Supplementary Information for

Efficient tumor targeting with high-affinity Designed Ankyrin Repeat Proteins (DARPins): Effects of affinity and molecular size

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Supplementary Materials and Methods

His-Tag-Specific ^{99m}Tc Labeling of the DARPins. The radioactive labeling of the DARPins with $^{99m}Tc(CO)_3$ at the his tag was performed essentially as described before (1). An Isolink kit vial (Mallinckrodt Medical B.V.), which contains in lyophilized form 8.5 mg sodium tartrate, 2.85 mg $Na_2B_4O_7 \cdot 10H_2O$, 7.15 mg of sodium carbonate, and 4.5 mg sodium boranocarbonate, was used to prepare $[^{99m}Tc(H_2O)_3(CO)_3]^+$. For this purpose, 1 ml of freshly eluted $^{99m}TcO_4^-$ from a commercial generator was added to the vial. The vial was placed in a boiling water bath for 20 minutes. A buffer mixture (pH 6.5, 2 parts 1 N HCl/ 3 parts 0.6 M phosphate buffer pH 7.0) (350 µl) was added to the prepared kit to neutralize the solution, and to decompose any residual boranocarbonate. Quality control of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ precursor was performed by TLC and reverse phase HPLC (C_{18} column). For labeling purposes all DARPins were first mixed with 1/10 volume of 1.0 M MES, pH 6.5, and concentrated to ~0.7–1.3 mg/ml before the required radioactivity of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ precursor was added. This mixture was incubated at 37°C for 1 h. Since incorporation was complete, preparative removal of any unincorporated $^{99m}Tc(CO)_3$ was not necessary. The labeled protein was checked by gel filtration analysis on a Superdex 75 gel filtration column (GE Healthcare) connected to a HPLC radioactivity monitor (LB 508, Berthold).

Tumor Cell Lines. For most *in vitro* cell binding experiments, *in viv*o tumor localization and biodistribution studies, the human ovarian carcinoma cell line SK-OV-3.ip was used (2) (kindly provided by Ellen Vitetta, University of Texas, Dallas, TX, USA), which is a derivative cell line isolated from the ascites that developed in mice given injections of human carcinoma SK-OV-3 cells. It was reported to express 2-fold higher levels of HER2 (2) than SK-OV-3. Nonetheless, the levels of tumor accumulation were comparable for SK-OV-3 and SK-OV-3.ip (Supplementary Table ST1). For some cell binding experiments, SK-OV-3 (3) (HTB77, ECACC, Salisbury, Wilts, UK) and BT474 cells (4) (HTB-20) were also used.

Biodistribution Studies of DARPins. Biodistribution studies of the various DARPin formats were performed in athymic mice to analyze their tumor localization, serum persistence, and body clearance. Mice were housed under controlled temperature (26°C), humidity (68%) and daily light cycle (12 h light/ 12 h dark). Mice experiments were approved by the Animal Ethics Committee of the Kanton of Aargau (Nr. 75528). Female 6–8 weeks old CD1-FOXn1/nu mice (Charles River, Germany) were engrafted with human SK-OV-3.ip tumor cells (or SK-OV-3 cells were indicated) subcutaneously injected at the lateral flanks (10^6 cells, mixed with BD Matrigel Matrix HC; BD Biosciences, Bedford, MA, USA). The studies were started two to three weeks after tumor inoculation, when the tumors had reached a size of 50–300 mg. Each mouse received intravenously (i.v.) a single dose of 8-10 µg of $^{99m}Tc(CO)_3$-labeled DARPin (37 MBq/mouse), administered in 100 µl. Mice (n = 3 per time point and construct) were sacrificed after 1 h, 4 h, 24 h, 48 h, and 72 h p.i. (as indicated in results), organs were removed and the accumulated radioactivity was measured in a γ-scintillation counter. The determined radioactivity of each organ and time point was normalized to the percentage of injected dose per gram tissue (%ID/g). The total amount of injected radioactivity was arbitrarily set to 100%.

Tumor Autoradiography. Twenty four h after 10 µg (37 MBq) of the $^{99m}Tc(CO)_3$-labeled DARPin G3 had been injected into tumor bearing mice, the SK-OV-3.ip tumors (0.5 cm diameter) were cut in 3 layers (rim, middle section, rim). The slices were put on Kodak X-Omat Blue (XB) film and exposed for 4 h. The slices were also analyzed with a Packard InstantImager.

SPECT Imaging Studies. Single photon emission computed tomography (SPECT) imaging experiments were performed with an X-SPECT^{TM}-system (Gamma Medica Inc.) with a single head SPECT-device and a computed tomography (CT) device, 22 to 26 h p.i. of the radiotracer, the $^{99m}Tc(CO)_3$-labeled DARPin G3 and G3-PEG20. The DARPin (555 MBq/ 200 µl) was administered via a lateral tail vein. The mice were anesthetized with an isoflurane/oxygen mixture and positioned in the SPECT-camera using the animal bed intended for this purpose. The depth of anesthesia was monitored by measuring respiratory frequency. Body temperature was controlled by a rectal probe and kept at 37°C by a thermo coupler and a heated air stream. In some cases imaging studies were performed post mortem. SPECT-data were acquired and reconstructed by the software LumaGEM (version 5.407 lum 10). CT data were acquired by the X-Ray CT-system Gamma Medica^{TM} and reconstructed by the software Cobra (version 4.5.1). Fusion of SPECT- and CT-data was performed by the software IDL Virtual Machine^{TM} (version 6.0). Images were generated by Amira^{TM} (version 3.1.1). Acquisition time varied between 32 min (G3-PEG20) and 53 min (G3).

For high resolution images, tumors were scanned with a pinhole collimator (tungsten based collimator on 1 mm diameter). The SPECT field of view was reduced to 30 mm x 30 mm and the acquisition time was increased to 4 h. The voxel limits were set to red $\geq$ 500 counts, voxel counts $\leq$ 100 were neglected.

Table ST1: Tissue distributions of DARPins and controls in SK-OV-3 or SK-OV-3.ip tumor bearing mice

