IgK constant region on bispecific antibodies, two bispecific antibodies targeting CD47 and PD-L1 were constructed, in which the antigen-binding portion targeting PD-L1 was an MHC-I element-modified antigen-binding portion, CH1/CL of the antibody HZ5G11 was replaced by MHCICα /MHCICβ, and the antigen-binding portion targeting CD47 was a Fab. Based on the construction of the antibody HZ14A9, the CL domain of the constant region of the Fab light chain was not mutated at F118 (EU numbering) site or carried the single-point mutation F118A. The CH2 domains of two heavy chains of both antibodies adopted LALA (L234A + L235A) double mutation to eliminate the potential Fc effector effect. The CH3 domain carried a KIH mutation to promote heterodimer formation. Thus, two bispecific antibodies targeting CD47 and PD-L1 at the same time were generated, which were MHL147-3322-IgG1-wt (molecular structures shown in FIG. 8) and MHL147-3322-IgG1-F118A (molecular structures shown in FIG. 9), respectively.

Table 12-1. Sequences of anti-PDL1*CD47 bispecific antibodies

Name of antibody	Polypeptide chain	Amino acid sequence
MHL147-3322-IgG1-wt	H2	(SEQ ID NO: 49)
	L2	(SEQ ID NO: 50)
	H3	(SEQ ID NO: 51)
	L3	DIQMTQSPSSLSASVGDRVTITCKASENVVSYVSWY QQKPGKAPKLLIYGASNRYTGVPSRFIGSGSSTDFTL TISSLQPEDFATYYCGQSYSYPLTFGQGTKLEIK RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 53)
MHL147-3322-F118A	H2	QITLKESGPTLVKPTQTLTLTCTVSGFSLSTYGVHWIR QPPGKALEWLGVIWRGVTTDYNAAFMSRLTITKDNS KNQVVLTMNNMDPVDTATYYCARLGFYAMDYWGQ GTLVTVSS GKETLQRADPPKTHVTHHPISRHEATLRCWALGFYP AEITLTWQRDGKDQTQKTELVETRPCGDRTFQKWAA VVVPSGEEQRYTCHVQHEGLPKPLTLRWEPS EPKSSDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVCTLPPSRDELTKN QVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPV LDSDGSFFLVSKLTVDKSRWQQGNVFSCSVMHEALH NRFTQKSLSLSPGK (SEQ ID NO: 49)
	L2	DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWY QQKPGKAPKLLIYYAANRYTGVPDRFSGSGYGTDFT FTISSLQPEDIATYFCQQDYTSPYTFGQGTKLEIK IQRTPKIQVYSCHPAENGKSNFLNCYVSGFHPSDIEV

		DLLKNGERIEKVEHSDLSFSKDWSFYLLYYTRFTPTE KDEYACRVNHVTLSQPKIVKWDRGP (SEQ ID NO: 50)
	H3	QVQLVQSGAEVKKPGASVKVSCKVSGFNIEDDYIEW VRQAPGQGLEWMGRIDPANDKTKYAQKFQGRVTMT GDTSTNTVYMELSSLRSEDTAVYYCARPGLRRYYSM DYWGQGTLVTVSS ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNST YRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGK (SEQ ID NO: 51)
	L3	DIQMTQSPSSLSASVGDRVTITCKASENVVSYVSWY QQKPGKAPKLLIYGASNRYTGVPSRFIGSGSSTDFTL TISSLQPEDFATYYCGQSYSYPLTFGQGTKLEIK RTVAAPSVFIAPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 52)

