

1 *Benchmarks*

2 **Preactivation Crosslinking – An Efficient Method for** 3 **the Oriented Immobilization of Antibodies**

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11 **Abstract:** Crosslinking of proteins for their irreversible immobilization on surfaces is a proven and
12 popular method. However, many protocols lead to random orientation and the formation of
13 undefined or even inactive by-products. Most concepts to obtain a more targeted conjugation or
14 immobilization requires the recombinant modification of at least one binding partner, which is often
15 impractical or prohibitively expensive. Here a novel method is presented, which is based on the
16 chemical preactivation of Protein A or G with selected conventional crosslinkers. In a second step,
17 the antibody is added, which is subsequently crosslinked in the Fc part. This leads to an oriented
18 and covalent immobilization of the immunoglobulin with a very high yield. Protocols for Protein A
19 and Protein G with murine and human IgG are presented. This method may be useful for the
20 preparation of columns for affinity chromatography, immunoprecipitation, antibodies conjugated
21 to magnetic particles, permanent and oriented immobilization of antibodies in biosensor systems,
22 microarrays, microtitration plates or any other system, where the loss of antibodies needs to be
23 avoided, and maximum binding capacity is desired. This method is directly applicable even to
24 antibodies in crude cell culture supernatants, raw sera or protein-stabilized antibody preparations
25 without any purification nor enrichment of the IgG. This new method delivered much higher signals
26 as a traditional method and, hence, seems to be preferable in many applications.

27 **Keywords:** Antibody coating, proximity-enhanced reaction, immunoglobulins, IgG, Protein A,
28 Protein G, bio-interaction, immunoprecipitation, pull-down assay, immunocapture, stabilization,
29 yield, regeneration, nanoparticles, microparticles, biochips, immunosensor, photochemical
30 crosslinker, click chemistry, Herceptin, Trastuzumab.

32 **1. Introduction**

33 Antibodies are one of the most important biochemical reagents. They can be used in
34 immunoassays [1,2], biosensors [3-7], microarrays [8,9], atomic force microscopy [10], surface
35 plasmon resonance [11,12], affinity chromatography [13,14], affinity purification-mass spectrometry
36 [15], mass spectrometric immunoassay [16], immunoprecipitation [17], and magnetic particle
37 separation [18] for the application in diagnostics, food and environmental analysis, medical and
38 biochemical research. Many of these techniques require the immobilization of the respective antibody
39 to a surface. Although the random attachment of the immunoreagent is common due to its simplicity,
40 oriented immobilization is usually considered to be preferable [12,19-23]. A multitude of techniques
41 has been proposed for the oriented immobilization of antibodies. However, only the use of secondary
42 antibodies, (strept)avidin, Protein A [24] or G [25] and the periodate method [26] have been used
43 more frequently. In some cases, the reversibility of such complexes is seen as an advantage since the
44 surface can be regenerated by the release of the primary binding reagent. However, for preparative
45 applications or sample preparation for mass spectrometry (e.g., immunocapture LC-MS/MS), the
46 elution of the immunoreagent leads to unwanted contamination of the sample or product. Besides,

47 the expensive antibody may be lost during the elution step. In these cases, either non-oriented
48 covalent techniques are used, or the oriented protein A/G/antibody complex needs to be stabilized
49 with crosslinking reagents. Unfortunately, with conventional crosslinkers, a targeted approach is
50 challenging, which leads to the random derivatization of many antibody side chains and amino-
51 termini. Since crosslinkers have been used heavily for the examination of protein-protein interactions
52 in general, these reactions have been studied in some detail. However, up to now, the random-
53 derivatization characteristics was accepted an inevitable consequence of this approach. It must be
54 noted that the N-termini of antibodies are quite near to their binding sites, which makes a potentially
55 negative influence of amino-reactive reagents quite likely. Since the variable region of antibodies
56 shows individual structures and properties, the prediction of such problems, e.g., the loss of binding
57 capacity, is nearly impossible today.

58 To overcome these limitations, we developed a novel two-step crosslinking method. In these
59 protocols, the antibody capturing molecule is pre-activated with “slow” crosslinkers, and
60 subsequently, any residual reagent is washed away to avoid any contact of the free crosslinking
61 reagent with the antibody. “Slow” in this context means the property that in a bifunctional
62 crosslinker, the first reaction does not lead to the hydrolysis or otherwise deactivation of the second
63 function. This concept shows some similarity with photochemical crosslinking [27], which has been
64 used in the exploration of nearly all types of bio-interactions. However, photochemical linkers have
65 some significant disadvantages, which may have limited their more widespread application. The
66 most obvious drawback is their light sensitivity, which requires appropriate countermeasures during
67 synthesis, purification, and use. Accidental exposure to light might reduce the conjugation yield in
68 an irreproducible way. Furthermore, the reaction yields of photochemical reactions often are low [27].
69 Also, the required setup for UV irradiation adds complexity to the experiments, the progression of
70 the reaction is difficult to monitor, and unwanted photochemical byproducts may be formed. Some
71 short wavelength lamps also need additional safety measures to avoid unwanted exposure of the
72 laboratory workers. Finally, the possibility of the direct introduction of a photo-inducible group in a
73 recombinant protein [28], leads to a complicated and expensive production, which might preclude
74 commercial availability even in the future.

75 One of the most popular applications for which chemical crosslinking plays an important role is
76 the immobilization of antibodies on magnetic or other beads. Particles pre-coated with Protein A or
77 G are readily available from many commercial suppliers. Most of the protocols delivered by the
78 manufacturer suggest crosslinking the protein G/IgG complex by use of chemical crosslinkers, such
79 as bis(sulfosuccinimidyl)suberate (BS3), to avoid co-elution of the antibody. However, the formation
80 of many byproducts and the potential inactivation of the antibodies is rarely considered at all.