organ	1h	4h	24h	48h
G3 in SK-OV-3				
Blood	0.89 ± 0.23	0.37 ± 0.04	0.13 ± 0.02	0.06 ± 0.00
Heart	0.81 ± 0.22	0.49 ± 0.07	0.26 ± 0.04	0.19 ± 0.02
Lung	1.22 ± 0.30	0.72 ± 0.07	0.36 ± 0.03	0.23 ± 0.03
Spleen	1.13 ± 0.33	1.08 ± 0.15	0.68 ± 0.13	0.44 ± 0.06
Kidney	162.02 ± 17.85	162.37 ± 18.04	91.04 ± 0.45	46.42 ± 9.74
Stomach	0.65 ± 0.18	0.64 ± 0.21	0.91 ± 0.57	0.32 ± 0.13
Colon	0.69 ± 0.18	0.65 ± 0.14	0.39 ± 0.03	0.19 ± 0.02
Liver	7.78 ± 2.30	8.34 ± 1.09	3.86 ± 0.72	2.94 ± 1.00
Muscle	0.58 ± 0.21	0.47 ± 0.17	0.18 ± 0.01	0.11 ± 0.02
Bone	1.38 ± 0.27	1.05 ± 0.21	0.78 ± 0.08	0.91 ± 0.23
Tumor	**8.27 ± 2.67**	**11.16 ± 1.36**	**8.13 ± 1.29**	**4.64 ± 0.38**
T:B	**9.29 ± 5.37**	**30.16 ± 6.94**	**62.54 ± 21.46**	**77.33 ± 11.52**
G3 in SK-OV-3.ip				
Blood		0.35 ± 0.03	0.11 ± 0.01	
Heart		0.48 ± 0.05	0.27 ± 0.03	
Lung		0.73 ± 0.15	0.34 ± 0.04	
Spleen		1.06 ± 0.14	0.65 ± 0.03	
Kidney		144.37 ± 9.17	73.33 ± 10.54	
Stomach		0.53 ± 0.18	0.37 ± 0.13	
Colon		0.58 ± 0.14	0.28 ± 0.06	
Liver		8.70 ± 1.27	4.24 ± 0.42	
Muscle		0.37 ± 0.08	0.17 ± 0.01	
Bone		1.37 ± 0.33	0.96 ± 0.38	
Tumor		**8.37 ± 1.71**	**6.21 ± 0.97**	
T:B		**23.91 ± 7.08**	**56.45 ± 12.76**	
E3_5 in SK-OV-3.ip				
Blood		0.42 ± 0.03	0.14 ± 0.01	
Heart		0.51 ± 0.07	0.24 ± 0.02	
Lung		0.64 ± 0.11	0.30 ± 0.04	
Spleen		0.82 ± 0.11	0.48 ± 0.09	
Kidney		170.46 ± 18.84	78.11 ± 6.99	
Stomach		3.39 ± 4.80	0.50 ± 0.07	
Colon		1.26 ± 1.30	0.29 ± 0.06	
Liver		4.12 ± 0.64	2.50 ± 0.09	
Muscle		0.36 ± 0.04	0.15 ± 0.01	
Bone		1.31 ± 0.46	0.91 ± 0.07	
Tumor		**1.18 ± 0.21**	**0.46 ± 0.05**	
T:B		**2.81 ± 0.72**	**3.29 ± 0.65**	
4D5scFv in SK-OV-3.ip				
Blood	3.03 ± 0.44	1.27 ± 0.41	0.50 ± 0.08	
Heart	1.50 ± 0.29	0.70 ± 0.22	0.42 ± 0.09	
Lung	2.70 ± 0.21	1.31 ± 0.47	0.84 ± 0.16	
Spleen	1.30 ± 0.16	0.85 ± 0.26	0.54 ± 0.16	
Kidney	124.77 ± 15.69	80.25 ± 32.93	51.73 ± 7.51	
Stomach	1.83 ± 0.85	6.43 ± 4.05	0.94 ± 0.10	
Colon	1.40 ± 0.21	1.32 ± 0.51	0.57 ± 0.11	
Liver	7.68 ± 1.63	5.72 ± 2.02	3.99 ± 2.27	
Muscle	0.64 ± 0.08	0.35 ± 0.14	0.25 ± 0.03	
Bone	0.88 ± 0.14	0.54 ± 0.21	0.34 ± 0.08	
Tumor	**2.92 ± 0.32**	**1.98 ± 0.73**	**1.86 ± 0.49**	
T:B	**0.96 ± 0.25**	**1.56 ± 1.08**	**3.72 ± 1.58**	

Biodistributions of DARPins and scFv were analyzed in nude mice, xenografted with human ovarian carcinoma SK-OV-3 tumors or SK-OV-3.ip tumors, as indicated. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3$-labeled constructs. Data are given as percentage of injected dose per gram tissue (% ID/g) and expressed as the mean ± S.D.

Table ST2: Tissue distributions of bivalent and bispecific DARPins in SK-OV-3.ip tumor bearing mice

Organ	1 h		4 h		24 h	
G3-E2_5						
Blood	0.70 ±	0.19	0.41 ±	0.06	0.14 ±	0.01
Heart	1.39 ±	0.38	1.00 ±	0.12	0.51 ±	0.02
Lung	1.31 ±	0.31	0.99 ±	0.21	0.45 ±	0.04
Spleen	3.05 ±	0.69	3.14 ±	0.36	1.26 ±	0.05
Kidney	178.91 ±	17.33	182.35 ±	27.84	79.10 ±	12.12
Stomach	3.73 ±	1.39	2.71 ±	0.38	0.98 ±	0.01
Colon	1.03 ±	0.14	0.80 ±	0.07	0.55 ±	0.03
Liver	10.95 ±	1.95	11.63 ±	1.34	6.14 ±	0.84
Muscle	0.55 ±	0.04	0.47 ±	0.11	0.25 ±	0.02
Bone	3.37 ±	0.52	1.72 ±	0.12	0.96 ±	0.02
Tumor	**3.37 ±**	**0.52**	**3.01 ±**	**0.09**	**1.77 ±**	**0.17**
T:B	**4.81 ±**	**2.05**	**7.34 ±**	**1.29**	**12.64 ±**	**2.12**
G3-G3						
Blood	0.72 ±	0.12	0.50 ±	0.15	0.18 ±	0.08
Heart	1.39 ±	0.25	1.48 ±	0.33	1.00 ±	0.23
Lung	1.09 ±	0.25	1.15 ±	0.19	0.56 ±	0.11
Spleen	3.43 ±	1.46	4.59 ±	0.88	2.37 ±	0.81
Kidney	86.42 ±	9.54	75.08 ±	22.81	41.29 ±	9.45
Stomach	2.32 ±	0.16	2.69 ±	0.72	0.75 ±	0.29
Colon	1.43 ±	0.13	1.37 ±	0.15	0.77 ±	0.31
Liver	16.29 ±	3.26	18.09 ±	2.48	9.24 ±	3.21
Muscle	0.79 ±	0.27	0.64 ±	0.24	0.41 ±	0.10
Bone	1.68 ±	0.50	2.35 ±	0.48	1.51 ±	0.38
Tumor	**2.76 ±**	**0.68**	**2.56 ±**	**0.48**	**1.58 ±**	**0.34**
T:B	**3.83 ±**	**1.58**	**5.12 ±**	**2.50**	**8.78 ±**	**5.79**

Biodistributions of a bivalent DARPin or bispecific DARPin (with one non-binding unit) were analyzed in nude mice, xenografted with human ovarian carcinoma SK-OV-3.ip tumors. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3^-$ labeled constructs. Data are given as percentage of injected dose per gram tissue (% ID/g) and expressed as the mean ± S.D.

Table ST3: Tissue distributions of DARPins with different PEG in SK-OV-3.ip tumor bearing mice

Organ	24 h	48 h
G3-PEG20		
Blood	2.19 ± 0.32	0.72 ± 0.10
Heart	1.12 ± 0.11	0.79 ± 0.24
Lung	1.48 ± 0.10	0.73 ± 0.12
Spleen	1.70 ± 0.17	1.39 ± 0.38
Kidney	26.30 ± 1.97	19.18 ± 3.94
Stomach	1.03 ± 0.40	0.59 ± 0.28
Colon	0.72 ± 0.12	0.51 ± 0.13
Liver	4.72 ± 0.03	3.54 ± 0.32
Muscle	0.67 ± 0.15	0.40 ± 0.10
Bone	0.77 ± 0.10	0.51 ± 0.15
Tumor	**14.77 ± 1.76**	**10.80 ± 2.41**
T:B	**6.74 ± 1.78**	**15.00 ± 5.53**
G3-PEG60		
Blood	8.48 ± 0.80	4.00 ± 1.30
Heart	2.89 ± 0.60	1.39 ± 0.42
Lung	4.00 ± 0.61	2.19 ± 0.44
Spleen	3.01 ± 1.30	2.37 ± 0.45
Kidney	8.23 ± 0.94	7.95 ± 1.31
Stomach	0.87 ± 0.15	0.44 ± 0.05
Colon	0.97 ± 0.18	0.52 ± 0.07
Liver	5.81 ± 1.05	4.21 ± 0.61
Muscle	0.90 ± 0.30	0.46 ± 0.09
Bone	1.24 ± 0.22	0.69 ± 0.09
Tumor	**13.66 ± 1.53**	**14.69 ± 5.93**
T:B	**1.61 ± 0.33**	**3.67 ± 2.67**

Biodistributions of PEGylated DARPins with different PEG molecules were analyzed in nude mice, xenografted with human ovarian carcinoma SK-OV-3.ip tumors. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3$-labeled constructs. Data are given as percentage of injected dose per gram tissue (% ID/g) and expressed as the mean ± S.D.

Table ST4: Tissue Distribution of DARPins with and without PEGylation in Balb/c mice

Organ	24 h	24 h
	G3	*G3-PEG20*
Blood	0.19 ± 0.02	7.24 ± 0.79
Heart	0.47 ± 0.08	3.53 ± 0.07
Lung	0.48 ± 0.07	4.65 ± 0.80
Spleen	0.95 ± 0.21	6.24 ± 0.66
Kidney	77.80 ± 13.53	74.94 ± 5.41
Stomach	0.43 ± 0.16	3.33 ± 0.33
Colon	0.27 ± 0.06	2.06 ± 0.32
Liver	4.00 ± 0.95	13.12 ± 1.42
Muscle	0.37 ± 0.05	1.78 ± 0.86
Bone	1.05 ± 0.14	2.30 ± 0.81

Biodistributions of a unmodified and PEGylated DARPin were analyzed in Balb/c mice. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3$-labeled constructs. Data are given as percentage of injected dose per gram tissue (%ID/g) and expressed as the mean ± S.D.

