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Research Notes

Functionalization Strategies for Antibodies: Lessons Learned

[1]Christian Schmidt, [1]Martin Geyer, [1]Jörg Bohrisch, [1]Verena Jentzen, [1]Alexander Gorczyza, [1]Karim Khalil, [1]Marina Volkert, [2]Mark A. Brown, [3]Saadettin Sel and [1]Joachim Storsberg

[1]Fraunhofer-Institute for Applied Polymer Research (IAP), Division of Life Science and Bioprocesses, Department of Biomaterials and Healthcare, Potsdam-Golm, Germany
[2]Department of Clinical Sciences and Colorado School of Public Health, Colorado State University, Fort Collins, CO, USA
[3]Department of Ophthalmology, Heidelberg University, Heidelberg, Germany

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Corresponding Authors:
Christian Schmidt and Joachim Storsberg
Department of Biomaterials and Healthcare, Division of Life Science and Bioprocesses, Fraunhofer Institute for Applied Polymer Research (IAP), Potsdam-Golm, Germany
Email: Christian.schmidt@iap.fraunhofer.de, joachim.storsberg@iap.fraunhofer.de

Abstract: Precious little is known about useful functionalization strategies of antibodies that will not impair either reactivity or specificity of this valuable reagent. We used an experimental approach to demonstrate our own experience with published protocols. The conjugates obtained were then tested with regard to their performance against commercially available detection kits. Our results are then viewed in light of published precedence to highlight areas where future effort is needed to refine such versatile tools.

Keywords: Chemistry, Immunology, Antibody, Reactivity, Specificity

Introduction

The emergence of antibodies as a reasonably economic solution to the conundrum faced by an organism of having to distinguish 'self' from invading pathogens provides the scientist with a versatile tool in pushing the boundaries of what and, equally important, how much, can be detected (Della Ventura *et al*., 2017). Most of these techniques, however, hinge on means to, say, immobilizing antibodies on a cantilever, or, to bring forth another versatile method, use enzymatic activities to indirectly localize recognized antigen; leaving aside issues of primary, secondary or tertiary detection schemes.

There is no gold standard when it comes to labeling a given molecule. For instance, metabolic labeling using radioisotopes is associated with the risk of inducing DNA fragmentation (Hu *et al*., 2001), whereas employment of non-canonical amino acids is most likely to alter the properties of the peptide (Johnson *et al*., 2010). As for immunoglobulins, the criteria are, to some extent, less stringent, provided that neither reactivity, nor specificity, or strength of recognition of the cognate antigen is altered as a result of the conjugation scheme. This leaves, for illustration, the approach of conjugating an antibody to nucleic acids to create a 'multianalyte immunoassay' within the realm of avenues to consider (Hendrickson *et al*., 1995).

As for wet chemistry on antibodies, the first consideration is directed to available reactive groups, primary amino groups, such as lysine residues and the N-terminus of the molecule, or, alternatively, a conveniently located cystine (Fig. 1), or, to name yet another strategy, primary carboxyl groups, e.g., glutamic acid and C-termini, even though those routs require elaborate intermediate steps, are seldom associated with high yield in comparison to the amino-functionalization and are omitted in further discussions (van Vught *et al*., 2014).

While it would seem prudent to consider that N-Hydroxysuccinimide (NHS) esters at first, their instability in aqueous media indicates their reactivity and suitability on the one hand, while, at the same time, achieving appropriate amounts of this reagent within close proximity of an antibody molecule within a cage of solvent (Stillinger and Rahman, 1972) may add further complexity to establishing batch-to-batch reliability of conjugated antisera.