81 In recent years, some quite smart concepts have been presented, to achieve “proximity-
82 enhanced” or “proximity-enabled” crosslinking reactions in biochemical complexes. Xiang et al. [29-
83 32] showed the introduction of haloalkane-modified tyrosine residues for this purpose. Very recently,
84 a similar concept was published based on haloalkane-modified lysines [33]. Furthermore, lysines
85 modified with a fluoroacetamide group were used in combination with a cysteine to introduce
86 defined crosslinks in proteins or protein complexes [34]. In addition, Furman et al. [35] and Xuan et
87 al. [36] presented other reactive groups for the same purpose. All of them require the site-specific
88 introduction of artificial amino acids [37,38], e.g., by tRNA-synthetases. This limits the applicability
89 to genetically modified proteins [39] and may be the reason for their lack of practical use. In contrast,
90 our approach can be used for any protein or peptide, irrespective of their source, if a favorable (bio-)
91 interaction can be formed.

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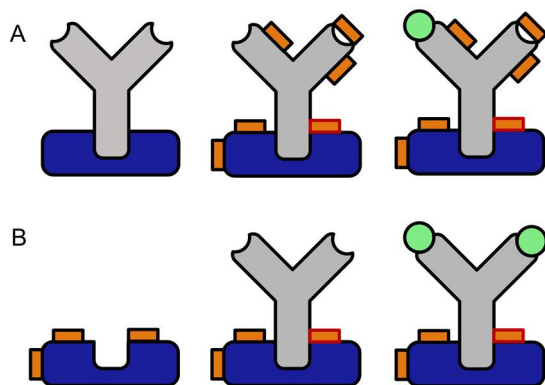


Fig 1: Comparison of conventional crosslinking (A) to the proposed preactivation crosslinking method (B). Please note the potentially higher binding capacity of the immobilized antibody and the complete lack of chemical modification in the F_{ab} region (blue: Protein A or G, grey: antibody, orange: crosslinker, orange with red rim: protein-protein crosslink, orange with dark rim: intramolecular or half crosslink, green: antigen).

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101 2. Materials and Methods

102 2.1 Chemicals and reagents

103 Laboratory water was obtained from a Milli-Q water purification system (Millipore, Bedford,
104 MA, USA). Dimethylsulfoxide (DMSO), was from AppliChem (Darmstadt, Germany), bovine serum
105 albumin (BSA), disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate dihydrate,
106 sodium chloride, citric acid monohydrate, and trisodium citrate dihydrate, sodium
107 cyanoborohydride, sulfuric acid, Tween 20, sodium tetraborate decahydrate were obtained from
108 Sigma-Aldrich (Steinheim, Germany). The reagents for crosslinking were obtained from the
109 following sources: Succinimidyl iodoacetate (SIA), bis(sulfosuccinimidyl) suberate, 1-ethyl-3-(3-
110 dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were
111 obtained from Thermo Scientific, succinimidyl(4-iodoacetyl) aminobenzoate (SIAB) and
112 sulfosuccinimidyl (4-iodoacetyl) aminobenzoate (Sulfo-SIAB) were obtained from Apollo Scientific
113 (Bredbury, UK). Glutaraldehyde, 1,3-butadiendiepoide and formaldehyde were obtained from
114 Sigma-Aldrich (Steinheim, Germany), disuccinimidyl tartrate was from CovaChem, glyoxal was
115 from Merck (Darmstadt, Germany) and tris(hydroxymethyl)phosphine was bought from abcr
116 (Karlsruhe, Germany). Protein G (pro-402-c) and recombinant Protein A (pro-774) were purchased
117 from ProSpec. The microtitration plates were from Greiner bio-one (Germany). Mouse Monoclonal
118 antibodies to horseradish peroxidase (HRP), clone HP-03 (IgG1), product No. 11-262-C100 were
119 obtained from EXBIO (Praha, Czech Republic). The humanized antibody Herceptin (Trastuzumab)
120 was kindly supplied by Roche. In this article, Herceptin is referred to as "human IgG1", due to its
121 human Fc domain. It was purified from any additives by Protein A chromatography. TMB substrate
122 was obtained from Seramun GmbH, Germany.

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124 2.2 Buffers

125 Phosphate-buffered saline (PBS), pH 7.4: 2.3 mM KH_2PO_4 , 10 mM $Na_2HPO_4 \cdot 2 H_2O$, 136.9 mM NaCl
126 Phosphate buffer, pH 6: 12.3 mM $Na_2HPO_4 \cdot 2 H_2O$, 61.0 mM $NaH_2PO_4 \cdot 2 H_2O$, Phosphate buffer, pH 7:
127 87.7 mM $Na_2HPO_4 \cdot 2 H_2O$, 39.0 mM $NaH_2PO_4 \cdot 2 H_2O$, Sodium borate buffer (SB), pH 8 and 9: 10.0 mM
128 $Na_2B_4O_7 \cdot 10 H_2O$, Citrate buffer, pH 5: 35.0 mM Citric Acid $\cdot H_2O$, 65.0 mM Trisodium citrate $\cdot H_2O$
129 Washing buffer (PBS-Tween), pH 7.4: pH 7.4: 1.3 mM KH_2PO_4 , 6.6 mM $Na_2HPO_4 \cdot 2 H_2O$, 0.5 mM
130 Tween 20, Elution buffer (Glycine/HCl), pH 2.3: 0.1 M Glycine, titrated to pH 2.3 with 0.1 N HCl.