Table ST5: Tissue Distribution of PEGylated DARPins in Balb/c mice

Organ	24 h	48 h	72 h
G3-PEG20			
Blood	3.87 ± 0.18	1.13 ± 0.16	0.60 ± 0.05
Heart	1.83 ± 0.20	0.92 ± 0.20	0.65 ± 0.08
Lung	2.47 ± 0.06	1.27 ± 0.17	0.77 ± 0.10
Spleen	4.19 ± 0.35	3.26 ± 0.06	3.32 ± 0.50
Kidney	43.05 ± 4.38	29.43 ± 2.69	19.57 ± 1.23
Stomach	1.40 ± 0.27	0.61 ± 0.11	0.68 ± 0.19
Colon	1.09 ± 0.12	0.63 ± 0.05	0.57 ± 0.05
Liver	7.38 ± 0.47	6.40 ± 1.18	5.13 ± 0.38
Muscle	0.58 ± 0.05	0.39 ± 0.09	0.20 ± 0.01
Bone	0.96 ± 0.05	0.62 ± 0.09	0.12 ± 0.13
G3-PEG40			
Blood	14.77 ± 1.34	10.77 ± 0.93	7.46 ± 1.37
Heart	4.53 ± 0.93	3.67 ± 1.09	2.04 ± 0.24
Lung	7.52 ± 1.00	5.70 ± 0.63	4.01 ± 1.10
Spleen	7.26 ± 0.60	7.64 ± 0.84	6.28 ± 0.78
Kidney	10.79 ± 0.98	9.58 ± 1.32	6.96 ± 1.57
Stomach	1.12 ± 0.37	1.67 ± 0.62	0.77 ± 0.50
Colon	1.35 ± 0.12	1.12 ± 0.04	0.68 ± 0.13
Liver	8.86 ± 1.12	9.69 ± 1.39	7.02 ± 1.39
Muscle	0.86 ± 0.09	0.72 ± 0.16	0.47 ± 0.20
Bone	1.54 ± 0.24	1.20 ± 0.14	0.80 ± 0.24
G3-PEG60			
Blood	18.60 ± 2.65	12.55 ± 1.40	8.25 ± 0.41
Heart	6.44 ± 1.28	3.78 ± 0.64	3.15 ± 0.42
Lung	9.07 ± 1.26	6.24 ± 1.17	4.74 ± 0.38
Spleen	9.30 ± 0.55	9.90 ± 1.74	9.85 ± 0.40
Kidney	17.88 ± 2.28	17.74 ± 1.13	14.54 ± 0.31
Stomach	2.00 ± 0.95	1.14 ± 0.31	1.40 ± 0.06
Colon	1.75 ± 0.38	1.10 ± 0.12	1.13 ± 0.20
Liver	10.69 ± 0.52	12.67 ± 2.13	11.23 ± 0.05
Muscle	1.12 ± 0.08	1.00 ± 0.04	0.63 ± 0.09
Bone	2.01 ± 0.31	1.67 ± 0.17	1.17 ± 0.46

Biodistributions of PEGylated DARPin with different PEG molecules were analyzed in Balb/c mice. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3$-labeled constructs. Data are given as percentage of injected dose per gram tissue (% ID/g) and expressed as the mean ± S.D.

Table ST6: Tissue distributions of DARPins in SK-OV-3.ip tumor bearing mice, followed up to 72 h

	PEG20 modified DARPins				
Organ	**1 h**	**4 h**	**24 h**	**48 h**	**72 h**
G3-PEG20					
Blood	27.00 ± 4.01	15.88 ± 3.04	3.55 ± 0.40	0.98 ± 0.08	0.71 ± 0.07
Heart	11.19 ± 0.16	5.74 ± 0.91	1.84 ± 0.12	0.97 ± 0.06	1.16 ± 0.19
Lung	11.32 ± 1.22	7.74 ± 1.94	2.32 ± 0.19	1.19 ± 0.27	1.03 ± 0.22
Spleen	6.13 ± 1.69	3.80 ± 1.04	3.77 ± 1.05	2.74 ± 0.13	4.11 ± 0.73
Kidney	24.68 ± 2.46	39.65 ± 5.16	56.36 ± 10.47	40.53 ± 2.66	36.04 ± 4.08
Stomach	3.18 ± 1.13	2.54 ± 0.25	1.08 ± 0.24	0.45 ± 0.19	1.17 ± 0.15
Colon	3.84 ± 0.51	2.29 ± 0.26	1.10 ± 0.09	0.54 ± 0.14	0.71 ± 0.00
Liver	11.75 ± 1.90	12.16 ± 2.51	10.33 ± 2.12	7.30 ± 0.65	11.49 ± 1.52
Muscle	1.00 ± 0.15	1.02 ± 0.19	0.80 ± 0.04	0.43 ± 0.03	0.63 ± 0.17
Bone	2.42 ± 0.35	2.28 ± 0.30	1.30 ± 0.14	0.90 ± 0.07	1.09 ± 0.06
Tumor	**3.24 ± 0.75**	**6.97 ± 1.66**	**11.55 ± 4.69**	**8.41 ± 1.84**	**11.88 ± 0.92**
T:B	**0.12 ± 0.05**	**0.44 ± 0.19**	**3.25 ± 1.69**	**8.58 ± 2.58**	**16.73 ± 2.95**

Biodistributions of PEGylated DARPins were analyzed in nude mice, xenografted with human ovarian carcinoma SK-OV-3.ip tumors. Mice (n=3 per time point) were sacrificed and organs excised at the times indicated after injection of the $^{99m}Tc(CO)_3$-labeled constructs. Data are given as percentage of injected dose per gram tissue (% ID/g) and expressed as the mean ± S.D.

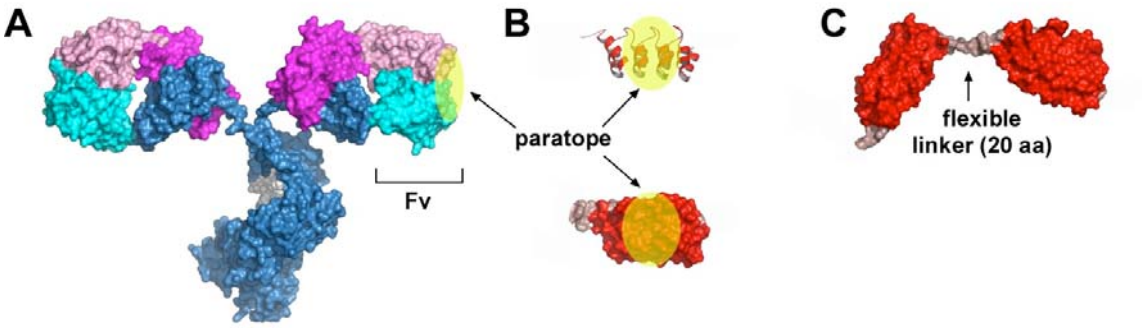


Figure S1: Comparison of the sizes of an antibody of the IgG type, and scFv, and a Designed Ankyrin Repeat Protein (DARPin) in monovalent and bivalent form, drawn to scale. **A**: Bivalent IgG (150 kDa), with the paratope overlaid in yellow, the Fv part is highlighted (the scFv would be identical in size with a linker connecting the two domains). **B**: DARPin structure shown as a ribbon model (above) and a space-filling model (below). While the MW of the DARPin G3 used in this study is only 14.5 kDa, the size of its paratope (overlaid in yellow) is comparable to that of an antibody. Each repeat of the DARPin consists of 33 amino acids forming a β-turn followed by two antiparallel α-helices. The internal repeats are randomized in the library and they can be evolved for new target specificity, while the repeats at the N- and C-terminus are important for maintaining the fold of the protein. **C**: Bivalent DARPin G3-linker-G3. The flexible linker of 20 aa allows the DARPin domains to adjust and permit independent binding to lead to a bivalent interaction. The antibody model is based on the PDB file 1IGT and the DARPin model was based on the PDB file 2JAB. The linkers and tags are modeled in random conformation. We thank Dr. Annemarie Honegger for providing these models.