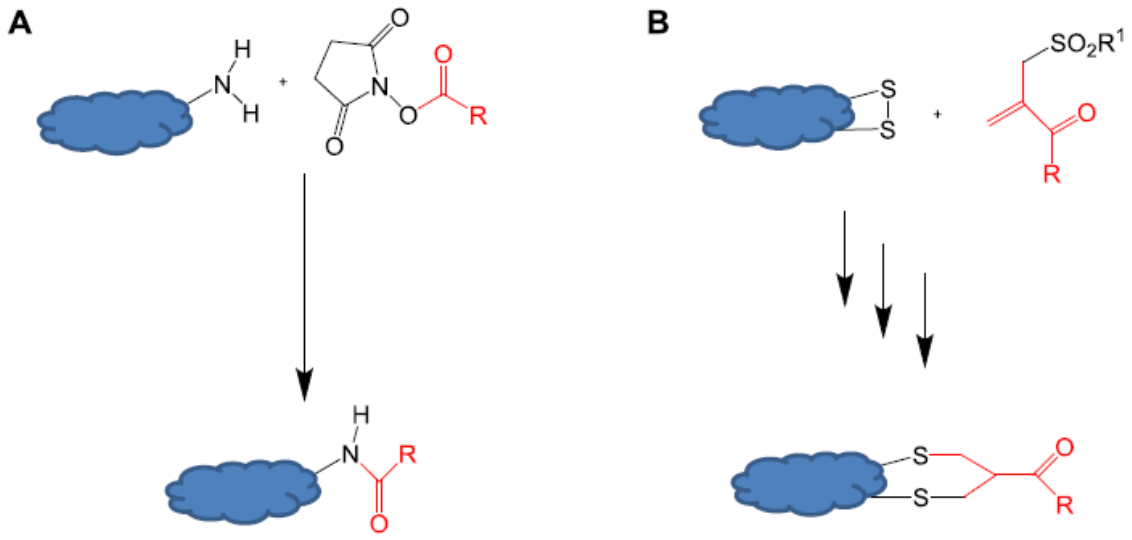

Fig. 1. Schematic illustration of two functionalization strategies of a peptide (depicted as a cloud) using (A) a free and primary amino-group and N-hydroxysuccinimide ester, or, (B) an available cystine and a monosulfone-enone reagent to transfer a substitution, indicated by the residue R in red

Alternatives to the above-described scenario, using, for instance cystine(s), appear less attractive because of an apparent vulnerability of γ species to structural and functional alterations a result of chemical alterations of their native disulfide bond related structural appearance (Liu and May, 2012). Considering that γ serves as backbone of licensed, used and newly evolving therapeutic antibodies (Irani *et al*., 2015), the sheer market penetration of γ would also add to the caution with regard to altering the structure and, at least by inference, the functionality of γ-based antisera.

Here, we report results of our own experimentation and discuss the results in the context of published precedence. To strengthen our arguments, we used our conjugates together with established and commercially available detection systems, based on the DAB staining method (Seligman *et al*., 1968). Our results show regardless of the means of detection, be it a newly synthesized system or avidin-biotin-based systems, robust and specific indication of proliferative activity in germinal centers in tonsillar tissue.

Results and Discussion

All molecular biology grade reagents, except antibodies, were obtained from Sigma Aldrich Biochemie GmbH, Hamburg, Germany. Commercially available kits for DAB-based histology and paraffin embedded sections of tonsillar tissue were graciously provided by Quartett GmbH, Berlin, Germany. Affinity purified γ (goat anti rabbit or goat anti mouse IgG(H+L)) were obtained from Dianova GmbH, Hamburg, Germany, whereas monoclonal anti-Ki-67 (rabbit, #EP5) was obtained from Epitonics, Burlingame, CA, USA, while Horseradish Peroxidase (HRP) was supplied by Kem En Tec (4120 A), Taastrup, Denmark. NMR studies were performed at the IAP facility.

At first, published precedence was used to pursue an activation of antibodies using a Michael-type addition of Di-Vinyl-Sulfone (DVS) to available hydroxyl groups (Ying-Sing *et al*., 2000), with the added convenience that a titration with thiosulfate would allow for the indirect determination of available vinyl-functionalities for further modification (Fig. 2).

Dextran was chosen as backbone for the first modification strategy, termed DVS-Dex. For this, 5 mmol of Dextran (500 kDa) were brought into solution in a total volume of 50 mL PBS and stirred for 10 to 15 min at room temperature, followed by an addition of 3.14 mmol $Na[BH_4]$, followed by a dropwise addition of 24.9 mmol DVS over a time of two minutes under constant agitation. An additional period of 32 min of constant agitation at room temperature followed, before the reaction was terminated by adjustment of the pH to 7 with 6.85 M HCl. The reaction mixture was then dialyzed against water using a dialysis bag with a cut off of 14 kDa until no changes in conductivity in comparison to fresh water was measurable. Subsequent trials to determine free vinyl groups using titration with thiosulfate failed to yield reproducible results. Instead, ^{1}H-NMR (500 MHz; D_2O as solvent) revealed a single substitution of statistically every third glucose unit in the dextran used (Fig. 3).