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134 2.3 Equipment

135 ELISA washer: 405 Select BioTek, ELISA reader: EPOCH 2 BioTek, multichannel pipettes: Eppendorf
136 Xplorer plus, Brand Transferpipette S, balance Mettler Toledo XS105 Dual Range, centrifuge Hettich
137 Mikro 220R, UV VIS spectrophotometer ThermoScientific Evolution 220

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139 2.4 Crosslinking protocol with SIAB or sulfo-SIAB

140 Crosslinking assays (Fig. 2) were performed in 96-well polystyrene microtitration plates (MTP).
141 Protein G was diluted in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 10 mg/L.
142 100 μ L of this solution was pipetted into each well of the MTP, which was shaken for at least 90 min
143 at 750 rpm. The plate was washed three times with PBS-Tween. In the next step, 300 μ L of a 1%
144 solution of bovine serum albumin (BSA) was added to each well. This blocking step was performed
145 for at least one hour at a shaking frequency of 750 rpm. The plate was subsequently washed three
146 times. Then 100 μ L of the crosslinker solution was added to the wells. Suitable concentrations have
147 been determined as follows: 0.25 mM for SIAB and 1 mM for sulfo-SIAB. For poorly soluble
148 crosslinkers, such as SIAB, DMSO can be used as solubilizer with a subsequent dilution in a buffer to
149 a maximum final concentration of 40% of solvent. After a reaction time of 15 min, residual crosslinker
150 was removed by three washing steps. The monoclonal anti-peroxidase antibody was diluted
151 1:100,000 (10 ng/L) in phosphate buffer (pH 6) and added to the wells (100 μ L per cavity). This
152 solution was incubated at 750 rpm for sixteen hours and removed by three washing steps with PBS-
153 Tween. A solution of 10 mg/L of horseradish peroxidase was prepared in PBS-Tween-BSA as
154 described above. 100 μ L was added to each well and shaken at 750 rpm for 15 min. Subsequently, the
155 plate was washed three times with PBS-Tween. Finally, 100 μ L per well of TMB substrate was added
156 and incubated for 1 to 30 minutes, as required to reach a sufficient absorbance. After this development
157 time, the reaction was stopped with 0.25 M sulfuric acid. The absorbances were recorded with a
158 microplate reader at 450 nm (650 nm reference wavelength).

159

160 2.5 Crosslinking protocol with glutaraldehyde

161 First, the protocol was performed as described in section 2.4 with protein G. Instead of SIAB
162 solution, 100 μ L of glutaraldehyde (2 mM) dissolved in sodium borate buffer (pH 8) was added to
163 the wells. After a reaction time of 15 min, residual crosslinker was removed by three washing steps.
164 The monoclonal anti-peroxidase antibody was diluted 1:100,000 (10 ng/L) in phosphate-buffer (pH 6)
165 with 0.1% of Tween 20 and 1% of bovine serum albumin (BSA) and added to the wells (100 μ L per
166 cavity). This solution was incubated at 750 rpm for one hour and removed by three washing steps
167 with PBS-Tween. In the following step, 100 μ L of NaCNBH₃ (200 μ g/ml) in PBS was added to the wells
168 to reduce imines to stable secondary amines. A one-hour incubation was required for the reduction
169 (750 rpm), and subsequently, the solution was removed by three washing steps with PBS-Tween. The
170 rest of the protocol followed the steps described in section 2.4.

171

172 2.6 Comparison of crosslinking protocols

173 The experiment was performed in analogy to the protocol 2.4. 100 μ L of a Protein G solution (10
174 mg/L, PBS pH 7.4) was pipetted to a microtitration plate and incubated for 90 min at room
175 temperature under shaking. After a washing step (PBS-Tween, pH 7.4), blocking was performed with
176 300 μ L of BSA solution (PBS pH 7.4) for one hour. After another washing step, 100 μ L of sulfo-SIAB
177 (100 μ L, 1 mM, PBS, pH 7.4) was incubated for 30 min. The wells for the BS3 crosslinker were supplied
178 with 100 μ L of PBS. After a subsequent washing step, the whole plate (except controls) was incubated
179 with a murine monoclonal antibody (HP-03, IgG1, 100 μ L, 1:100,000, phosphate buffer, pH 6.0) for 16
180 hours. After the next washing step, the sulfo-SIAB wells were supplied with 100 μ L of PBS, and the
181 BS3 wells were incubated with 100 μ L of BS3 in PBS (pH 7.4) for 30 min under shaking. After the next
182 washing step, any non-crosslinked antibody was removed with elution buffer (pH 2.3, glycine/HCl,
183 30 min) under shaking. After a further washing step, the plate was supplied with 100 μ L of
184 horseradish peroxidase (HRP, 10 mg/L, PBS-Tween + 1% BSA) and incubated for 15 minutes. After a
185 final washing step, the 100 μ L of TMB substrate was added, incubated for 10 minutes and stopped

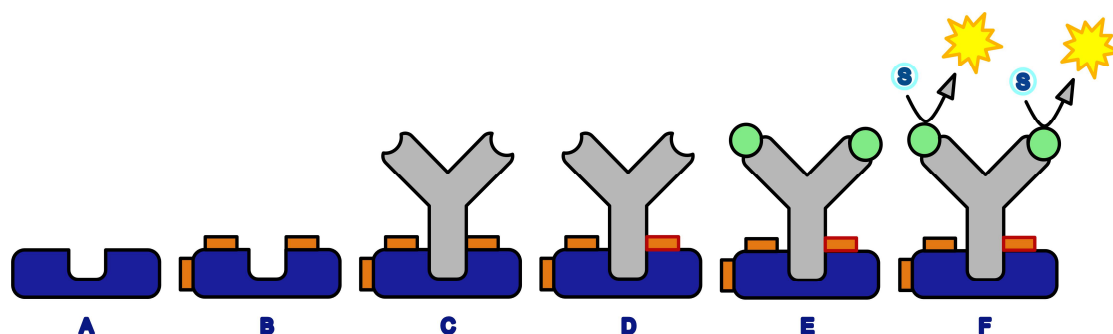
186 with diluted sulfuric acid. The signal was recorded at 450 nm. A blank value (without crosslinker)
 187 was subtracted.
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189 3. Results

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191 3.1. Crosslinking assay based on Protein G–Mouse IgG Interaction

