SUPPORTING INFORMATION for

Site specific discrete PEGylation of ^{124}I-labeled Fab' fragments improves tumor microPET/CT imaging in mice

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Contents

Preparation of Fab'-A, Fab'-B, and Fab'-C (Scheme 1)

mCC49, a full length mouse IgG, was reacted with the ficin,[S-1] a cysteine endopeptidase from figs, to produce the F(ab')$_2$ fragment. Ficin is purified prior to the fragmentation reaction by ion exchange chromatography to remove protein fragments that could co-purify with the F(ab')$_2$ product. The final concentration of cysteine in the reaction was 0.013 mM. This concentration was found to be sufficient to keep ficin active while low enough so that the F(ab')$_2$ product was not reduced.

Attempts were made to produce F(ab')$_2$ using pepsin, as described in the literature.[25] The pepsin-produced F(ab')$_2$ was reduced, reoxidized, and reacted with activated dPEGs as described below. In this case SDS-PAGE analysis (data not shown) of the conjugation product revealed the band corresponding to a single dPEG addition plus two higher molecular weight bands that we tentatively attributed to di- and tri-dPEG adducts. These higher molecular weight bands were not present when mCC49 was fragmented with ficin. We therefore used ficin to prepare the F(ab')$_2$.

The mCC49 F(ab')$_2$ was analyzed on a SDS-PAGE on a 4 – 20% gradient gel under non-reducing conditions. The product contained a single major band (Figure S1. Lanes: L-1 = mCC49, L-2 = mCC49 F(ab')$_2$). While the theoretical MWs of mCC49 F(ab')$_2$ and mCC49 are ~100 kDa and ~150 kDa respectively, the molecular weights determined from commercial standards on the gel were ~120 kDa and ~180 kDa. Proteins that contain disulfide bridges, such as mCC49 and mCC49 F(ab')$_2$, do not always bind their expected amount of SDS when they are not reduced and hence show less mobility on the gel and a higher apparent molecular weight.

The first step in the conjugation reaction is the reduction of mCC49 F(ab')$_2$ to mCC49 Fab'. This is done using 4 mM dithiothreitol at pH 7.4, conditions that reduce the disulfide bond between the heavy and light chains as well as the disulfide bonds in the hinge region between the two heavy chain fragments (Figure S1, Lane 3).

The first two Fab' conjugates (mCC49 Fab'-dPEG®$_{12}$-(dPEG®$_{24}$)$_3$ acid = Fab'-A and mCC49 Fab'-dPEG®$_{12}$-(dPEG®$_{12}$)$_3$) acid = Fab'-B) were prepared by reducing the F(ab')$_2$ fragments and then using an air oxidation step to reform the disulfide bonds between the heavy and light chains and a disulfide bond between two of the three cysteines in the heavy chain hinge region. This method leaves a single unpaired cysteine thiol in the hinge region that is free for conjugation to the Mal-dPEG®s.

The air oxidation method proved to be highly variable and was replaced by reoxidation of the disulfides using sodium tetrathionate. The single unpaired cysteine in the hinge region is protected by this method (Figure S1, Lane 4); we speculate that the protecting group may be a sulfenylthiosulfate. This protected cysteine is converted to a free thiol by treatment with a low concentration of mercaptoethylamine (Figure S1, Lane 5). This intermediate contains a small amount of F(ab')$_2$ as well as the Fab' product. Purification was not performed at this step, however, because the thiol containing Fab' is intrinsically unstable and the F(ab')$_2$ impurity is easily separated from the final product. We therefore used this product mix without purification to react with MAL-dPEGs and purified the final dPEGylated products. This method was used successfully for the preparation of mCC49 Fab'-NEM, and mCC49 Fab'-dPEG®$_{12}$-(m-dPEG®$_{24}$)$_3$ (Fab'-dPEG®-C).