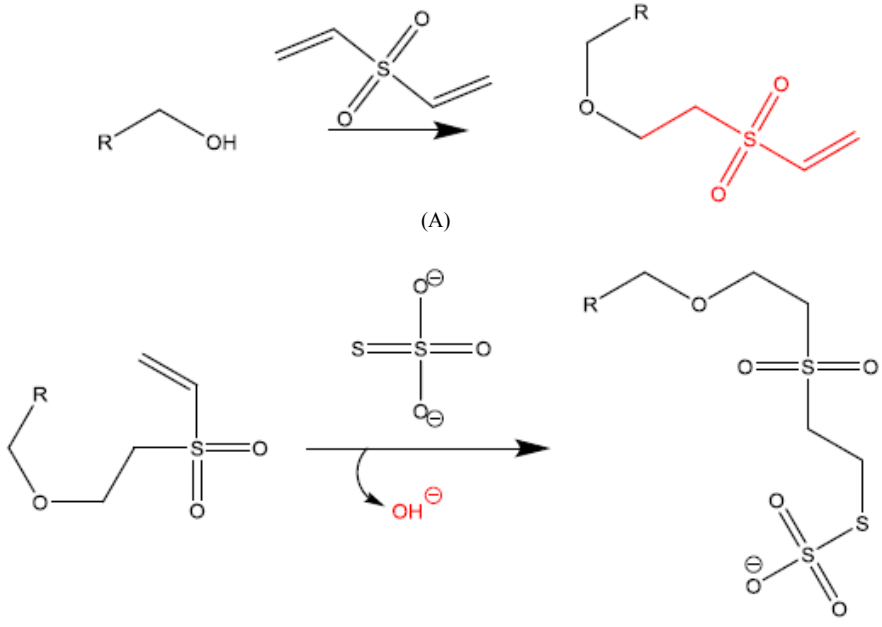

Fig. 2. Schematic illustration of the functionalization of a residue R with available hydroxyl group and di-vinyl-sulfone. As depicted in (A), reaction of an available hydroxyl group with the residue R with di-vinyl-sulfone yields a functionalization with one free vinyl group for additional modification. The scheme illustrated in (B) illustrates the reaction of free vinyl groups in the product of reaction (A) with thiosulfate, resulting in the release of hydroxyl ions, which can be utilized to determine freely available vinyl groups

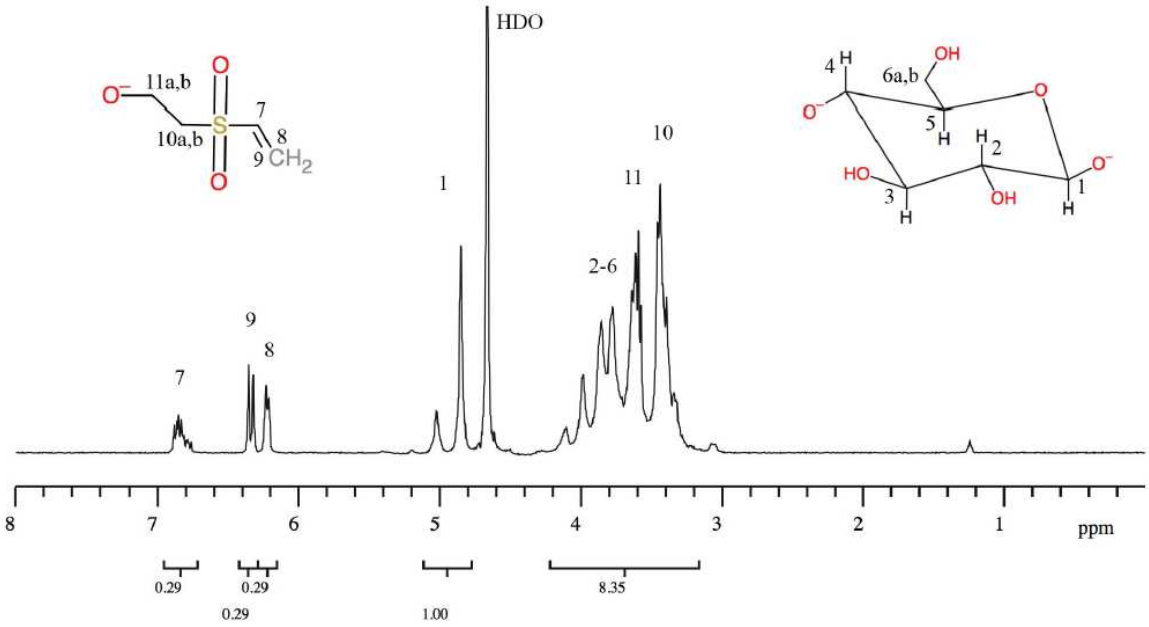

Fig. 3. On average, roughly one in every three glucose units in the dextran used was modified with a vinyl-function. Shown here is a representative 1H NMR analysis (500 MHz) with heavy water (D_2O) as solvent and plot of the chemical shift δ in ppm in reference to tetra-methyl-silan $(CH_3)_4$ Si. Inserted are structural formulas of a glucose unit from dextran and di-vinyl-sulfone with non-equivalent hydrogen atoms numbered, whilst equivalent hydrogen atoms are indicated with lower-case letters. The following chemical shifts are observed: δ = 6.83 (ddd, $^3J_{7,9}$ = 16.5, $^3J_{7,8}$ = 10.3 Hz, 0.3H, H-7), 6.34 (d, $^3J_{9,7}$ = 16.6 Hz, 0.3H, H-9), 6.22 (dd, $^3J_{8,7}$ = 9.6, $^2J_{8,9}$ = 3.4 Hz, 0.3H, H-8), 4.94 (d, 1H, H-1), 4.12-3.69 (m, 5H, H-2, H-3, H-4, H-5, H-$6_{A,B}$), 3.69-3.55 (m, 2H, H-$11_{A,B}$), 3.50-3.30 (m, 2H, H-$10_{A,B}$)

The 1H NMR was measured at a frequency of 500 MHz in deuterium oxide. The CH-acidic protons of the glucose hydroxyl groups are replaced by deuterium, resulting in an HDO signal. Evaluation of the NMR result was eased by disregarding signal splitting caused by magnetic interactions of other, non-equivalent protons in the glucose unit of Dextran. The protons of the system CH_2 = CH-SO_2- are detected at a chemical shift between 6 to 7 ppm with multiplicity and coupling constants according to theory with the protons appearing to be distinctly separated from one another. Magnetic interactions (cis: $^3J_{8.7}$ = 9.6 Hz; trans: $^3J_{7.9}$ = 16.5 Hz; germinal: $^2J_{8.9}$ = 3.4 Hz) can be detected in this system in accordance with predications. Furthermore, the anomeric proton (H-1) of the dextran molecule appears, as predicted, not to be superimposed on the HDO signal. The other signals of the sugar protons (H-2, H-3, H-4, H-5, H-$6_{A,B}$) overlap with one another and are detected as multiples' in the range from 4.12 to 3.69 ppm. The two methylene protons H-11A, B and H-$10_{A,B}$ are also found as multiples' in the ^{1}H NMR spectrum (Fig. 3).