DNA sequences encoding and constituting each polypeptide chain were inserted into vectors (e.g., pcDNA3.1 vector disclosed in CN107001463A, pCHO1.0 vector disclosed in CN109422811A, etc.), respectively, to obtain expression vectors expressing the corresponding polypeptide chain. According to a mass ratio in equal proportion, the expression vectors encoding each polypeptide chain were co-transfected into ExpiCHO-S cells (manufacturer: Shanghai Institute of Pharmaceutical Industry, Cat No. 127200005) at a transfection expression volume of 0.1-1 L and a cell density of 6E+06 cells/mL. After transfection, the cells were placed in ExpiCHO^{TM} medium (manufacturer: Thermo, Cat No. A2910001), and expressed and cultured at 32 °C with 5% CO_2 at 130 rpm. On day 10, the cell supernatant was collected, and protein A affinity chromatography was performed on the target protein using AKTA Pure 25 L system from GE and MabSelect SuRe LX packing. After elution, the concentration of the eluted protein was measured using NanoDrop Lite (Thermo Fisher Scientific). The purity of the eluted protein was measured by SEC-HPLC.

Table 12-2. Expression amount and purity of anti-PDL1*CD47 bispecific antibodies

Sample	Purification method	Expression amount (mg/L)	HPLC-SEC purity (%)
MHL147-3322-IgG1-wt	Protein A affinity chromatography	22	82.94

MHL147-3322-IgG1-F118A	Protein A affinity chromatography	38	92.85

The purification results of one-step protein A affinity chromatography showed that MHL147-3322-IgG1-F118A had higher expression amount and purity than MHL147-3322-IgG1-wt (Table 12-2), and the single-point mutation F118A (EU numbering) in the CL domain could improve the assembly and pairing process of the bispecific antibody in cells and reduce the formation of mispaired products, thereby improving the expression amount and purity of the bispecific antibody.

5.2. Thermal stability

The thermal stability of HZ14A9, HZ5G11, MHL147-3322-IgG1-wt, and MHL147-3322-IgG1-F118A was characterized by Nano DSF (Nano Temper). The concentration of the sample to be tested was adjusted to about 1.0 mg/mL, then a small amount of the sample was sucked using a silica capillary and placed in a Nano DSF sample suspension. The temperature was raised in a range of 20.0 °C to 95.0 °C at a constant speed of 1.0 °C/min. The optical signal change of the protein sample was collected, and the thermal stability of the protein was analyzed.

Table 13. Thermal stability of anti-PDL1*CD47 bispecific antibodies

Sample	Melting temperature Tm1 (°C)
MHL147-3322-IgG1-wt	66.4
MHL147-3322-IgG1-F118A	67
HZ14A9	68.3
HZ5G11	67

The Nano DSF experimental results showed that HZ14A9, HZ5G11, MHL147-3322-IgG1-wt, and MHL147-3322-IgG1-F118A all had good thermal stability, and the melting temperature Tm1 of all samples was higher than 65 °C (Table 13). The single-point mutation F118A (EU numbering) in the CL domain had no significant effect on the thermal stability of the bispecific antibodies.

5.3. Affinity

The affinity properties of the HZ5G11 monoclonal antibody, the HZ14A9 monoclonal antibody, MHL147-3322-IgG1-wt and MHL147-3322-IgG1-F118A bispecific antibodies were characterized by Biacore. The HZ5G11 monoclonal antibody, the HZ14A9 monoclonal antibody, MHL147-3322-IgG1-wt and MHL147-3322-IgG1-F118A bispecific antibodies were respectively immobilized onto a sensing chip by a capture method, and a human PDL1 (manufacturer: ACROBiosystems, Cat. No. PD1-H52H3) or human CD47 (manufacturer: ACROBiosystems, Cat. No. CD7-H5227) protein antigen solution with a certain concentration gradient was injected into the sample. Association/dissociation curves were acquired in real time by Biacore 8K Control Software 3.0, and data analysis was performed by Biacore Insight Evaluation Software 3.0. An association rate constant Ka, a dissociation rate constant Kd, and an equilibrium constant KD were obtained.

Table 14. Binding affinity of anti-PDL1*CD47 bispecific antibodies to human CD47 antigen

Sample	ka (1/Ms)	kd (1/s)	KD (M)
HZ14A9 monoclonal antibody	2.45E+06	1.43E-02	5.85E-09