Figure S2 shows the gel analysis of the conjugation of Fab' and Mal-dPEG-C (Lane 3). Lanes 4 – 10 are Fractions 49 – 55 from the Superdex 200 purification. Fractions 51 – 55 were combined as the final product, which was subsequently characterized by MALDI (Table 1 manuscript) and radiolabeled. MALDI analysis (Table 1 paper) confirmed that a single Mal-dPEG was added in each case to a Fab' fragment. The relative affinities were determined by ELISA using plates coated with Bovine Submaxillary Mucin (Table 1 paper). The Fab'-NEM standard had a Ka that was ~ 40 X lower than the Ka for mCC49. Most of the samples in this ELISA were stored for several months at 4°C prior to analysis and their stabilities under these storage conditions have not been determined.

Tables S1 and S2 contain the biodistribution data as % ID/organ, and the biodistribution data as ratio of tumor to other organs. Tables S3 – S5 contain data based upon quantitative analysis of the PET images. Figure S3 contains the pharmacokinetic parameter calculations.

References

S-1. Irvin E. Liener, Bernard Friedenson (1970) Ficin, *Methods in Enzymology*, *Volume 19*, 261-273.

Figure S1. SDS-PAGE GEL (4-20%, Non-Reducing)
Preparation of Fab'

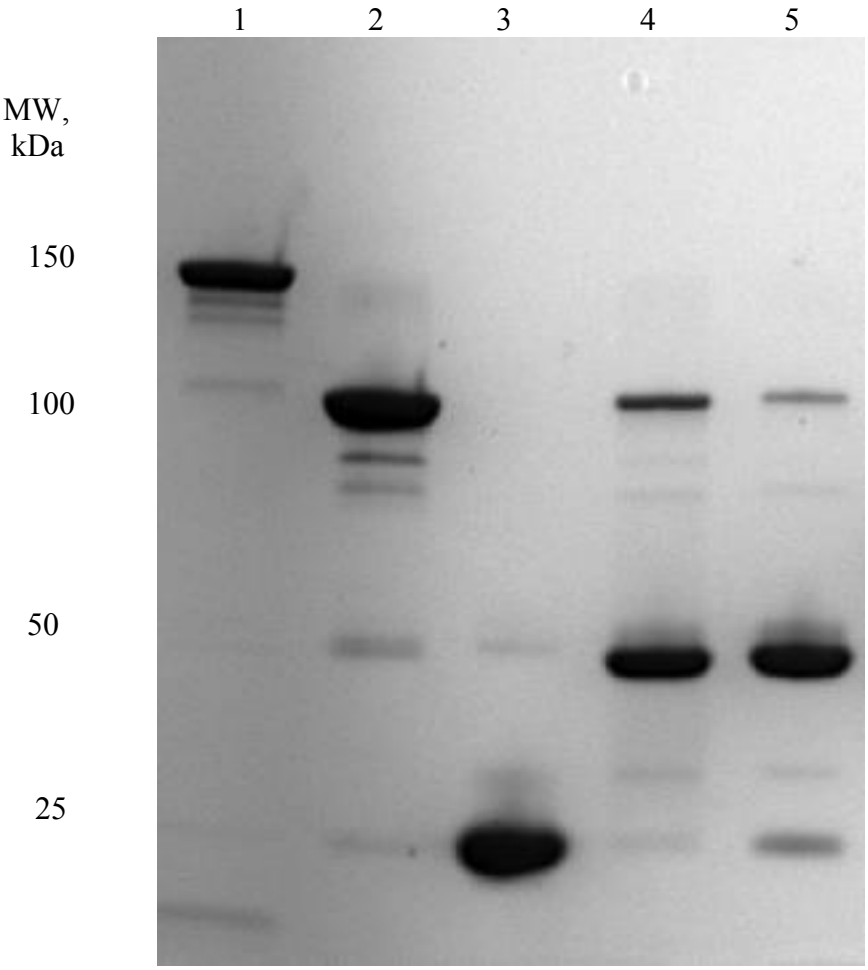
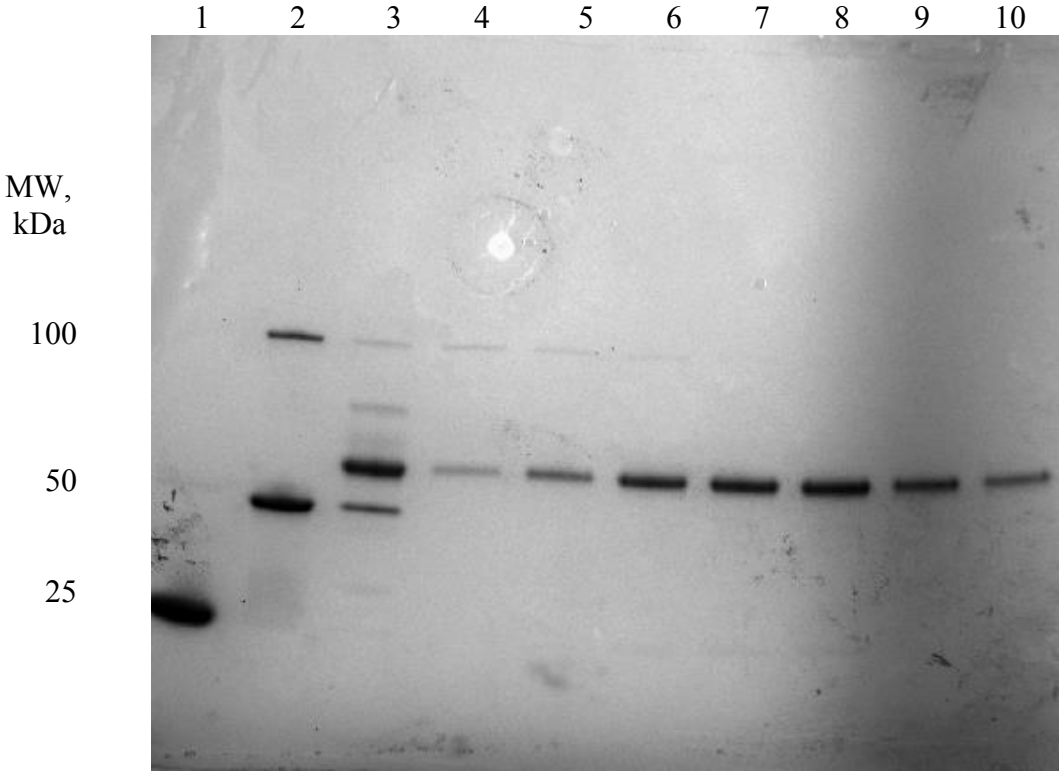