On the basis of the normalization of the integral of the anomeric H-1 proton, the Degree of Substitution (DS) of 0.3 can be calculated from the integral for the system CH_2 = CH-SO_2. Expressed alternatively, on average, every third glucose unit in the dextran used was modified with a single a vinyl group. Given that the dextran used had an average molecular weight of 500 kDa, of the circa 2700 glucose units in a given dextran molecule, around 900 vinyl groups are available for further functionalization with Horseradish Peroxidase (HRP) and then coupled with immunoglobulin, in this case γ (Fig. 4).

The enzymatic activity of the HRP was determined colorimetrically and expressed as Units (U) of enzyme according to Ferrari *et al*. (2014), who define one unit of HRP as the quantity of material, capable to generating 1 mg of purpurogallin from pyrogallol at 20°C and pH 6 in a time span of 20 sec (Fig. 5).

The results of the measurement of the enzymatic activity of the HRP are shown in Fig. 6. Here, the optical density of the generated purpurogallin at 420 nm is plotted against time in five independent experiments. We used a 100 mM phosphate buffer (pH 6) and 100 µg HRP at 20°C room temperature. A calibration curve was generated using the optical density of purpurogallin at 420 nm. According to literature, a time point of 20 sec lies within a linear range of the reaction graph and was, therefore used to determine the enzymatic activity of the HRP. Our experimentation revealed an averaged enzymatic activity of 425±25 U per mg of solid powder. Of note is that the manufacturer denoted an activity of 268 U/mg, which, according to our own measurements, is a very conservative estimate (Fig. 6).

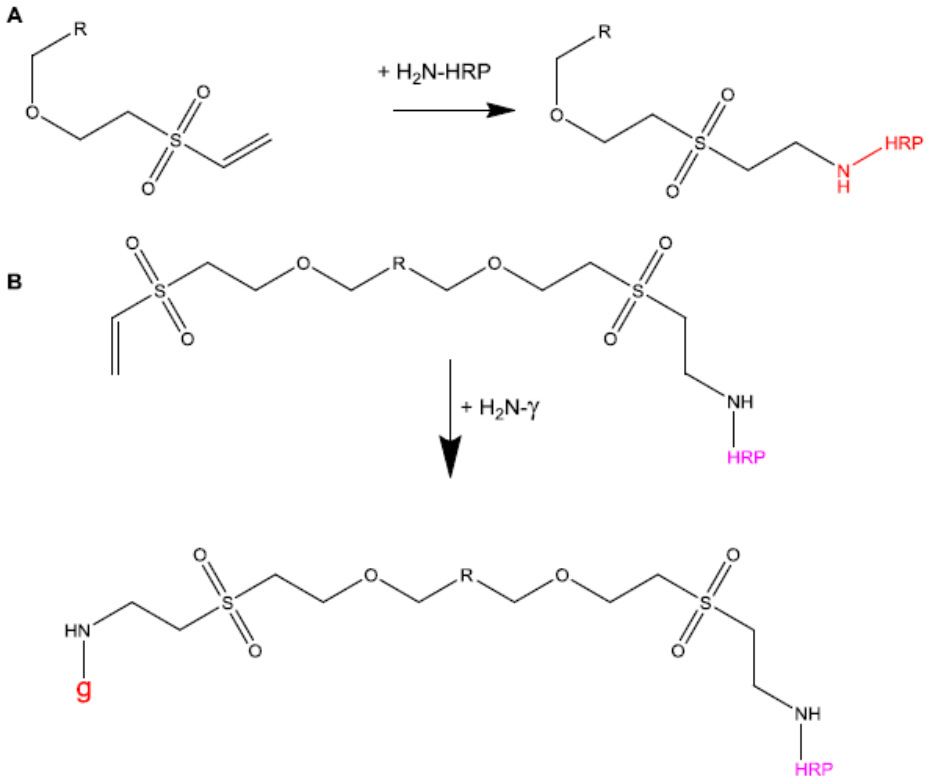

Fig. 4. Graphical illustration of the functionalization strategy of a multivalent residue R consecutively with di-vinyl-sulfone, followed by coupling of HRP (panel A) and utilization of available vinyl-functions to add g immunoglobulin (panel B)

Fig. 5. Schematic illustration of the HRP-catalyzed transformation of pyrogallol to purpurogallin. The optical density of purpurogallin was measured to establish a calibration curve for our assays

