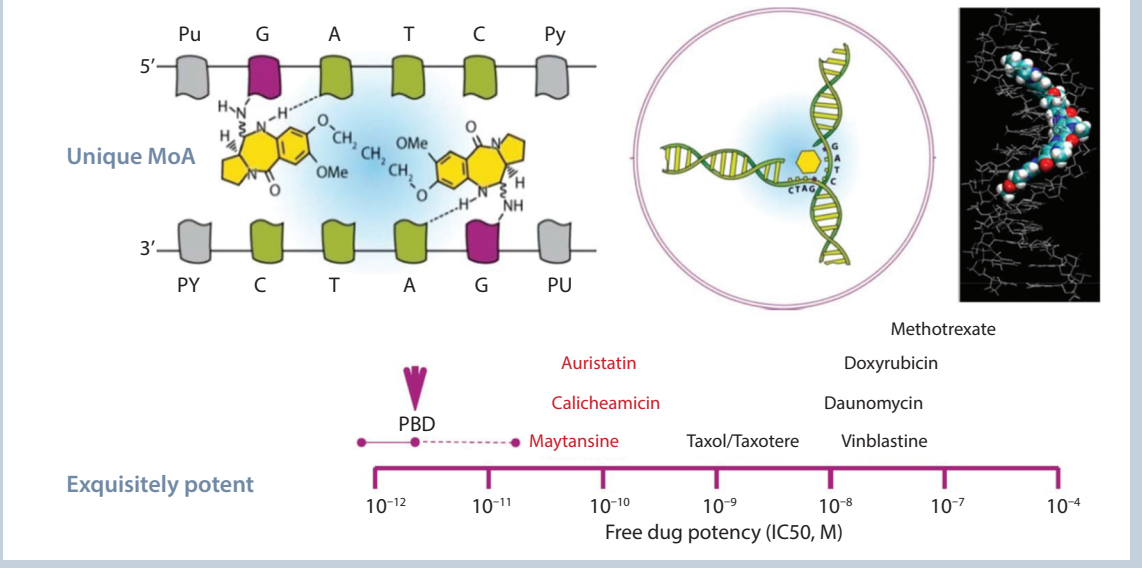
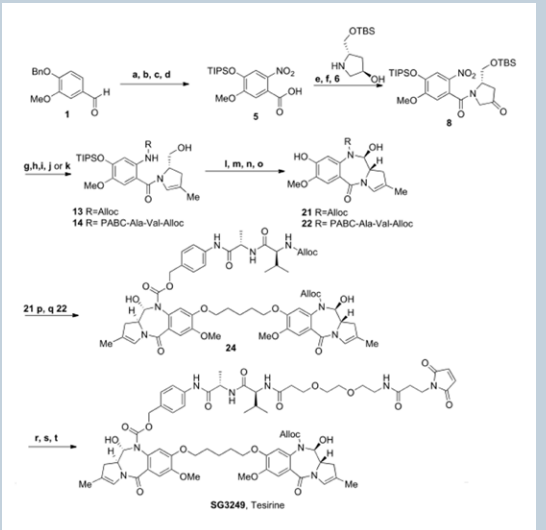


Figure 3: PBD-dimer payload synthesis reported by Spirogen. Reported synthesis is 30 steps long from benzylvanilin with an overall yield of 0.54% (**2**).