192 Well-known protein interaction pairs were chosen for this study. Recombinant Protein G, a
 193 protein from *Streptococcus*, (or Protein A), and a murine monoclonal antibody (IgG₁) against
 194 horseradish peroxidase (HRP) were used. The advantage of the latter is its antigen, which can be
 195 easily determined in a microtitration plate (MTP) format and hence is ideally suited for screening
 196 purposes. Protein G (or A) were adsorbed to the MTP, washed and subsequently activated with the
 197 respective crosslinker. Any excess of the reagents was easily removed by washing steps; this is a big
 198 plus of any heterogeneous format. Furthermore, this setup simplifies any pH variation by complete
 199 buffer exchange. After the activation step, the antibody was added in a suitable conjugation buffer.
 200 After a suitable conjugation time, the conjugation yield was determined by elution of the antibody
 201 by acidic buffer (glycine/HCl). Any non-conjugated antibody will be released; the conjugated fraction
 202 will stay immobilized on the plate surface. In the next step, horseradish peroxidase was added and
 203 incubated. After the next washing step, a chromogenic substrate based on tetramethylbenzidine and
 204 hydrogen peroxide were added. After a suitable development time, the color reaction was stopped
 205 by acid and absorbance was measured with an MTP-reader. This assay (Fig. 2) was designed for the
 206 convenient examination of the preactivation crosslinking procedure.
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210 Fig 2: Crosslinking assay for the screening of potential crosslinkers (blue: Protein A or G, grey:
 211 anti-horseradish peroxidase antibody, orange: crosslinker, orange with red rim: protein-protein
 212 crosslink, orange with black rim: intramolecular or half crosslink, green: horseradish peroxidase
 213 (antigen), yellow: chromogenic product. A: Coating with Protein G, B: Preactivation with the
 214 crosslinker, C: Antibody binding to Protein G, D: Formation of crosslink, E: Binding of antigen
 215 (enzyme), F: Enzymatic formation of chromogenic product (washing steps are not shown).
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218 3.2 Crosslinker Screening

219 A considerable number of crosslinkers have been proposed, and quite a few of them are
 220 commercially available. The most frequently used ones seem to be based on N-hydroxysuccinimide
 221 (NHS) chemistry or their sulfo-derivatives [40-42], targeted against the ϵ -amino group of the lysine
 222 side chain and the N-terminus of a peptide or protein. We have chosen a series of crosslinkers based
 223 on different chemistries for a prescreening. There are several criteria, which are relevant for the
 224 selection of a suitable crosslinker, for example, the chemical reactivity, the linker length and
 225 flexibility, the hydrophobicity, the solubility and stability of the reactive groups in aqueous buffers,
 226 their pH preference and many more. In Table 1, a list of crosslinkers is shown, which have been used
 227 for the screening.

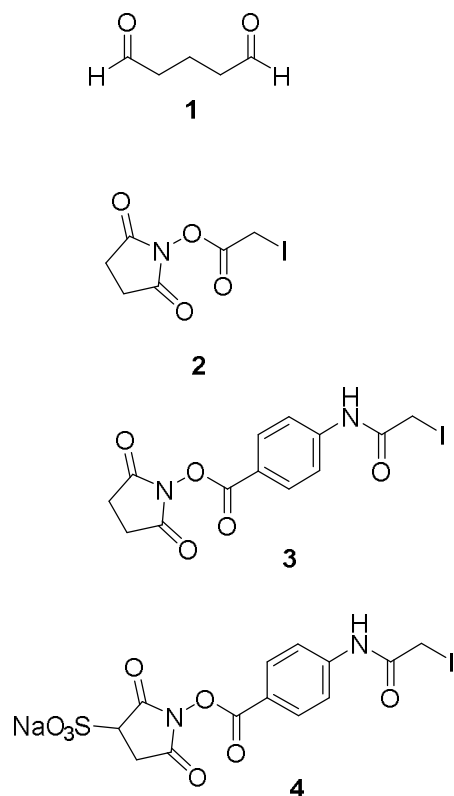
228 Table 1: Compounds used for the preactivation crosslinker screening with Protein G at pH 7.4.
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Crosslinker	Abbr.	Efficiency
Formaldehyde [43]	FA	-
Disuccinimidyl tartrate [44]	DST	-
Tris(hydroxymethyl) phosphine	THP	-
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide [45] / N-Hydroxysuccinimide	EDC/NHS	-
Bis(sulfosuccinimidyl)suberate [44]	BS3	+
1,3-Butadiendiepoide	BDDE	+
Glutaraldehyde [46-48]	GA	++
Succinimidyl iodoacetate [49]	SIA	++
Succinimidyl (4-iodoacetyl)aminobenzoate [44]	SIAB	+++
Sulfosuccinimidyl (4-iodoacetyl)aminobenzoate [44,50]	Sulfo-SIAB	+++

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231 In Table 1, the results are summarized as – to +++, where – stands for no crosslinking and +++ denotes
 232 a very high signal caused by crosslinked Protein G/antibody complex. It has to be noted that many
 233 crosslinkers, which are perfectly suitable for normal crosslinking protocols (reaction with the
 234 preformed complex), such as BS3, are not or only weakly active in the new format. In the novel
 235 preactivation format only a few crosslinkers have proven to be suitable: Particularly glutaraldehyde
 236 (GA) [46,51,52], succinimidyl iodoacetate (SIA) [49], sulfosuccinimidyl iodoacetate (sulfo-SIA),
 237 succinimidyl (4-iodoacetyl)aminobenzoate (SIAB) [44] and sulfosuccinimidyl (4-
 238 iodoacetyl)aminobenzoate (sulfo-SIAB) [50] can be recommended. All further experiments have been
 239 focused on these pre-selected reagents (Fig. 3).

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242 Fig. 3: Chemical structures of crosslinkers, which have been found particularly suitable for
 243 preactivation protocols: Glutaraldehyde (1), Succinimidyl iodoacetate (2), Succinimidyl(4-iodoacetyl)
 244 aminobenzoate (3), Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (4).