For the bivalent molecules, two DARPins were cloned in series, connected by a flexible Gly-Ser linker, resulting in the connecting sequence …AEILQKL$(G_4S)_4$RSDLG… , where the first underlined sequence is from the C-cap of the upstream DARPin G3 and the second underlined sequence is from the N-cap of the downstream DARPin (either G3 or the non-selected control DARPin E2_5 (5)) (Kawe et al., manuscript in preparation).

The nomenclature of the DARPins follows that of Figure 1 in ref. (6), where also the full sequences are given. Briefly, the amino acids exchanged are appended to the name of the clone, G3. *E.g.*, G3-D refers to a point mutant in which an Asp of G3 is exchanged. For DARPin-DARPin conjugates, the names are connected by a hyphen, such as G3-G3.

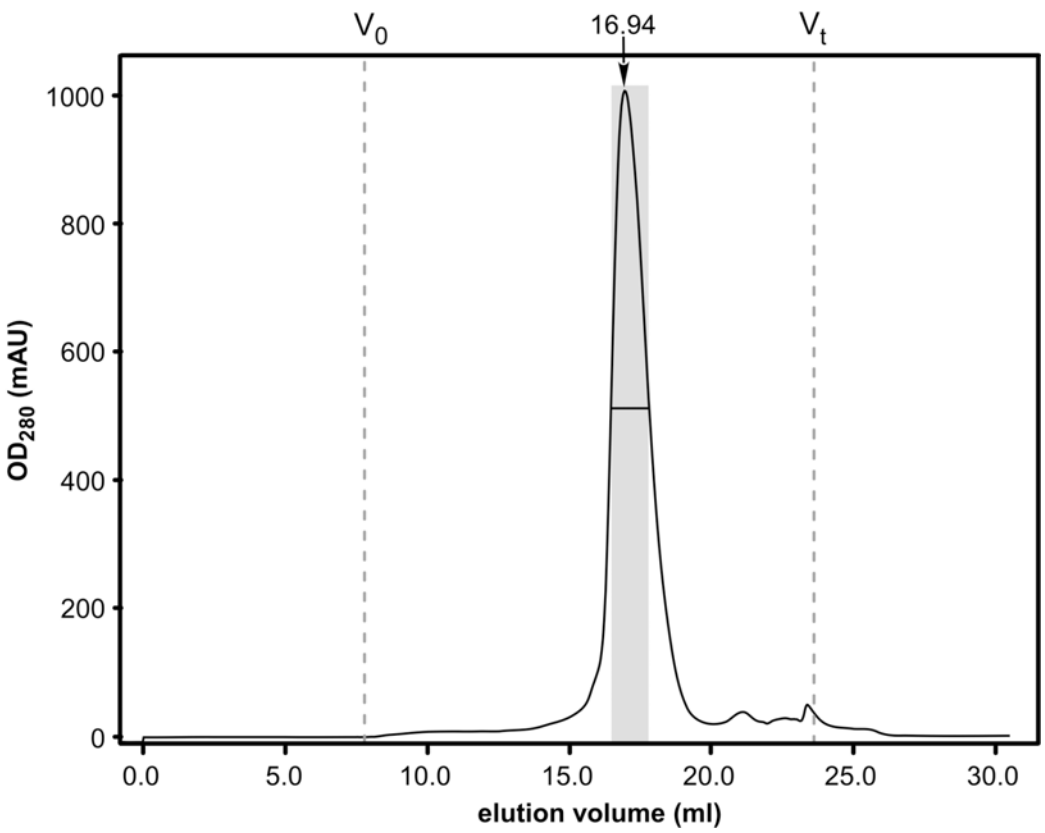


Figure S2: Typical preparative size-exclusion chromatography of an unmodified DARPin variant. This elution profile was obtained after IMAC purification of an unmodified DARPin variant (here G3-D) which was afterwards used in the animal experiments (or, for the cys-containing variants, modified with a PEG moiety). The DARPin elutes entirely within a range of 2-3 ml, showing a sharp elution profile. Fractions within the half-width range of the corresponding elution signal, indicated by a grey box, were combined yielding >99% pure protein samples as judged by 15% SDS-PAGE analysis. The void volume (V_0 = 7.7 ml) and the total column volume (V_t = 23.56 ml) are indicated by broken gray lines in the graph. About 1.5 ml of the respective samples were loaded each on a Superdex 200 10/30 column (GE healthcare) and run at a flow rate of 0.5 ml/min in PBS at 10°C. The elution volume of the main signal is indicated by an arrow.

The DARPins G3 and the point mutants G3-D, G3-AVD and G3-HAVD were expressed in *E. coli* XL1-Blue and purified essentially as previously described (6, 7). To assure high purity of the protein samples for the animal experiments, the size exclusion chromatography (SEC) above was always carried out. Fractions within the half-width range of the corresponding elution signal were combined yielding >99% pure protein samples as judged by 15% SDS-PAGE analysis.

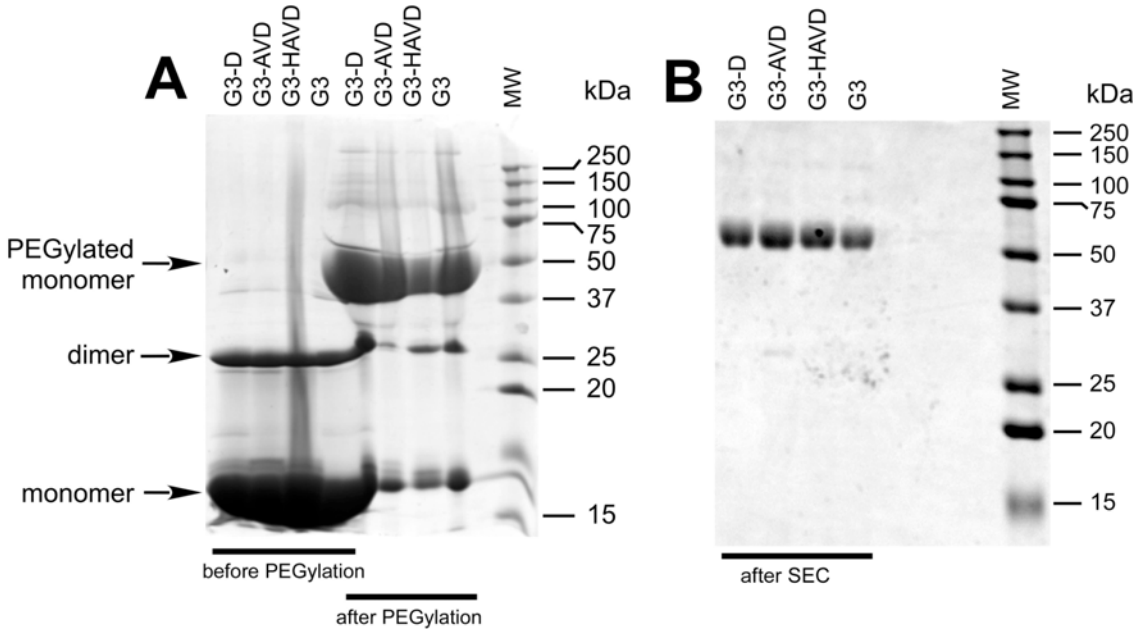


Figure S3: PEGylation and purification analysis of DARPin variants. The proteins were monitored on a 15% non-reducing SDS-PAGE stained with Coomassie brilliant blue. **A**: IMAC- and SEC-purified DARPin variants before and after the PEGylation reaction (see Material and Methods for details). **B**: PEGylated DARPin variants after a second SEC-purification step. The size marker is indicated in kDa. The monomeric, dimeric and PEGylated monomeric formats of the DARPin variants are indicated by arrows.

For PEGylating the DARPins, a Cys was introduced at the penultimate position (aa 136), changing the end of the C-cap from AEILQKLN to AEILQKLCN. Earlier experiments with PEG40 and PEG60 variants had been carried out with a construct ending in AEILQKCN.