Reagents and conditions: (a) HNO_3, 12 °C, 95%; (b) TFA, 85 °C, 50%; (c) TIPSCl, imidazole, 100 °C, 88%; (d) $NaClO_2$, NaH_2PO_4, H_2O_2, THF, –78 °C to rt, 100%; (e) DCC, HOBt, Et_3N, DCM, –10 °C to rt, 90%; (f) TEMPO, TCCA, DCM, 100%; (g) Tf_2O, 2,6-lutidine, DCM, –45°C, 78%; (h) $MeB(OH)_2$, Ag_2O, $AsPh_3$, $Pd(PHCN)_2Cl_2$, 70 °C, 70%; (i) Zn 30 equiv, HCOOH/EtOH 5/95, 30 °C, 80%; (j) Alloc-Cl, pyridine, DCM, –78 °C to rt, 100%; (k) triphosgene, Et_3N, THF, 5 °C, then Alloc-Val-Ala-PAB-OH, Et_3N, THF, 40 °C, 50%; (l) AcOH/MeOH/THF/water 7/1/1/2, rt, 71%–80%; (m) DMSO, $(COCl)_2$, Et_3N, DCM, –78 °C to rt, 66%–60%; (n) TBS-OTf, 2,6-lutidine, DCM, 0 °C, 85%–65%; (o) LiOAc, DMF/water, 95/5, rt, 100%–100%; (p) 1,5-diiodopentane, K_2CO_3, acetone, 60 °C, 90%; (q) 22, K_2CO_3, acetone, 65 °C, 86%; (r) TBAF/AcOH, THF, rt, 80%; (s) $Pd(PPh_3)_4$, pyrrolidine, DCM, rt, 100%; (t) Mal-dPEG8-acid, EDCI, DCM, rt, 73%

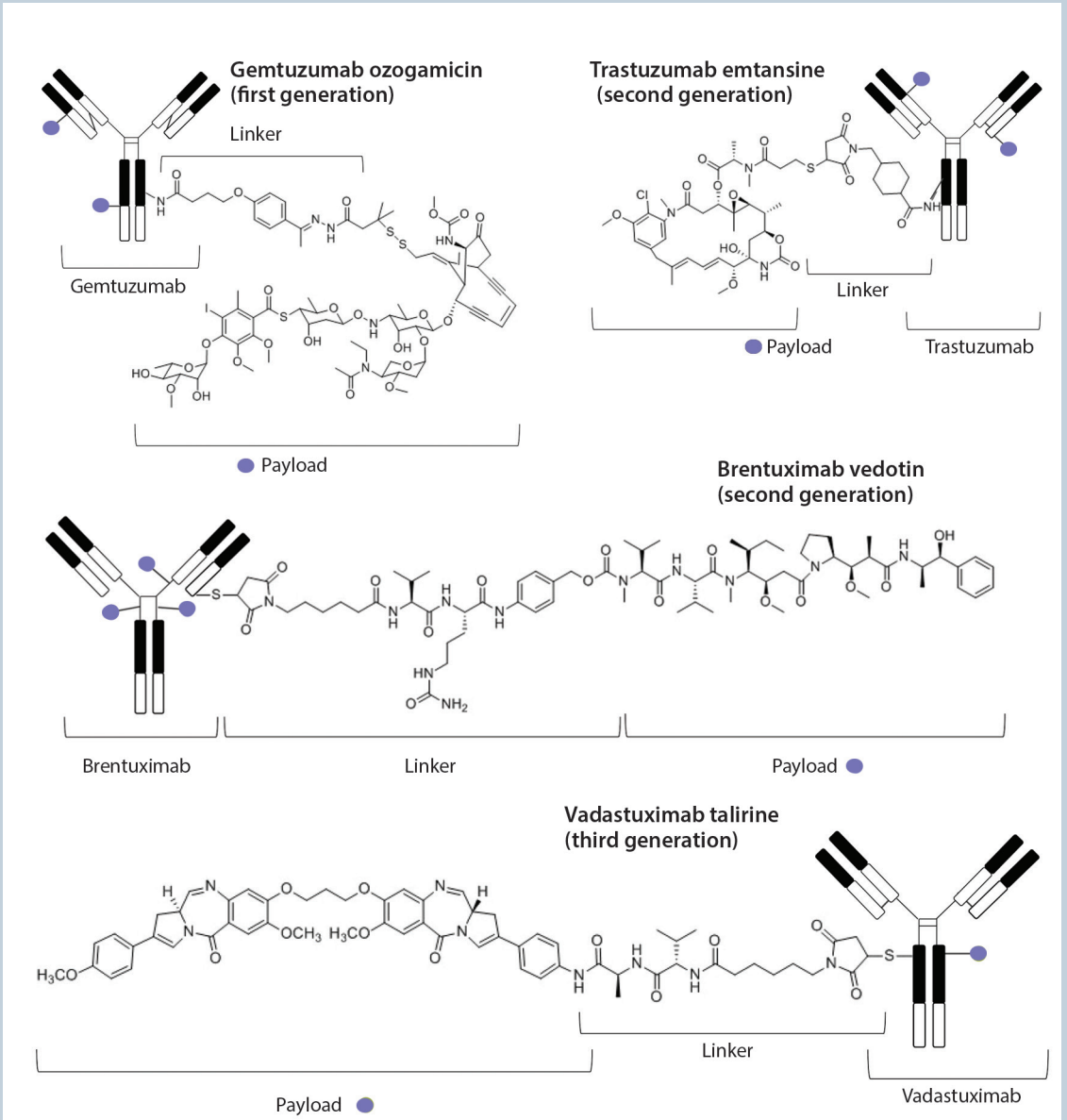
purity/aggregation. DAR is controlled during process development by carefully investigating the amounts of linker or reductive agent and toxin; aggregation is a product of DAR, payload hydrophobicity, and process (buffer type, cosolvent, concentration, and temperature). Novasep routinely designs processes for controlled DAR and aggregation using a DoE approach.