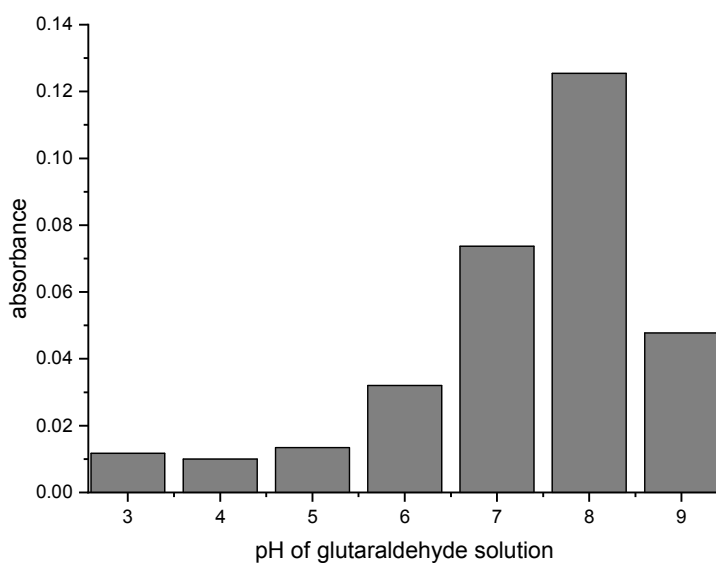
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246 3.3 Influence of pH on the crosslinking of Protein G with murine IgG₁

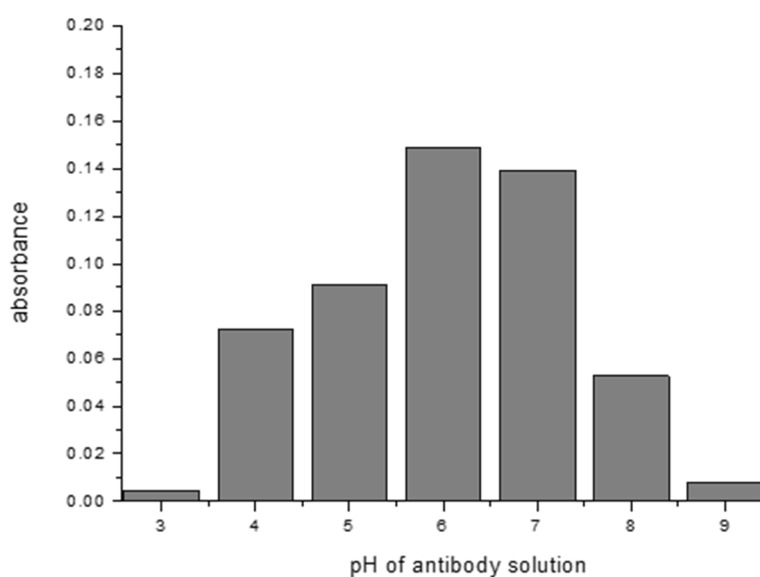
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248 Most crosslinking reactions are highly pH dependent. This was explored with the examples
249 glutaraldehyde/mouse IgG₁/Protein G, and SIAB/mouse IgG₁/Protein G. For glutaraldehyde, it could
250 be shown that a pH of 8 seems to be optimal for preactivation (Fig. 4). This means that a standard
251 buffer, such as PBS of a pH 7.4 is a suitable option. In the second step, the addition of the mouse IgG₁,
252 a pH of 6 seems to be preferable (Fig. 5). This might be dominated by the binding optimum of the
253 murine IgG₁/Protein G pair, which has been determined as pH 4-6 [53,54]. In addition, it was
254 observed that increasing the incubation time of the glutaraldehyde leads to increasing immobilization
255 yields (data not shown).

256 (A)



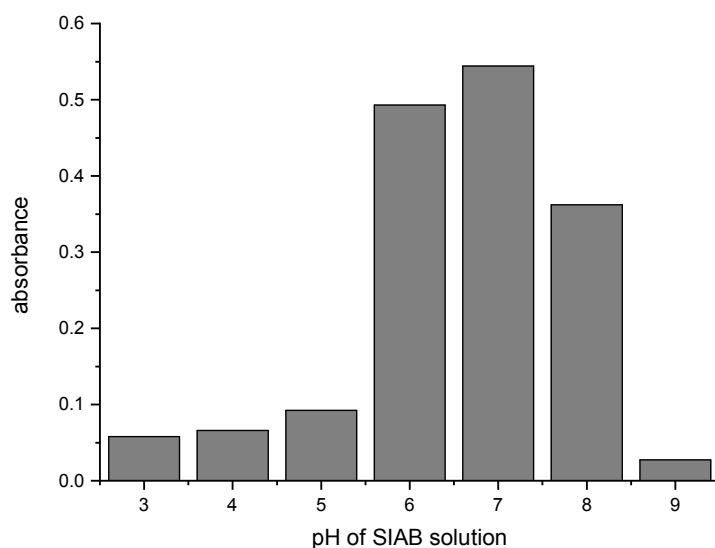
257 (B)



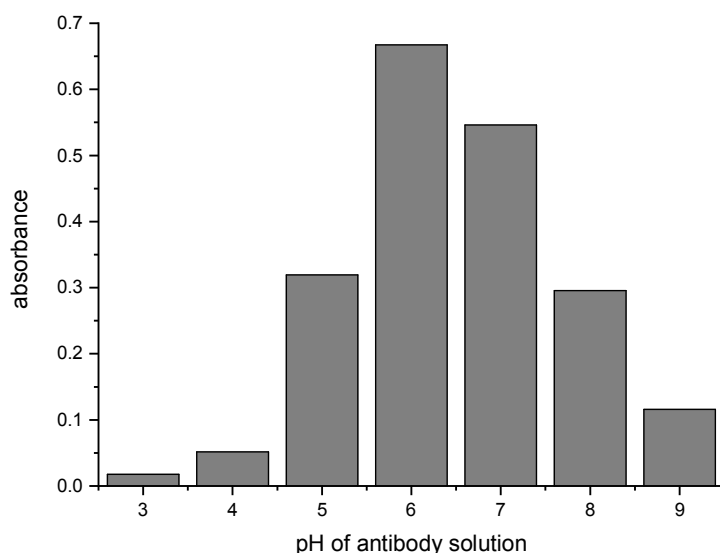
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259 Fig. 4 (A): Protein G: pH influence of glutaraldehyde solution on the preactivation immobilization of
260 mouse IgG₁. (B): pH influence of antibody solution in the same experiment.

