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COMMENTARY

Commentary regarding: "Activity determination of FAD-dependent glucose dehydrogenase immobilized in PEDOT: PSS-PVA composite films for biosensor applications"

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Abstract

In the light of continuous improvement and optimization, recent experiments that the authors conducted give new insights into the applied evaluation method of Riegel et al. [1]: Thorough investigations of the previous results regarding the usage of the Lowry Assay showed discrepancies in the determination of the released amount of protein in the analysis solution. The accurate quantification of this parameter is crucial as it directly influences the calculation of the residual enzymatic activity. In concrete terms, this finding has a major impact on the presented and discussed results in the article "Activity determination of FAD-dependent glucose dehydrogenase immobilized in PEDOT: PSS-PVA composite films for biosensor applications" [1]. Thus, this commentary addresses the new insights concerning the applied evaluation method, explains necessary revisions and discusses new conclusions derived from the adjusted evaluation method.

KEYWORDS

applicability, BCA assay, FAD-GDH, Lowry assay, PVA

As mentioned in the supporting information of the article [1] in question, the presence of the conducting polymer poly(3,4-ethylenedioxythiophene:polystyrene sulfonate) (PEDOT:PSS) led to an interference of the complexation between protein and the dye Coomassie Brilliant Blue G which the protein assay according to Bradford [2] is based on. Since such disturbances could not be found with Folin-Ciocalteu's phenol reagent in the presence of PEDOT:PSS, the Lowry assay [3] was applied. However, upon adding PVA to the system, no further interferences were experimentally assessed because this polymer is considered as a rather inert material [4] which is often chemically modified in order to improve its reactivity [4–6]. Besides, research on analyzing and reviewing components that the Folin-Ciocalteu's phenol reagent interferes with gained tremendous momentum over the past decades [7–11]. Among many different investigated categories, such as phenolics, detergents, buffers, vitamins, amines, aldehydes, and thiols, some solvents, no hint could be found that PVA contributes to the protein absorbance in der Lowry assay as well.

Nevertheless, in the course of further development, optimization, and improvement of the research, the applied evaluation methods presented in the published article were supplemented by the bicinchoninic acid (BCA) assay [12]. Like the Lowry assay, this protein quantification method is based on the biuret reaction but easier to handle, more sensitive, and tolerant to many detergents and buffers [13]. Thus, the BCA assay was meant to validate the published results and simplify the film analysis. However, the outcome

Abbreviations: BCA, bicinchoninic acid; C-F, Folin-Ciocalteu's phenol; FAD-GDH, flavin adenine dinucleotide-dependent glucose dehydrogenase; PB, phosphate buffer; PEDOT:PSS, poly(3,4-ethylenedioxythiophene: polystyrene sulfonate); PVA, poly(vinyl alcohol); TS, test system.