With more than 16 PBD-ADCs entering the clinic, it is clear that satisfactory PBD-ADC can be produced and purified. Nevertheless the PBD platform presents some unique challenges in term of payload hydrophobicity (Table 1).

Hydrophobicity is linked favorably with DNA minor groove binding efficiency and therefore with payload efficacy. Recent studies by Seattle Genetics (**3**) recently linked high payload and ADC hydrophobicity with increased off-target toxicity. From a chemistry, manufacturing, and controls (CMC) perspective, increased hydrophobicity can lead to increased ADC aggregation as well as increased free payload carryover and removal issues. For example, ADC-bound payloads interact with either free payloads or other ADC-bound payloads. Payload hydrophobicity therefore is a balance between efficacy, toxicity, and manufacturability.

Seattle Genetics reported that conjugation of its highly hydrophobic SGD-1910 yields adequate ADC only on site-specific engineered antibodies. Similarly, on stochastic ADC, the higher loadings (DAR 4, DAR 6) lead to nonmanageable aggregate levels (**4**). Moreover, Seattle Genetics

Figure 4: Conjugation technologies for the four commercially available ADCs; (TOP) stochastic lysine conjugation (Mylotarg, Besponsa, Kadcyla); (CENTER) stochastic cysteine conjugation (Adcetris); (BOTTOM) the same as TOP and CENTER, but with more advanced site-specific cysteine conjugation (Seattle Generics' vadastuximab talirine)



also disclosed a specific purification method based on C-18 filtration to remove unbound SGD-1910 from ADCs (**5**).

Using SG3249, multiple ADCs have been produced for clinical trials on stochastic formats (Abbvie/StemCentrx Rova-T, ADCT/Genmab ADCT-301) or site-specific formats (ADCT-402), with no limitations or issues reported either in aggregation control or free payload removal.

Having identified the two main issues of PBD-dimer payloads conjugation to be aggregation control and free toxin removal, Novasep can propose a large range of solutions to solve those issues. Such solutions include quickly identifying best-process parameters to control aggregation using DoE, tangential-flow filtration (TFF) optimization for optimal payload clearance, or chromatographic purification for removal of ADC aggregates or residual payloads (Table 2).

ADC Aggregates or Residual Payloads

ADCs are complicated biologics with added chemical toxins, and as such they are some of the

Figure 5: Drug-to-antibody ratio (DAR) and conjugation process (**4**). REPRINTED (ADAPTED) WITH PERMISSION FROM JEFFREY SC ET AL. A POTENT ANTI-CD70 ANTIBODY–DRUG CONJUGATE COMBINING A DIMERIC PYRROLOBENZODIAZEPINE DRUG WITH SITE-SPECIFIC CONJUGATION TECHNOLOGY. BIOCONJUGATE CHEM. 24 (7) 2013, PP 1256–1263. COPYRIGHT © 2013, AMERICAN CHEMICAL SOCIETY.