261 For SIAB a pH around 7 was found to be optimal for preactivation (Fig. 6). This also means that a
 262 standard buffer, such as PBS pH 7.4 might be a good option. Similar to the situation with
 263 glutaraldehyde, a pH of 6 was found to be preferable (Fig. 7) for the antibody (mouse IgG1). In
 264 experiments with Protein A, a preferable pH of 7.4 was found (not shown), in accordance with the
 265 reported IgG/Protein A optimum. This also supports the notion that not the linker, but the protein-
 266 protein interaction governs the second step. This has to be taken into consideration when new
 267 crosslinking pairs should be explored.
 268 (A)

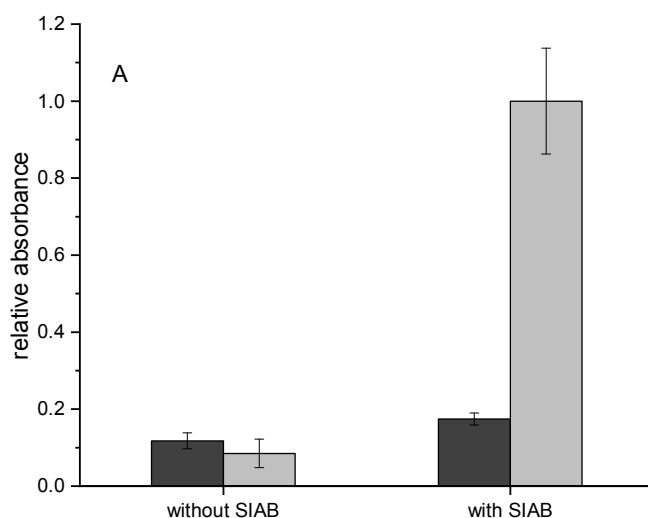


269 (B)
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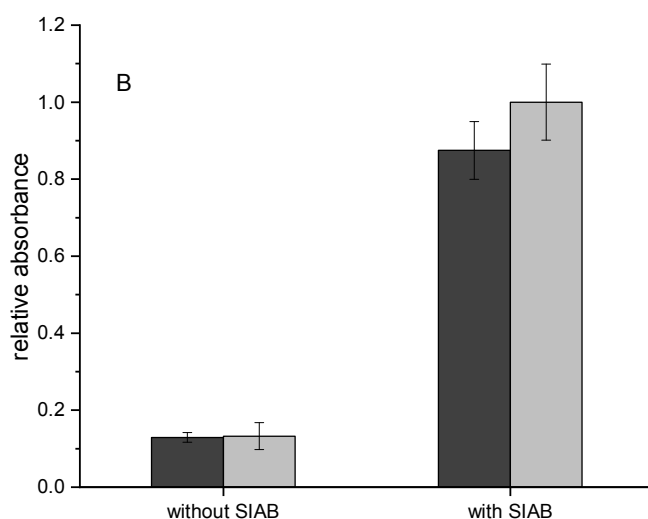


271 Fig. 5(A): Protein G: pH influence of SIAB solution on the preactivation immobilization of mouse
 272 IgG₁. (B) pH influence of antibody solution in the same experiment.
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275 In the next step, the sequential crosslinking with the systems Protein A and Protein G in combination
 276 with murine IgG₁ and human IgG was examined. In the case of IgG₁ from mouse, a monoclonal
 277 antibody against horseradish peroxidase was used as a model system. In Fig. 8A it could be shown
 278 that Protein A/IgG₁ leads to a much lower signal than Protein G/IgG₁. In the case of human IgG,
 279 Protein A and Protein G lead to very similar immobilization results (Fig. 8B).



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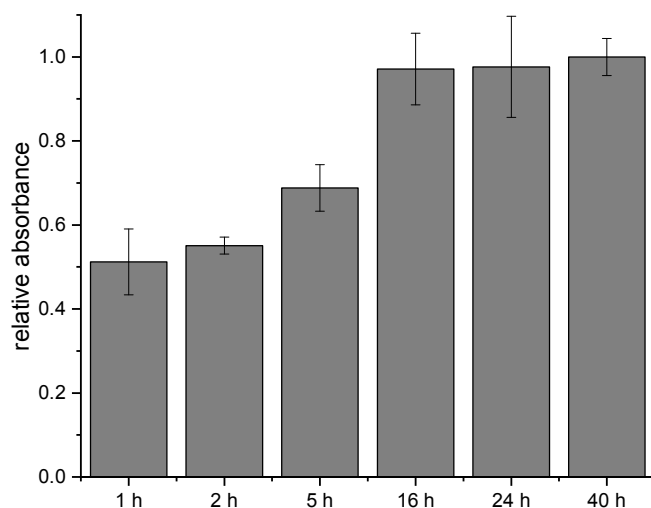
Fig. 8A: Preactivation immobilization of *mouse* IgG₁ with Protein A (dark grey) or Protein G (light grey), 8B: Preactivation immobilization of *human* IgG with Protein A (dark grey) or Protein G (light grey).