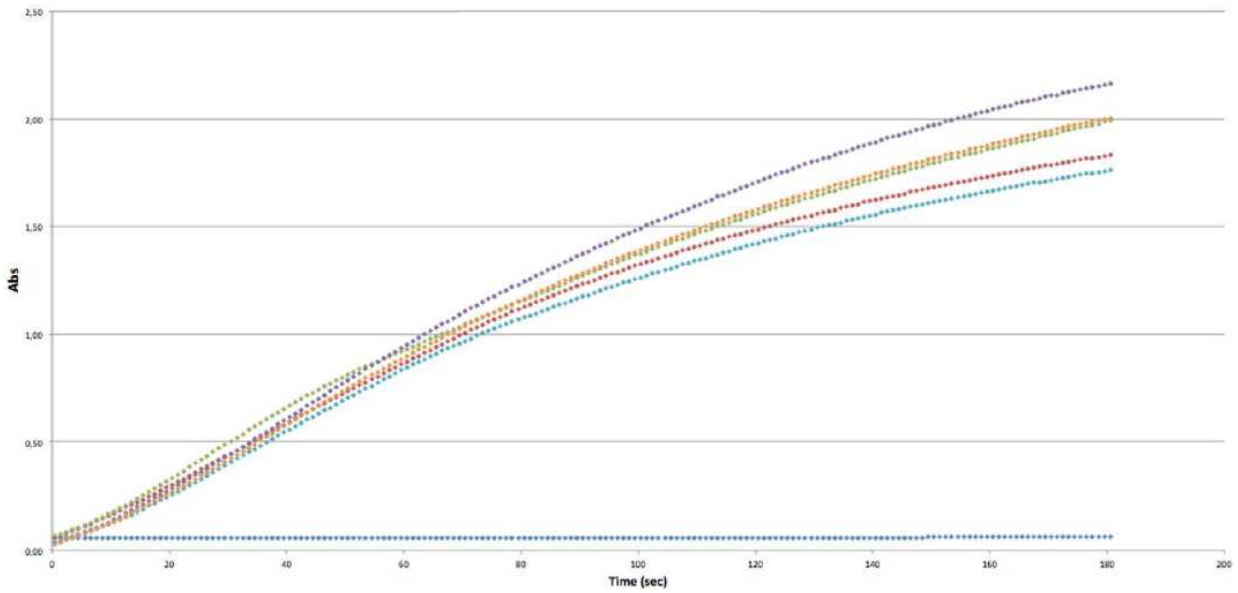

Fig. 6. Plot of the optical density of purpurogallin (abbreviated as Abs) over time (in seconds) as a result of the HRP-catalyzed transformation of pyrogallol at pH 6 and 20°C. The dark blue line is the result of measurements of the reaction without HRP, whereas light blue, red, green, yellow and purple lines, respectively, show the result of individual experiments

For the coupling of DVS-DEX with γ, we added to 100 µL of both, anti-rabbit and anti-mouse, (≙ 230 µg of each γ species) 158 µL DVS-DEX (≙ 2.64 mg) and 558 µL of 500 mM PBS pH 10, yielding a total of 916 µL. The mixture was incubated for 20 h at 30°C in an incubator. The reaction was stopped by an addition of 16.75 mg of glycine and spun at 12,000 rpm for 10 min at 4°C. The resulting pellet was washed twice with 500 mM PBS pH 10 and used immediately for immunohistochemistry using anti-Ki67 and paraffin-embedded tonsillar cancer tissue using standard staining techniques, including antigen retrieval and nuclear staining with Hematoxylin (Hasui *et al*., 2002).

With a constant dilution of anti-Ki67 of 1/100, a commercial detection system was used as reference for evaluation of the generated system (Fig. 7 and 8). As shown in Fig. 7, the reference system (Dako, Envision; Hasui *et al*., 2002) yielded a robust staining of ellipsoid areas, with the synthesized system resulting in a similar pattern, albeit lower in intensity (Fig. 8). We conclude that a simple centrifugation of the reaction mixture and washing with reaction buffer was sufficient to generate a system, using the chemistry described above, that can produce staining patterns similar to a commercially available detection kit, albeit lower in intensity.

Microscopic analysis was performed using an Olympus CKX41 with a Peltier-cooled digital camera and an automatic generation of scale bars using internal computation using the magnification used in the respective setting. Automatic correction for exposure (brightness and contrast) was chosen to generate images with comparable brightness, contrast and magnification, to assist in analysis and comparison of the staining results.

Tonsillar tissue was selected because of a highly proliferative area, the germinal center, with an area of low proliferative activity surrounding germinal centers (Soares *et al*., 2004; Cattoretti *et al*., 1992). This stark contrast in those two areas was seen as a suitable study object to assay for precision, accuracy and sensitivity of the newly synthesized detection kit in comparison to a commercially available alternative.