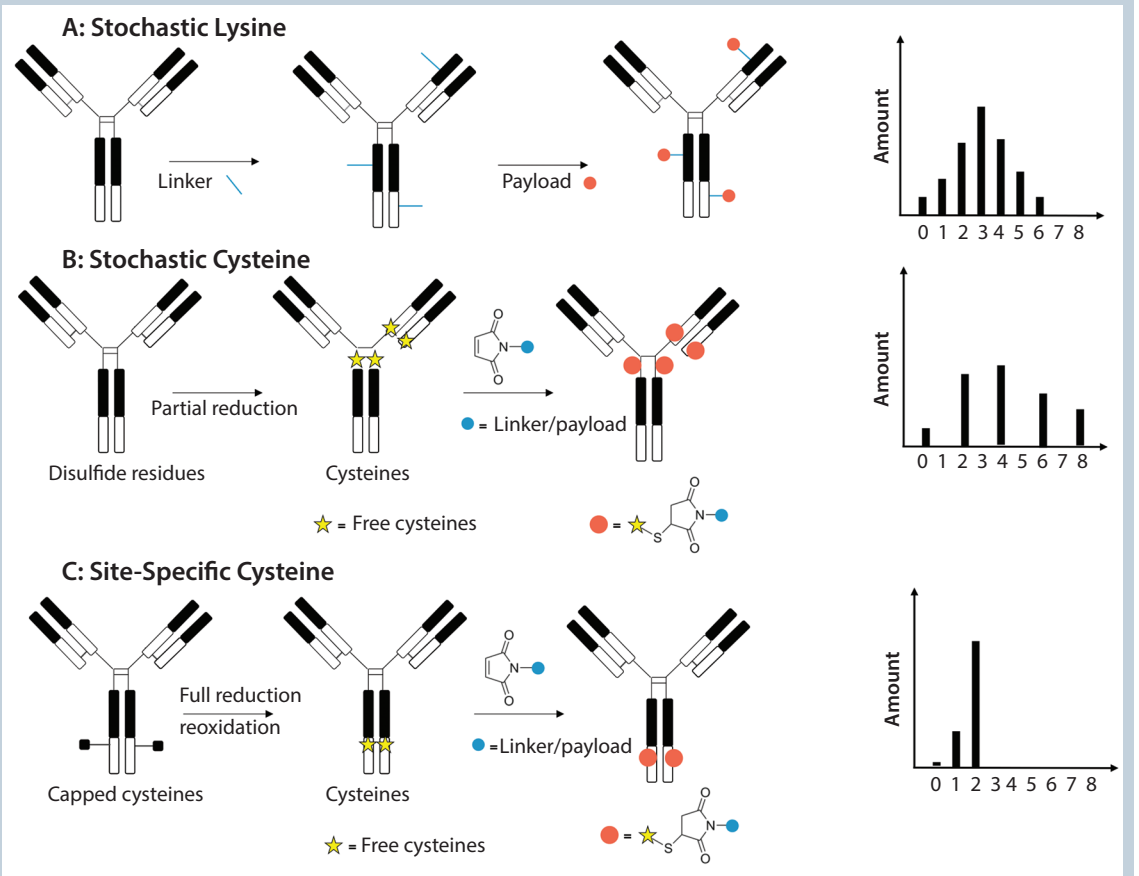
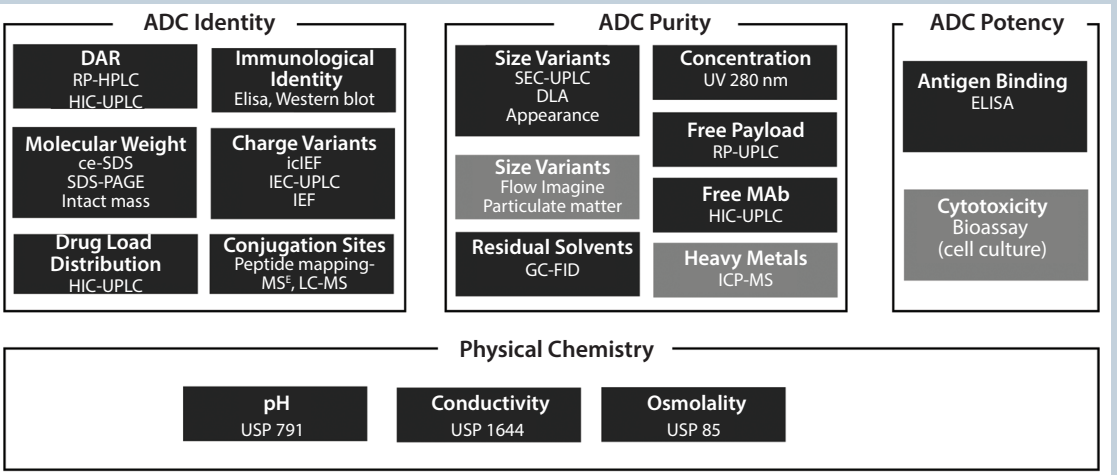