The species specificity of Protein A, G, and other IgG binding molecules had been explored in detail [55]. Hence, it is well-known that mouse IgG₁ binds only weakly to Protein A, in contrast to human IgG, which is a strong binder. These properties could be confirmed in our system. This is also clear support of the selectivity of this crosslinking procedure. If the crosslinker alone would be responsible for the immobilization, no such behavior would be expected. Furthermore, the addition of BSA to the antibodies did not influence the immobilization significantly (data not shown). This also substantiates the highly selective mechanism and contradicts any simple protein crosslinking hypothesis. In this case, any presence of any irrelevant protein should heavily compete with the desired immobilization process, which is a frequent problem in conventional immobilization procedures.

298 3.4 Incubation time of SIAB-activated Protein G with mouse IgG₁

299

300 In the next experiments, the time-dependency of the crosslinking process with SIAB was explored. It
301 could be shown that some of the crosslinkers seem to bind relatively fast, in contrast to others, which
302 need several hours to reach a maximum signal. After one hour of antibody incubation, about 50% of
303 the maximum was achieved already. After 16 hours, the signal doubled. Further extension of the
304 incubation time did not increase the response anymore. The non-linear increase indicates that at least
305 two different rate constants, and hence two different crosslinking sites may be involved. In general,
306 24 hours should be more than sufficient to reach a maximal signal (Fig. 9).



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308 Fig. 9: Influence of the incubation time on the SIAB-based preactivation immobilization of mouse
309 IgG₁ on Protein G.

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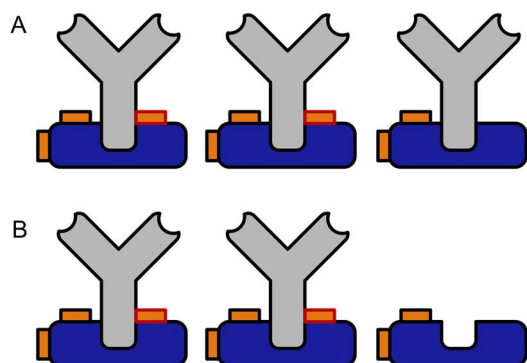
311 3.5 Influence of solvents on the crosslinking process

312 The water solubility of different crosslinkers varies widely. Particularly, SIAB is poorly water soluble.
313 Hence, concentrated SIAB solutions cannot be prepared in the usual buffers. Hence, SIAB should be
314 pre-dissolved in organic solvents such as methanol or DMSO. Interestingly, we found that DMSO
315 leads to more efficient activation of Protein G than methanol. With 40% of DMSO, a concentration of
316 only 0.25 mM of SIAB is sufficient to obtain a maximum signal in the model system. In contrast, with
317 40% of methanol, more than 2.5 mM of SIAB is necessary (data not shown). This leads to the
318 conclusion that the activation of Protein G with SIAB should be preferentially performed in PBS pH
319 7.4 with 40% of DMSO. In the case of SIA, the exceptionally high reactivity even with methanol has
320 to be taken into consideration [49]. For SIA, a stock solution in acetonitrile seems to be preferable.

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322 3.6 Crosslinking yield of the Protein A/G IgG system

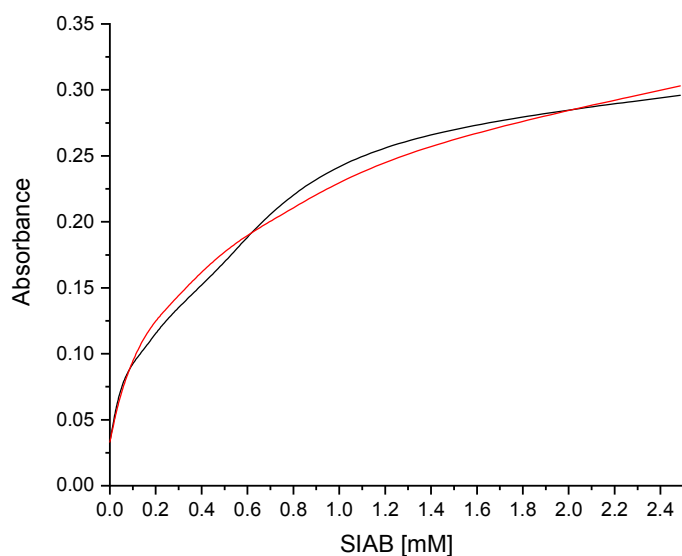
323 The crosslinking yield of the method was determined by an additional dissociative elution step with
324 acidic glycine/HCl buffer, which is a proven approach to elute IgG from Protein A or G columns. Any
325 non-crosslinked IgG should be lost during this step, leading to a loss of binding capacity. Fig. 10
326 shows a schematic representation of this test.



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328 Fig. 10: Elution test for the determination of the crosslinking yield. (A): Bound antibodies before the
 329 elution step (B): Bound antibodies after the elution step. Any non-crosslinked antibody is lost in a
 330 subsequent washing step.

331 In contrast to most photochemical crosslinking protocols, the crosslinking yield with SIAB seems to
 332 be quantitative (Fig. 11), at least in systems of sufficient binding strength of the protein-protein
 333 complex. However, even in the case of incomplete crosslinking, a simple pre-elution step easily gets
 334 rid of any traces of non-crosslinked antibody. This avoids leakage of antibodies into the affinity-
 335 purified sample.