For producing PEGylated proteins, the protein was first IMAC purified (**A**) and then polished by a SEC-step using a SD 200 HR 26/60 column (GE Healthcare) with PBS as running buffer. Fractions belonging to the main elution peak were combined and further concentrated using Amicon filtration devices (Millipore; Billerica, MA). Afterwards any air-oxidized protein was reduced with 5 mM DTT at room temperature for 1-3 h, and the DTT removed with a NAP-5 column (GE Healthcare). The protein was then incubated with a 1.5 molar excess of maleimide-PEG (Nektar Therapeutic; Huntsville USA) (either PEG-20 i.e. with a MW of 20 kDa, branched PEG-40 (2 · 20 kDa) or branched PEG-60 (2 · 30 kDa), (see Results) in 100 mM Na-phosphate buffer, pH 7.5. PEGylated protein was separated from unreacted PEG and unreacted protein by SEC using a SD 200 HR 10/30 column (GE Healthcare) with PBS as running buffer. Purity (>98%) and homogeneity of the protein samples were confirmed by non-reducing SDS-PAGE (**B**) and analytical size exclusion chromatography (**Figure S4**).

The purified yield with this approach was thus typically 10-20 mg after IMAC and one additional SEC step from a 1 l culture, and 3-10 mg when the protein was PEGylated and two times SEC purified. Note that in these procedures, all emphasis was placed on maximizing purity and eliminating potential traces of aggregated species, even though none could be detected, at the expense of discarding large fractions of the proteins.

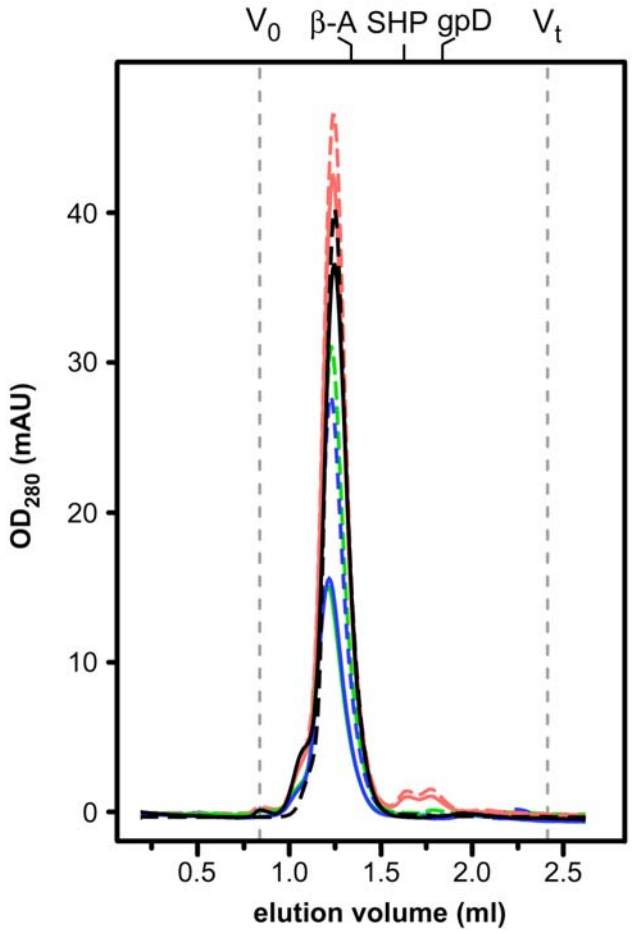
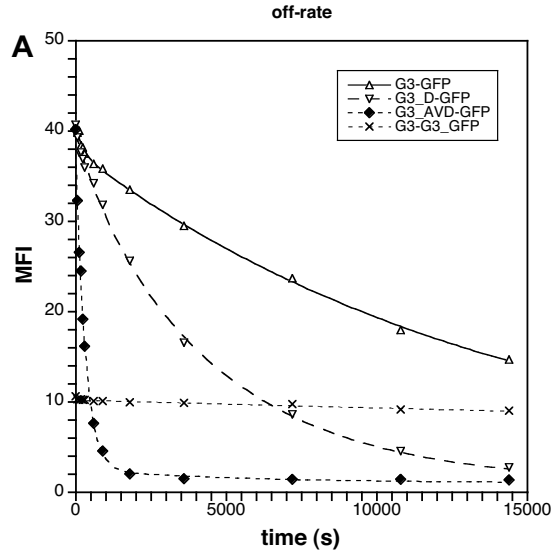
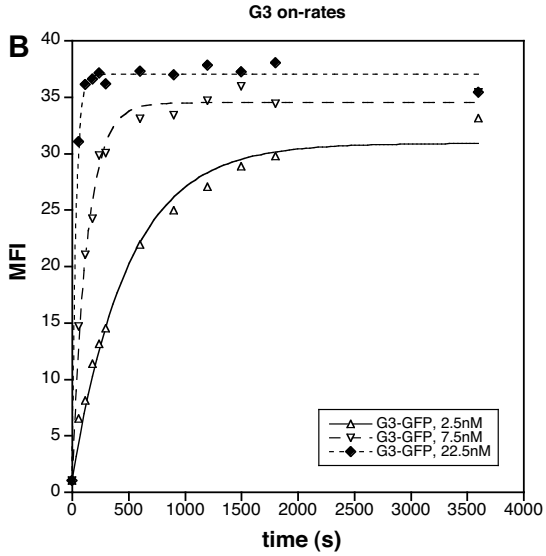
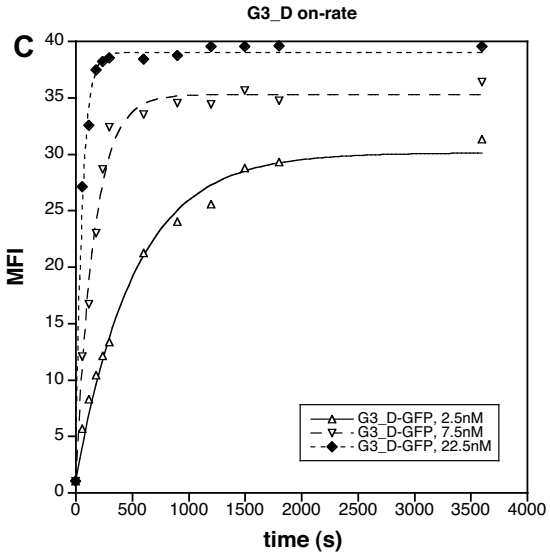
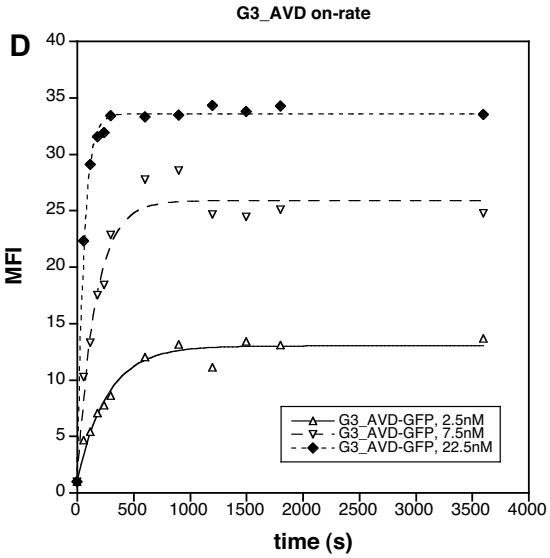


Figure S4: Analytical size-exclusion chromatography of all PEGylated DARPin variants. The elution profiles of all PEGylated DARPin variants, purified as described in Supplemental Methods and used in the animal experiments are shown (black: G3; green: G3-D; red: G3-AVD; blue G3-HAVD). The protein concentrations were not normalized. Elution profiles given by a dashed line refer to molecules incubated for 3 h at 37°C; elution profiles given by solid line refer to freshly purified proteins or to proteins stored at -80°C. All molecules give defined symmetric peaks and do not show higher molecular aggregates. The void volume (V_0 = 0.8 ml) and the total column volume (V_t = 2.4 ml) are indicated by broken gray lines in the graph. Lines indicate the elution volume of the marker proteins (β-amylase (β-A) 200 kDa; SHP 49 kDa; gpD 17.3 kDa). Samples were loaded at a concentration of 700 nM on a Superdex 200 PC column and run at a flow rate of 60 µl/min in PBS at RT. The apparent molecular weights of the PEGylated DARPin variants are ~320 kDa each, consistent with previous data (8).