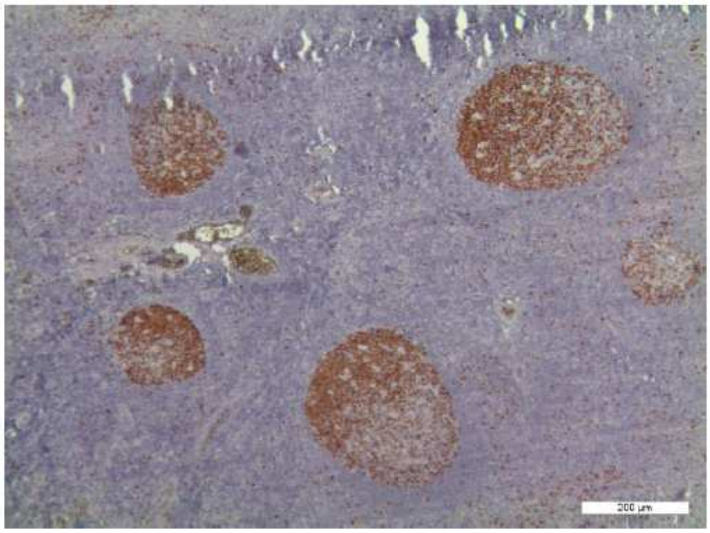

Fig. 7. Robust staining of proliferative active centers using the reference kit from Dako. Shown here are representative photograph of an anti-Ki67 stained tonsillar tissue using antigen retrieval, DAB chromogenic development and counterstain with Hematoxylin

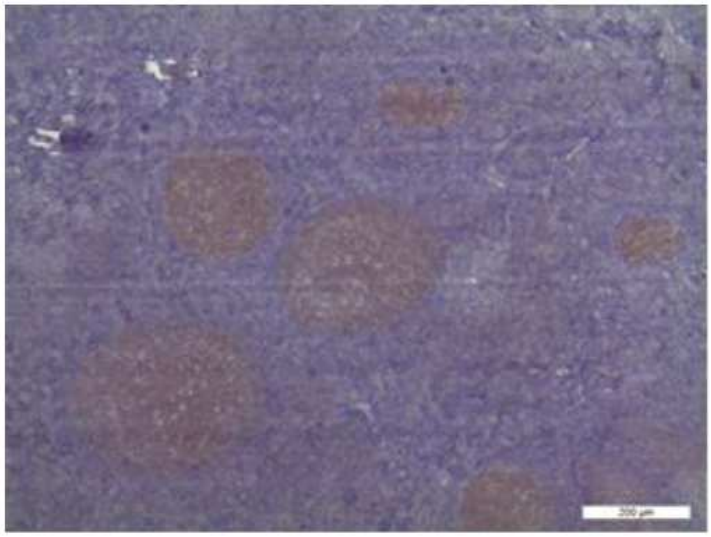

Fig. 8. Robust staining of proliferative active centers using the self-synthesized detection kit. Shown here are representative photograph of an anti-Ki67 using DAB in analogy to Fig. 7

Against the background that Ki-67 serves as marker for proliferating cells, it is in accordance with published precedence that germinal centers are marked with Ki-67 due to a considerable proliferative activity in germinal centers in tonsils. Against this background, we concluded that our newly synthesized detection system performs comparably with a commercially available system with regard to precision and accuracy of the staining, albeit not with regard of the intensity of the detection.

Next, we wanted to exclude the possibility of false-positive staining, described, for instance, in Buchwalow *et al*. (2011), using the same primary antibody, Ki-67 (see above) and different commercially available staining kits. At first, we used two commercially available detection systems, designated A and B, respectively. Processing the tissue and using the reagents according to the manufacturer's recommendation, including antigen retrieval, proprietary blocking and development reagents, respectively, revealed a detectable staining without primary antibody in either scenario (Fig. 9 and 10). Comparison with our previous results revealed that the only difference between either Dako kit or the newly synthesized system was the absence of what was termed 'antibody enhancer.'

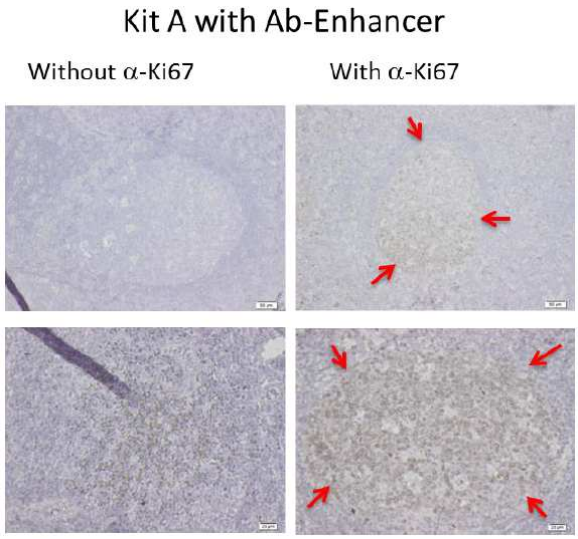

Fig. 9. Diffuse staining of proliferative centers without primary antibodies using a commercially available detection kit A including antibody enhancer (abbreviated as Ab-Enhancer)