Figure S2. SDS-PAGE Gel (4 – 20%, Non-Reducing) Preparation of Fab'-C.



L1 = Reduced Fab'

L2 = Reoxidized Fab'

L3 = MAL-dPEG-C Conjugation Product

L4 – L10 = Fractions 49 – 55 of the Superdex 200 Purification of Fab'-C

Table S1. Comparison of biodistribution (%ID/organ) of ^{124}I-mCC49Fab'(Fab'-NEM, n=5), ^{124}I-mCC49Fab'-dPEG®$_{12}$-(dPEG®$_{24}$COOH)$_3$ (Fab'-A, n=5), ^{124}I-mCC49 Fab'-dPEG®$_{12}$-(dPEG®$_{12}$COOH)$_3$ (Fab'-B, n=4), and ^{124}I-mCC49 Fab'-dPEG®$_{12}$-(m-dPEG®$_{24}$)$_3$ (Fab'-C, n=3) in nude mice at 72 h.

	Fab'-NEM	**Fab'-A**	**Fab'-B**	**Fab'-C**
lungs	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
heart	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
liver	0.24 ± 0.19	0.99 ± 0.74	0.17 ± 0.04	0.19 ± 0.11
spleen	0.01 ± 0.01	0.03 ± 0.02	0.00 ± 0.00	0.01 ± 0.00
pancreas	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
GI	0.38 ± 0.31	0.37 ± 0.12	0.15 ± 0.08	0.39 ± 0.07
Kidneys	0.04 ± 0.02	0.13 ± 0.05	0.01 ± 0.00	0.02 ± 0.00
tumor	3.13 ± 1.94	9.71 ± 3.16	1.87 ± 0.55	1.01 ± 0.71

Table S2. Comparison of biodistribution ratio of tumor/organ of ^{124}I-mCC49Fab'(Fab'-NEM, n=5), ^{124}I-mCC49Fab'-dPEG®$_{12}$-(dPEG®$_{24}$COOH)$_3$ (Fab'-A, n=5), ^{124}I-mCC49 Fab'-dPEG®$_{12}$-(dPEG®COOH$_{12}$)$_3$ (Fab'-B, n=4), and ^{124}I-mCC49 Fab'-dPEG®$_{12}$-(m-dPEG®$_{24}$)$_3$ (Fab'-C, n=3) in nude mice at 72 h.