Figure 6: Analytical methods available at Novasep — in-house capabilities in black, outsourced activities in gray



most complex biological molecules to analyze. It is extremely important to have appropriate analytical capacities to enable fast and efficient bioconjugation process development. Novasep is using state-of-the-art ultraperformance and high-performance liquid chromatography (UPLC and HPLC) systems for fast results, along with a fully equipped quality control laboratory for current good manufacturing practice (cGMP) in-process controls, drug-substance release, characterization, and stability (Figure 6).

Table 1: Hydrophobicity table Log D calculation of Log D methodology; payloads and payload linkers were drawn using Chemdraw, either as maleimides or methyl amide for amine reactive molecules. Log D at pH 7.4 then was calculated using the Chemicalize website from ChemAxon Ltd. (https://chemicalize.com), DAR = drug-to-antibody ratio

		Log D (pH = 7.4)			
Class	ADC Payload	Payload	Payload Linker	Conjugation Technique	Example DAR
Auristatins	Vedotin (mc-vc-PAB-MMAE)	2.01	4.13	stochastic cysteine	4.0
	Mafodotin (mc-MMAF)	1.22	0.81	stochastic cysteine	4.0
Maytansins	Emtansine (SMCC-DM1)	3.70	3.56	stochastic lysine	3.5
	Soravtansine (sPDB-DM4)	4.47	1.31	stochastic lysine	3.5
Calichaemicin	Ozogamicin (hydrazone calicheamicin)	4.07	5.43	stochastic lysine	2.5
	Anthramycin	0.55	NA	NA	NA
	SG2000	2.24	NA	NA	NA
	Talirine (SGD-1910)	4.12	4.71	site-specific cysteine only	2.0
PDB dimers	Tesirine (SG3249)	3.52	2.11	stochastic lysine site-specific cysteine	2.5 2.0
	IMGN462	7.53	4.40	stochastic lysine	3.0
	IMGN549	5.68	4.22	site-specific cysteine	2.0

Table 2: Solutions for ADC purification; DoE = design of experiments; TFF = tangential flow filtration

Issue	First Solution	Second Solution
ADC aggregation	DoE-based process optimization	Chromatographic purification
Free payload	DoE-based TFF optimization	Chromatographic purification

The Potential of PBD-Dimers

In the past five years, PBD-dimers have emerged as one of the most interesting classes of ADC payloads, as judged by the number of PBD-dimer ADCs in clinical trials (17) as well as the interest of the industry as a whole: More than 50% of companies with more than two ADCs in development currently are working with PBD-dimers, either through licenses or in-house modifications. Although PBD-dimers present specific challenges in term of synthesis, purification, and conjugation, such ADC projects can proceed forward smoothly with the support of an expert contract development and manufacturing organization (CDMO) such as Novasep.

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Bertrand Cottineau *is R&D Manager, HPAPI and Bioconjugation;* ***Rachel De Luca*** *is Site Director, Le Mans;* ***Mélanie Derde*** *is Analytical Manager, Bioconjugation; and* ***Francois D'hooge*** *is Scientific Director, Bioconjugation, at Novasep, 1 Rue Démocrite, 72000 Le Mans, France; +33 2 43 39 94 40; adc@novasep.com; www.novasep.com.*

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