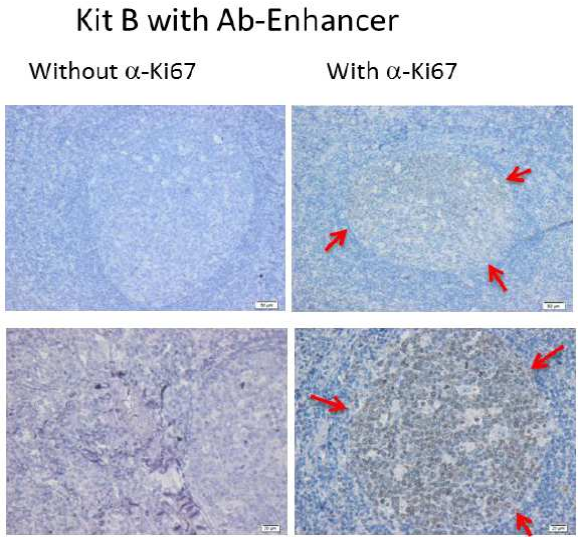

Fig. 10. Diffuse staining of proliferative centers without primary antibodies using a commercially available detection kit B including antibody enhancer (abbreviated as Ab-Enhancer)

Under the conditions of using the commercially available kits A and B, respectively, along with the reagents and protocols provided by the suppliers and those materials alone and identical microscopic settings for documentation of the staining results, our results strongly support the notion that we were unable to discern whether 'antibody enhancer' of kit A were comparable to 'antibody enhancer' of kit B, nor that presence of these materials decreases the probability of false-positive staining results. On the contrary, our results can be used to argue that presence of materials, referred to as 'antibody enhancer' increases the likelihood of false-positive staining results (Fig. 9 and 10). We, therefore, avoided any further use or development of routes that would involve or constitute in any way materials with similar staining properties than the 'antibody enhancer' reagents supplied with the commercially available staining kits A and B.

A free energy 83.68 kJ/mol for the formation of a biotin-avidin complex was calculated by Green (1963). Expressed differently, the dissociation constant for this complex (single binding site) was shown to be 10-15 M by Green (1963). This observation paved the way for usage of this phenomenon for the development of protocols and products for cytology, enzymology and histology (Guesdon *et al*., 1979; Livnah *et al*., 1993). Later studies dampened the enthusiasm by calling to attention endogenous affinity for avidin in human lymphoid tissue, such as tonsillar material (Wood and Warnke, 1981; Banerjee and Pettit, 1984).

To test whether histology involving avidin-HRP yield staining results similar to Wood and Warnke (1981) and Banerjee and Pettit (1984), we prepared biotinylated goat-anti-rabbit with a 4.unit polyethylene oxide linker between the biotin moiety and the Ig molecule according to established protocols to achieve a modification of around 3 biotin residues per Ig molecule using a 4-fold molar excess of biotin in relation to the amount of Ig (Smith, 2006; Newton-Northup and Deutscher, 2017). Freshly prepared biotin-Ig conjugate was used in our histology assays. Commercially available Avidin-HRP was used to assay for presence of an affinity for avidin in tonsillar tissue that had not been subjected to either blocking of endogenous biotin or HRP.

As shown in Fig. 11, our results show no staining of tonsillar tissue with avidin-HRP. This apparent absence of avidin-affinity in the paraffin-embedded tissue seems to be at variance with Banerjee and Pettit (1984). Closer examination of Banerjee and Pettit (1984) reveals that the authors stored the explanted material at -70°C until cryosectioning was performed, followed by methanol-acetone fixation and lyophilization, whereas the material in our study was subjected to formalin fixation and embedded into paraffin. With this argument appearing to be of obvious convenience, neither logic parsimony (Borden and Linklater, 2013) nor evidence presented in this study provides sufficient basis for a conclusion that the different methods of tissue processing are sufficient to explain the phenomenon reported. Further effort is required to resolve this matter.

Consistent with previous experimentation (Fig. 7 and 8) as well as precedence cited (Soares *et al*., 2004; Cattoretti *et al*., 1992), anti-Ki-67 staining resulted in a robust marking of proliferative activity in germinal centers in the tonsillar tissue used (Fig. 12).