336

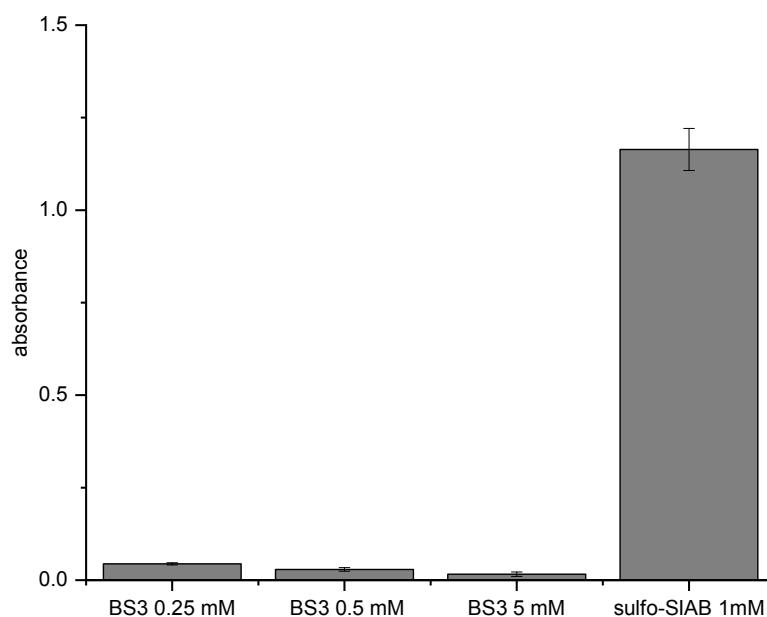
337 Fig. 11: Examination of the crosslinking yield of SIAB-activated Protein G with the recombinant
 338 antibody Herceptin. Residual non-crosslinked human IgG was eluted with a glycine/HCl buffer at
 339 pH 2.2 (black: before elution, red: after elution). The crosslinking yield of the protein G/ human IgG
 340 system was apparently quantitative.

341

342 3.7 Comparison of the efficiency of the traditional and the novel immobilization method

343 A traditional crosslinking protocol with the reagent bis(sulfosuccinimidyl)suberate (BS3) was
 344 compared to the proposed 2-step (preactivation) method based on sulfo-SIAB (Fig. 12). It is evident
 345 that the novel method leads to a much higher signal in this model assay. Besides, the experiment
 346 shows that a higher concentration of BS3 leads to lower signals, which is most likely caused by the
 347 unwanted chemical modification of the antibody binding site. The used concentration range of BS3

348 is based on the manufacturer's recommendation. The concentration of sulfo-SIAB in the preactivation
349 step was derived from our optimization experiments.



350 Fig. 12: Comparison of the traditional with the novel immobilization method. BS3: Post-crosslinking
351 of Protein G/antibody complex according to the manufacturer's recommendation [56], sulfo-SIAB:
352 Preactivation of Protein G with the subsequent addition of the antibody. Any non-crosslinked
353 antibody was removed by elution buffer (glycine/HCl pH 2.2).
354

355 4. Discussion

356 It could be shown that some known crosslinkers can be used in a novel, 2-step protocol for
357 oriented antibody immobilization. Up to now, protein G/IgG or protein A/IgG complexes have been
358 treated with crosslinkers *after* the protein-complex had been formed, which inevitably leads to
359 chemical changes in and near the variable region of the antibody, which is critical for selective
360 binding and preservation of binding capacity. In our approach, Protein A or G is chemically pre-
361 activated by an excess of homo- or heterobifunctional reagents. This did not eliminate the bioselective
362 interaction between Protein A or G and the immunoglobulin. It can be assumed that this 2-step
363 reaction is generally applicable for most immunoglobulins, which have some affinity to Protein A or
364 G. A further advantage of this approach is the flexibility of the conjugation conditions, such as pH,
365 salt concentration, additives and so on. The resulting conjugate should show no loss of binding
366 capacity by the chemical crosslinking step since any covalent bonds are restricted to the Fc part of the
367 antibody far away from the antigen binding site. Also, it can be assumed that no optimization of the
368 conjugation should be required for known IgG subclasses since the regions involved in binding to
369 Protein A or G are highly conserved. We noticed that the presence of other proteins (such as albumin)
370 did not significantly influence the conjugation efficiency and hence, neither a pre-purification nor
371 preconcentration of the antibody or serum is necessary. Even very raw or diluted antibody
372 preparations might be used directly for the conjugation, which is in strong contrast to common
373 products with pre-activated surfaces.

374 This selective and covalent immobilization protocol should be useful in many fields: The
375 preparation of immunoaffinity columns, magnetic beads, the coating of nanoparticles, such as
376 quantum dots or gold particles, the activation of glass or other slides for microarray technology, the

377 robust coating of immunosensor surfaces, the oriented and irreversible immobilization of antibodies
378 on microtitration plates and even homogeneous variants, such as the labeling of antibodies might be
379 feasible. It should be stressed that in contrast to most other recent concepts, neither the production of
380 genetically modified proteins [57] nor the introduction of synthetic amino acids is required. Most
381 buffers, preservatives or protein additives do not limit the applicability of this approach. However,
382 the transfer of this protocol to other biochemical binding pairs has still to be explored.

383 This approach might be even useful for crosslinking experiments in solution, which are highly
384 popular in proteomics [58] and structural biology [59,60]. All experiments, which are performed with
385 traditional thermal or photochemical crosslinkers today, could be alternatively performed with
386 protocols analogous to those presented here. The crosslinking site might be more restricted and hence
387 better to control.

388 Regarding the reaction mechanism, it seems to be evident that crosslinkers suitable for this
389 approach need at least one active group, which does not hydrolyze or otherwise deactivate too fast.
390 Therefore, bifunctional NHS esters [41] seem to be suboptimal. In contrast, haloacetyl-residues, such
391 as SIA or SIAB possess a good balance between stability and reactivity towards nucleophiles. We
392 assume that at neutral pH values, primarily histidine residues are involved in the crosslinking with
393 haloacetyl groups, in contrast to the mechanism with glutaraldehyde, which should be dominated
394 by lysines [48]. Due to the slow reaction, even complexes with a relatively low affinity at low protein
395 concentrations may be accessible, in contrary to photochemical groups, which have very short
396 reactive lifetimes and hence often low reaction yields [27] with various side reactions. Finally, we
397 want to stress that all required reagents necessary for this novel approach are commercially available
398 from standard suppliers. Considering the much lower signals obtained with the old method, in most
399 applications the proposed approach should be preferred.

400

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406 **Conflicts of Interest:** The authors declare no conflict of interest.



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