off-rate
A
MFI
time (s)
G3-GFP
G3_D-GFP
G3_AVD-GFP
G3-G3_GFP


G3 on-rates
B
MFI
time (s)
G3-GFP, 2.5nM
G3-GFP, 7.5nM
G3-GFP, 22.5nM


G3_D on-rate
C
MFI
time (s)
G3_D-GFP, 2.5nM
G3_D-GFP, 7.5nM
G3_D-GFP, 22.5nM


G3_AVD on-rate
D
MFI
time (s)
G3_AVD-GFP, 2.5nM
G3_AVD-GFP, 7.5nM
G3_AVD-GFP, 22.5nM

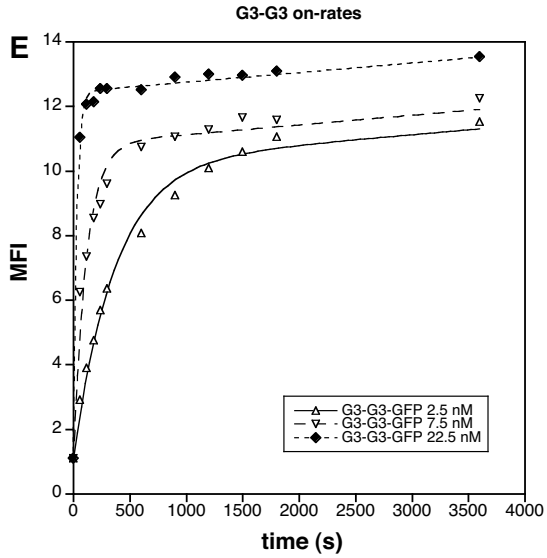

G3-G3 on-rates
E
MFI
time (s)
G3-G3-GFP 2.5 nM
G3-G3-GFP 7.5 nM
G3-G3-GFP 22.5 nM

Figure S5. Binding and dissociation kinetics of monomeric DARPin-GFP fusion proteins with different affinities on BT474 cells as well as the dimeric fusion protein G3-G3-GFP. The mean fluorescence intensity (MFI) is recorded as a function of time. (**A**) Dissociation of cells preloaded with DARPin after dilution in fresh buffer in the presence of unlabeled competitor G3, (**B**)-(**E**) association phase, (**B**) G3-GFP, (**C**) G3-D-GFP, (**D**) G3-AVD-GFP, (**E**) bivalent G3-G3-GFP at concentrations indicated.

DARPins were fused to superfolder-GFP (9) with a short linker, resulting in the connecting sequence …AEILQKL$(G_4S)_2$RSMSKGEEL…, where the first underlined sequence is from the C-cap of the upstream DARPin G3 and the second underlined sequence is from superfolder GFP (abbreviated for simplicity throughout as GFP). The fusion proteins were expressed in *E. coli* and IMAC-purified using the N-terminal $MRGSH_6$ tag, as described for the unfused DARPins.

To obtain correct values for binding and dissociation rates from surface HER2, internalization has to be minimized. We thus preincubated the cells with 0.2% sodium azide for 30 min at 37° C before incubating with DARPins, and have sodium azide present during all steps. It was found that under these conditions, DARPins completely dissociate over time, with almost no fluorescence remaining associated with the cells after extended time, suggesting that internalization was completely prevented. To obtain accurate concentration values for the DARPin-GFP fusions, they were determined by measuring the absorbance at 485 nm, the maximum of GFP, using the reported molar extinction coefficient for determining concentrations (9).

For on-rate determinations, BT474 cells were incubated at concentration of $1 \cdot 10^6$ cells/ml (as determined using a CASY TT cell counter (Innovatis)) with 2.5, 7.5 and 22.5 nM DARPin-GFP fusions in PBS containing 1 mg/ml BSA and 0.2% sodium azide (abbreviated PBSBA) at room temperature. For each time point, an aliquot of cells was withdrawn and directly used for FACS analysis on a FC500 instrument (Beckman Coulter) equipped with 488 nm argon laser, without further washing, to maintain time resolution. For each time point, 10^4 cells were counted, and the mean fluorescence intensity (MFI) was recorded. Data were fitted to a monoexponential reaction (approach to equilibrium), where $k_{obs} = k_{on} \cdot [\text{DARPin}] + k_{off}$. The molar concentration of HER2 in these experiments was determined by direct titration with G3-GFP fusions on cells (**Figure S6**).

For off-rate determinations, BT474 cells at a concentration of $1 \cdot 10^6$ cells/ 100 µl were saturated with DARPins for 60 min on ice with 50 nM G3-GFP, G3-D-GFP, G3-G3-GFP or 5 µM G3-AVD-GFP (because of its lower affinity), in PBSBA. The cells were washed with PBSBA extensively. The cells were then diluted 10-fold to a final concentration of $1 \cdot 10^6$ cells/ml in PBSBA at room temperature. To prevent reassociation of dissociated DARPin-GFP, an excess of competitor (50 nM unlabeled DARPin G3) was added. Under these conditions, complete loss of fluorescence over time is seen, indicating that both rebinding and receptor internalization could be successfully prevented. Before FACS measurements, the cells were briefly washed once with ice-cold PBS.

The experiment was also carried out in the reverse fashion, by prelabeling the cells ($1 \cdot 10^6$ cells/ 100 µl) with unlabeled 100 nM G3 or G3-G3. Cells were washed three times to removed unbound DARPins, and dissociation was started by competing with 100 nM of labeled G3-GFP or G3-G3-GFP. In this case, the dissociation of the unlabeled DARPin is measured by the increase of fluorescence, as dissociation is much slower than the very rapid association at the GFP-labeled DARPin under these conditions. These experiments confirm the off-rates of GFP-labeled DARPins within a factor 2 (data not shown).

To exclude effects of GFP, the DARPins G3, G3-G3 and G3-E2_5 were also labeled with Alexa488 NHS ester. To obtain mono-labeled DARPins, the labeling reaction were carried out at pH 7.2 in PBS, which favors labeling of the N-terminal amino group, and the resulting species were separated by anion-exchange HPLC. Titration data on human BT474 cells analyzed by FACS are consistent with stoichiometric binding of HER2 on cells (data not shown). Thus, we can exclude the possibility that G3-G3 or G3-E2_5 may have fewer accessible epitopes than G3.

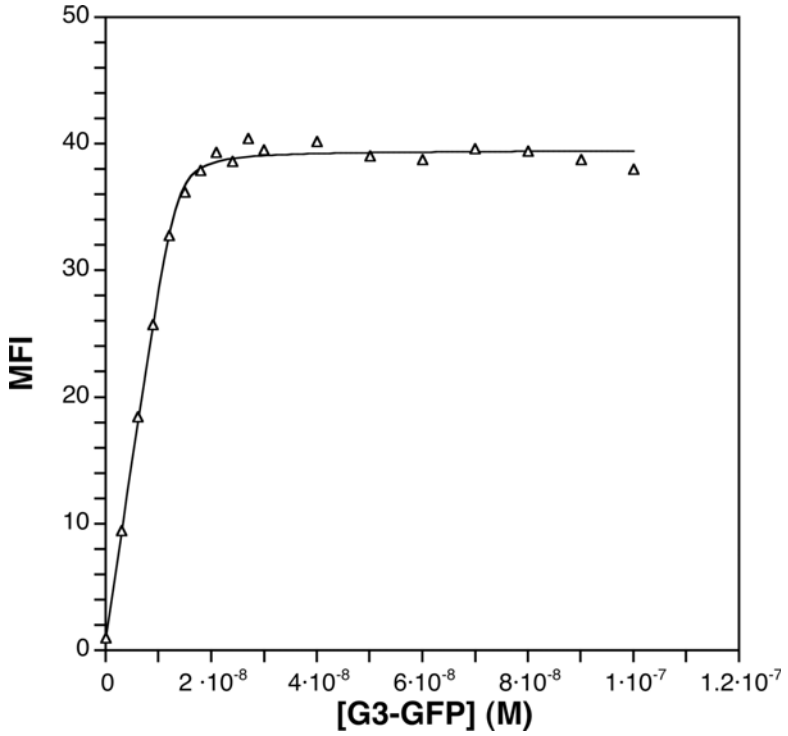


Figure S6: Equilibrium titration of HER2 on BT474 cells with the G3-GFP fusion protein. An equivalence point can be determined, and thus the molar concentration of HER, since the concentration of the G3-GFP stock solution is known and since with $1 \cdot 10^6$ cells/ml (determined with a Innovatis CASY TT cell counter), the molar concentration of HER2 is far above K_D. The equivalence point was determined by fitting the data to