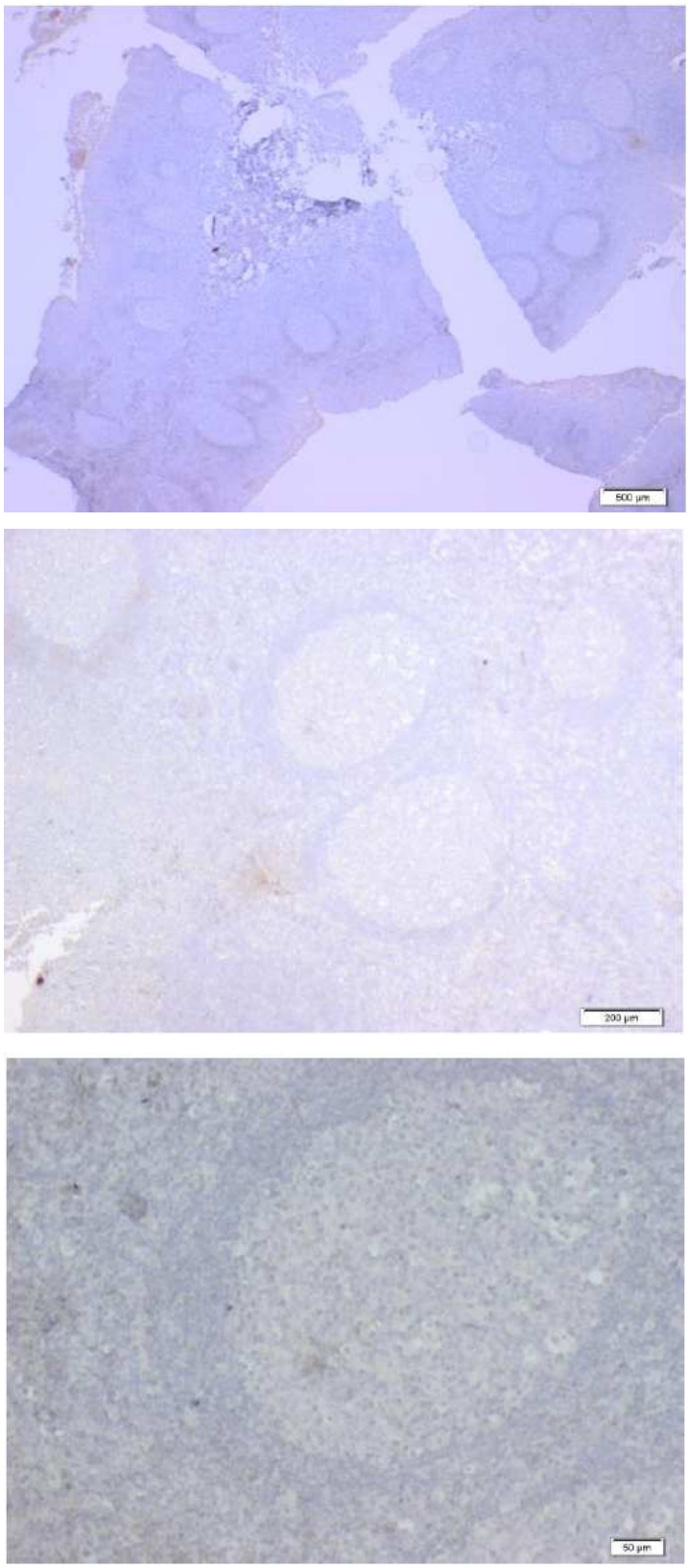

Fig 11. Absence of positive DAB staining of tonsillar tissue with an avidin-HRP conjugate. Shown here are representative photographs using increased magnifications as indicated by the scale bars

Fig. 12. Robust positive DAB staining of tonsillar tissue with high proliferative activity an avidin-HRP conjugate, freshly biotinylated goat-anti-rabbit as secondary antiserum and Ki-67 as primary antibody. Shown here are representative photographs using increased magnifications as indicated by the scale bars

Conclusion

The issue surrounding processing of tissue for diagnostic purposes is described in many publications (Haines and Chelack, 1991; Shi *et al*., 1991; Werner *et al*., 2000). Here, we report absence of avidin-affinity in lymphatic tissue after formalin fixation and antigen retrieval without prior blockage of endogenous activities, such as biotin or HRP. Furthermore, we synthesized a detection system completely independent of the biotin-approach with robust markings of proliferative activity using anti-Ki-67 antiserum.

The sensitivity of γ to either conformational changes as a result of modification of the molecule itself (Irani *et al*., 2015; Storsberg and Schmidt, 2015; Liu and May, 2012), or as a result of recognition of the cognate antigen (Rosen *et al*., 2005; Stanfield *et al*., 2004; Saphire *et al*., 2001) highlights an area of biomedical sciences that is not fully understood. Consequently, any efforts in providing a universal strategy for modification of a given Ig molecule must be accompanied by a lengthy and expensive iterative process to eliminate modification schemes that would spoil its activity until systematic studies involving structural and functional interrogation were to yield guides to aid colleagues to embark in a more efficient and less costly derivatization of antibodies.

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Author's Contributions

Christian Schmidt: Wrote the first draft of the paper.

Martin Geyer, Verena Jentzen, Alexander Gorczyza, Karim Khalil and Marina Volkert: Performed assays and assisted in the interpretation of the results.

Christian Schmidt, Jörg Bohrisch and Joachim Storsberg: Conceived of this study, planned and designed experiments, assisted in the interpretation of the results.

Christian Schmidt, Mark A. Brown, Saadettin Sel and Joachim Storsberg: Provided critical input and assisted in improving and revising the paper.

Ethics

Christian Schmidt, Martin Geyer, Jörg Bohrisch, Verena Jentzen, Alexander Gorczyza, Karim Khalil, Marina Volkert, Mark A. Brown, Saadettin Sel and Joachim Storsberg: The authors declare that no competing interests exist.

Mark A. Brown and Christian Schmidt: Both are members of the Editorial Board of The American Journal of Immunology, are waived from the Article Processing fee for this contribution and receive no remuneration for the editorial work, neither individually nor collectively.

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