	Fab'-NEM	**Fab'-A**	**Fab'-B**	**Fab'-C**
blood	46 ± 24	75 ± 70	81 ± 9.7	25 ± 6.8
lungs	33 ± 10	42 ± 18	52 ± 7.1	29 ± 2.3
heart	56 ± 23	64 ± 37	171 ± 13	62 ± 10
liver	26 ± 20	8.8 ± 5.2	9.6 ± 3.4	9.6 ± 7.2
spleen	23 ± 4.9	23 ± 8.5	29 ± 11	10 ± 4.5
pancreas	80 ± 50	93 ± 53	241 ± 44	60 ± 11
GI	46 ± 39	59 ± 22	48 ± 30	12 ± 0.68
Kidneys	30 ± 15	17 ± 6.8	39 ± 3.9	24 ± 8.1
muscle	61 ± 12	50 ± 35	154 ± 47	62 ± 7.2
skin	26 ± 12	43 ± 23	25 ± 3.8	19 ± 0.80
carcass	47 ± 24	41 ± 19	64 ± 10	43 ± 4.9

Table S3. SUV (PET Tumor ROI imaging intensity, MBq/mL) normalized by injected dose in MBq and body weight in g)

	Fab'	Fab′-A	Fab′-B	Fab′-C
5 h	0.39 ± 0.18	0.60 ± 0.23	0.29 ± 0.06	0.27 ± 0.08
24 h	0.16 ± 0.10	0.39 ± 0.08	0.15 ± 0.04	0.12 ± 0.04
72 h	ND*	0.17 ± 0.05	0.05 ± 0.02	0.06 ± 0.02

*ND: not determined

Table S4. Comparison of Tumor SUV (from Table S3) between Fab'-A and other conjugates

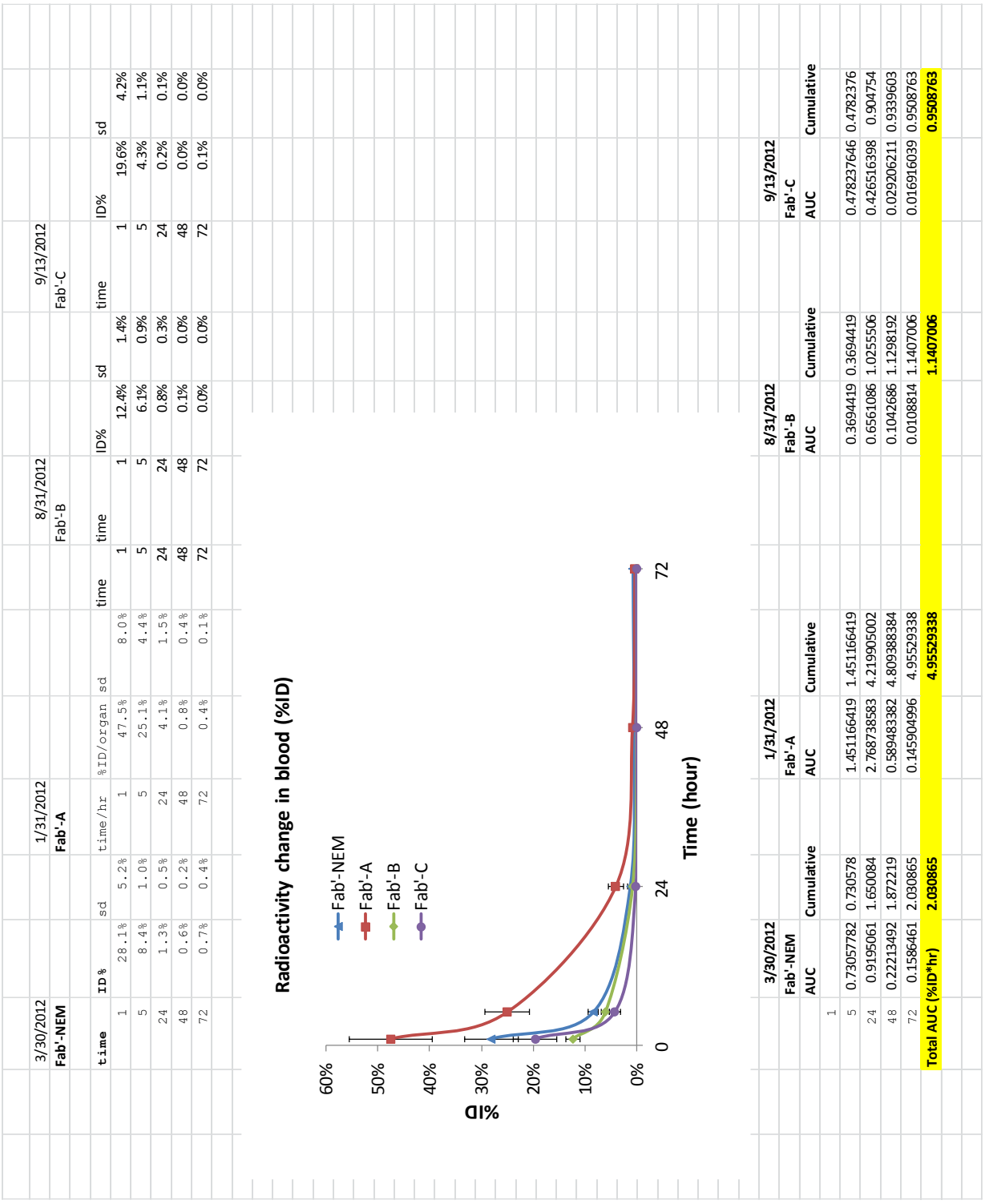
	Fab'-A /Fab'	Fab'-A /Fab'-B	Fab'-A /Fab'-C
5 h	1.52	2.09	2.25
24 h	2.38	2.70	3.24
72 h	ND*	3.41	2.72