$$MFI = \frac{MFI_{\max}}{[G3 \cdot HER2]_{\max}} \cdot \left(-\frac{[G3]+[HER2]+K_D}{2} + \sqrt{\left(\frac{[G3]+[HER2]+K_D}{2}\right)^2 - [G3]\cdot[HER2]} \right)$$

where *MFI* is the measured mean fluorescence intensity (corrected for background), MFI_{max} the background-corrected plateau value, [G3] the concentration of the GFP-labeled DARPin, [HER2] the concentration to be determined by the fit, and K_D the dissociation constant (which does not significantly influence the fit under these conditions). The value $MFI_{max}/[G3 \cdot HER2]_{max}$ is a proportionality constant determined from the fit. The equivalence point is at 13.4 nM G3-GFP, which translates to $8.7 \cdot 10^5$ HER2 molecules per cell.

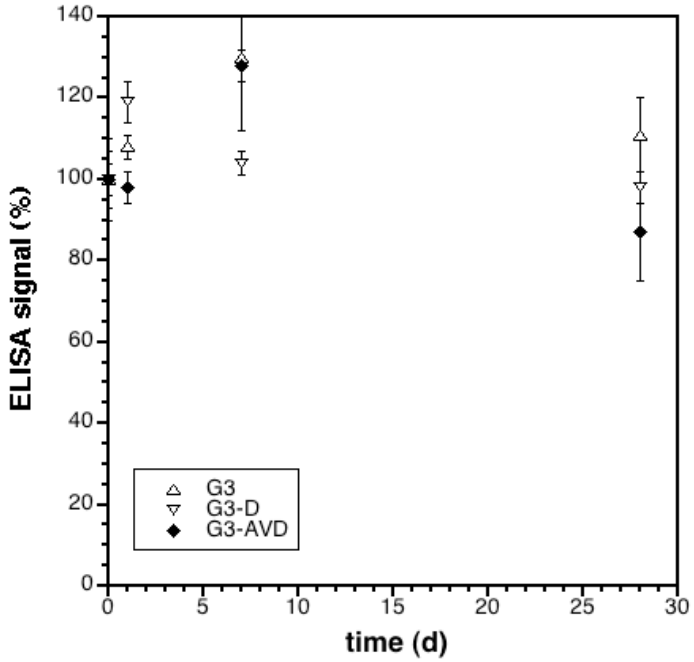
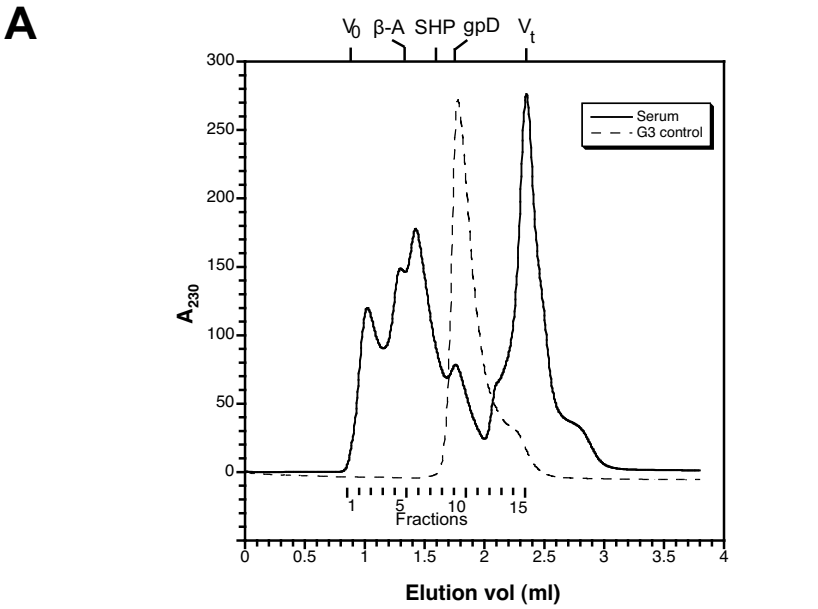
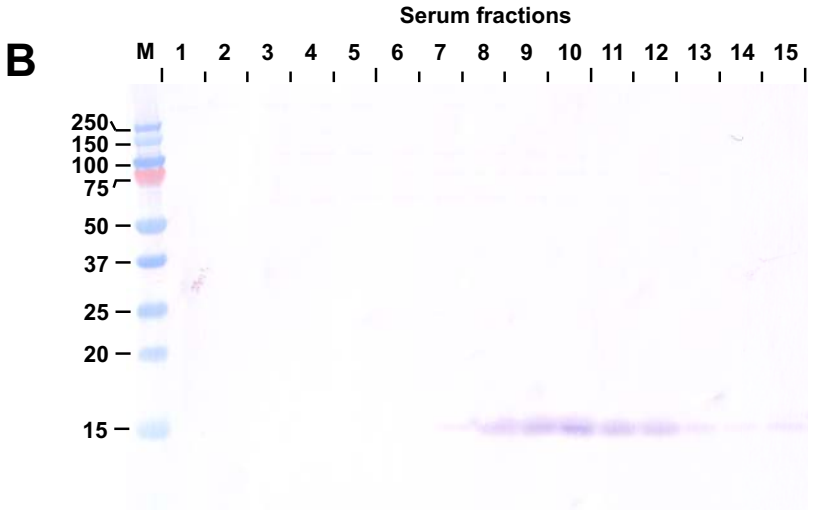


Figure S7: Stability of DARPins in human serum. DARPins were incubated at 15 μM in human serum for up to four weeks at 37°C. Samples were withdrawn and frozen at day 2, 7 and 28. Dilution series were made for each DARPin (because of the different affinities) and ELISAs with coated HER2 were carried out. To be able to measure active DARPin concentrations, values in the linear range of the ELISA are plotted (0.1 -1 nM, depending on the mutant).


A
V0 β-A SHP gpD Vt
300
250
200
150
100
50
0
A230
Serum
G3 control
1 5 10 15
Fractions
0 0.5 1 1.5 2 2.5 3 3.5 4
Elution vol (ml)


Serum fractions
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
250
150
100
75
50
37
25
20
15

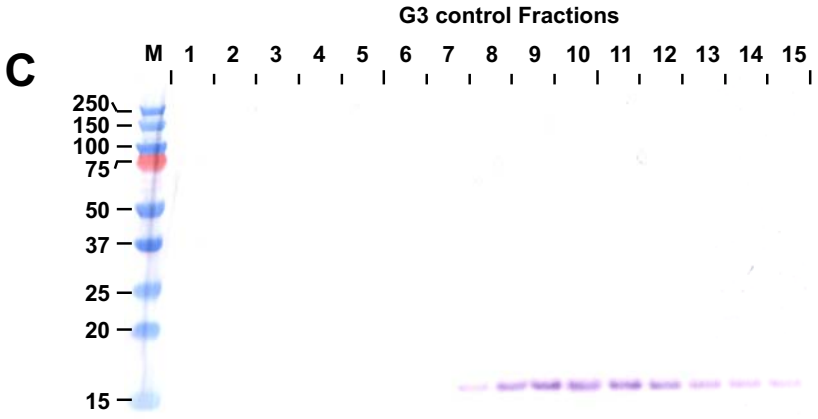

G3 control Fractions
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
250
150
100
75
50
37
25
20
15

Figure S8: Stability of DARPins after a 4 week incubation in human serum at 37°C. As an example, G3 is shown. The DARPins were incubated at about 15 μM in human serum at 37 °C, and after 4 weeks, the serum was analyzed. To investigate whether the DARPin might have aggregated, DARPins were enriched from the serum by IMAC and the elution fraction analyzed by analytical size exclusion chromatography on a Superdex 200 PC column (**A**). The void volume V_0 and total column volume V_t are indicated above the graph, as are the elution volumes of the marker proteins (β-amylase (β-A) 200 kDa; SHP 49 kDa; gpD 17.3 kDa). The DARPin enriched by IMAC from serum is still accompanied by many other proteins, and thus the DARPin was detected by western blot (**B**). The fractions correspond to those in (**A**). As a control, fresh DARPin G3 was analyzed on a Superdex 200 PC column in the same way (**C**). It can be seen that the DARPin elutes in the same fractions as before the serum incubation.