*ND: not determined

Table S5. SUV Tumor / SUV arm muscle. (Tumor/Background, T/B)

	Fab'	Fab′-A	Fab′-B	Fab′-C
5 h	3.10 ± 1.33	4.49 ± 1.71	3.02 ± 0.70	3.21 ± 0.63
24 h	21.76 ± 16.62	16.79 ± 7.15	14.07 ± 7.14	26.01 ± 9.64

Figure 3S. Data and calculations for pharmacokinetics in blood of mice.



	3/30/2012			1/31/2012				8/31/2012				9/13/2012			
	Fab'-NEM			Fab'-A				Fab'-B				Fab'-C			
time	ID%	sd		time/hr	%ID/organ	sd		time	ID%	sd		time	ID%	sd	
1	28.1%	5.2%		1	47.5%		8.0%	1	12.4%	1.4%		1	19.6%		4.2%
5	8.4%	1.0%		5	25.1%		4.4%	5	6.1%	0.9%		5	4.3%		1.1%
24	1.3%	0.5%		24	4.1%		1.5%	24	0.8%	0.3%		24	0.2%		0.1%
48	0.6%	0.2%		48	0.8%		0.4%	48	0.1%	0.0%		48	0.0%		0.0%
72	0.7%	0.4%		72	0.4%		0.1%	72	0.0%	0.0%		72	0.1%		0.0%

	3/30/2012			1/31/2012			8/31/2012			9/13/2012		
	Fab'-NEM			Fab'-A			Fab'-B			Fab'-C		
	AUC	Cumulative		AUC	Cumulative		AUC	Cumulative		AUC	Cumulative	
1												
5	0.73057782	0.730578		1.451166419	1.451166419		0.3694419	0.3694419		0.478237646	0.4782376	
24	0.9195061	1.650084		2.768738583	4.219905002		0.6561086	1.0255506		0.426516398	0.904754	
48	0.22213492	1.872219		0.589483382	4.809388384		0.1042686	1.1298192		0.029206211	0.9339603	
72	0.1586461	2.030865		0.145904996	4.95529338		0.0108814	1.1407006		0.016916039	0.9508763	
Total AUC (%ID*hr)		2.030865			4.95529338			1.1407006			0.9508763	