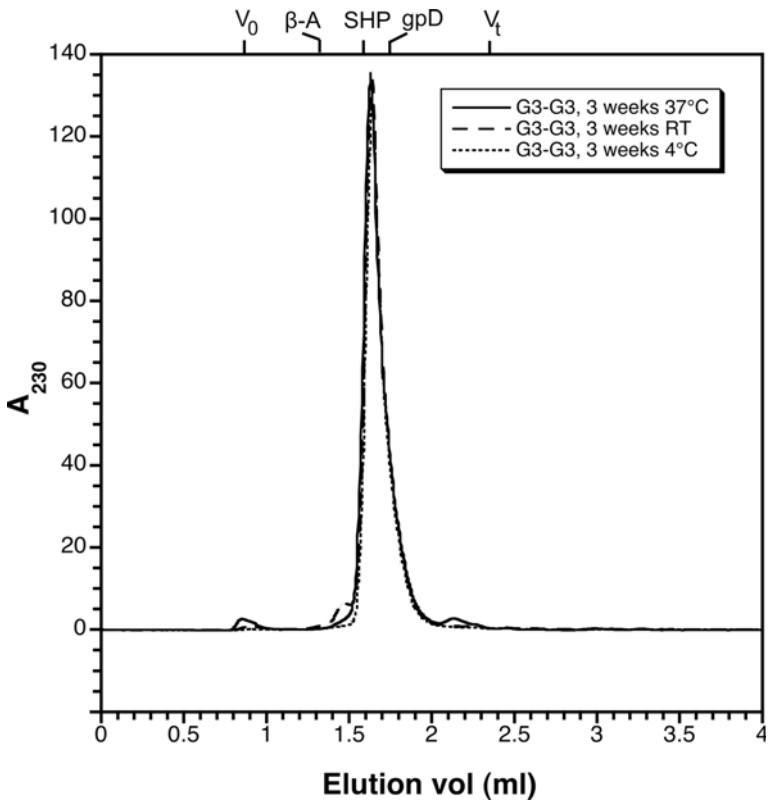


Figure S9: Stability of the bivalent DARPin G3-G3. The bivalent DARPin G3-G3 was incubated at about 280 μM in PBS buffer for 3 weeks at either 4°C, room temperature (RT) or 37°C. After this time, the samples were analyzed at 10 μM by analytical size exclusion chromatography on a Superdex 200 PC column. The void volume V_0 and total column volume V_t are indicated above the graph, as are the elution volumes of the marker proteins (β-amylase (β-A) 200 kDa; SHP 49 kDa; gpD 17.3 kDa).

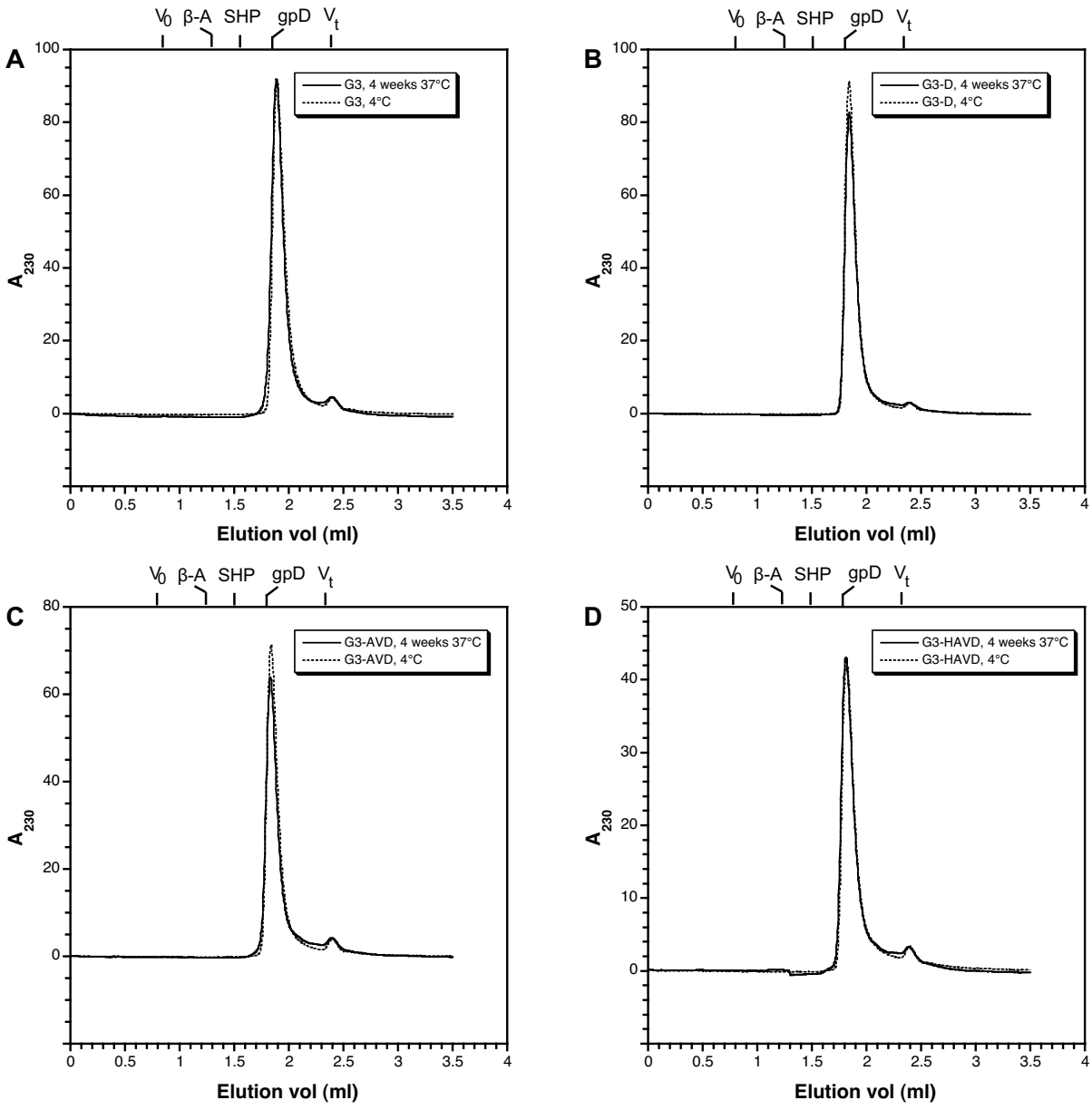

A
B
C
D
V_0 β-A SHP gpD V_t
G3, 4 weeks 37°C
G3, 4°C
G3-D, 4 weeks 37°C
G3-D, 4°C
G3-AVD, 4 weeks 37°C
G3-AVD, 4°C
G3-HAVD, 4 weeks 37°C
G3-HAVD, 4°C
A_{230}
Elution vol (ml)

Figure S10: The monovalent DARPins G3, G3-D, G3-AVD and G3-HAVD were incubated at about 15 μM in PBS buffer for 4 weeks at 37°C. After this time, the samples were analyzed by analytical size exclusion chromatography on a Superdex 200 PC column and compared to a sample that had been kept at 4°C. The void volume V_0 and total column volume V_t are indicated above the graph, as are the elution volumes of the marker proteins (β-amylase (β-A) 200 kDa; SHP 49 kDa; gpD 17.3 kDa). The small peak at the total volume is residual imidazole, as these samples were purified only by IMAC. Gel filtration was carried out on a Superdex-200 column on an ETTAN system or ÄKTAmicro system (GE Healthcare).

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