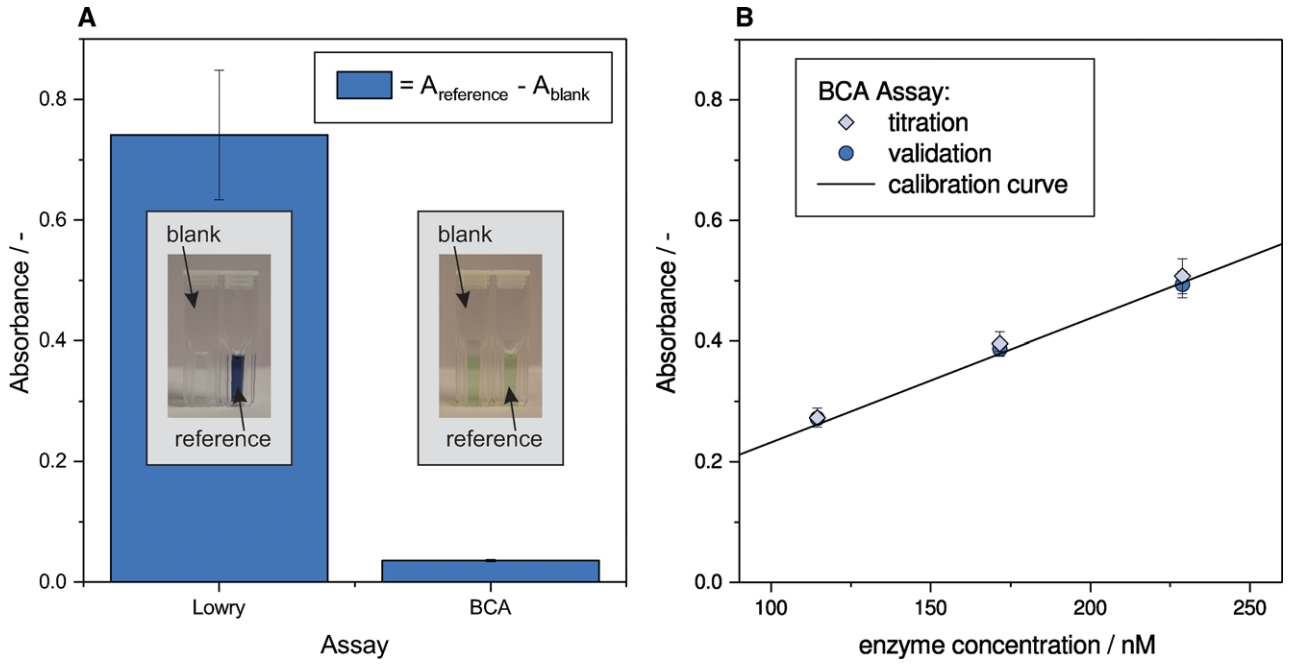

FIGURE 1 Evaluation of the applicability of the employed protein assays—comparison between Lowry and BCA Assay (A) and validation of BCA assay by means of titration (B). (A) The measured absorbance difference between the analysis solution of reference film 3, prepared without enzyme, and the corresponding blank is shown for both applied methods, additionally visualized by photography ($n = 14–25$, $p = 0.95$). (B) A known amount of enzyme is titrated to the analysis solution of the reference film 3 (diamonds) as well as to PB (circles) to validate its accurate determination ($n = 3$, $p = 0.95$) and compared to the independently conducted calibration curve (solid line)

was not consistent with the previous results. Consequently, a thorough analysis was performed in order to reveal the origin of the found discrepancy.

First, a comparison between Lowry and BCA Assay was conducted to evaluate their applicability. Following the methods for coping with interfering substances proposed by Peterson [11], reference samples were prepared to incorporate the incompatible components into the reagent blank. These so-called 'reference films' have the same composition as their corresponding test system (TS), only without adding the enzyme. Hereto, the solid content and the ratio of each component to one another are kept constant. Next, reference films according to the composition of TS3–the best performing TS–but without protein were prepared and analyzed as described in the experimental section of the respective article [1] with minor adjustments: after the enzyme release procedure, two protein assays and no activity assay were performed. Figure 1A shows the measured absorbance of the analysis solution of the reference film in relation to the respective blank for each assay. Since the reference sample contained no enzyme, no protein was expected to be found. However, a strong signal was recorded for the Lowry assay as indicated by the blue colored sample in the image that compares blank and reference. Regarding the BCA assay, blank and reference cannot be distinguished optically; in term of numbers, the measured absorbance difference is below 0.04. These findings explain the observed discrepancy mentioned in the beginning and declare the Lowry assay unsuitably to quantify the dissolved protein.

Taking into consideration that the Lowry assay worked well with no PVA added to the films, it can easily be assumed that PVA is responsible for the discrepancies. However, such an observation is neither reported nor could a clear explanation be found in literature. Thus, it is only possible to speculate about the reason of this issue since tracing it back is not the focus of this article:

One hypothesis can be the presence of methanol in the prepared PVA solution, a potential inferencing component [14–17]. According to the manufacturer [18], methanol is used when producing this polymer. Its residual content in the commercially available polymer is stated to be below 3% [19]. However, any interference with the F-C reagent seems rather unlikely since a compatibility of the solvent between 1–4 v-% or even up to 10%, depending on the source, has been documented [14–17].

Any interactions of the F-C reagent with functional groups of PVA might be a second possible explanation. PVA consists of hydroxyl and acetate groups whose ratio is determined by the degree of saponification. Since the F-C reagent has shown a certain affinity to hydroxyl groups [9,10,14] such as of phenolics, polyphenolics, and some alcohols, a complex reaction could have taken place leading to the observed color change. A final conclusion is not intended to be provided as it is out of scope of this commentary.

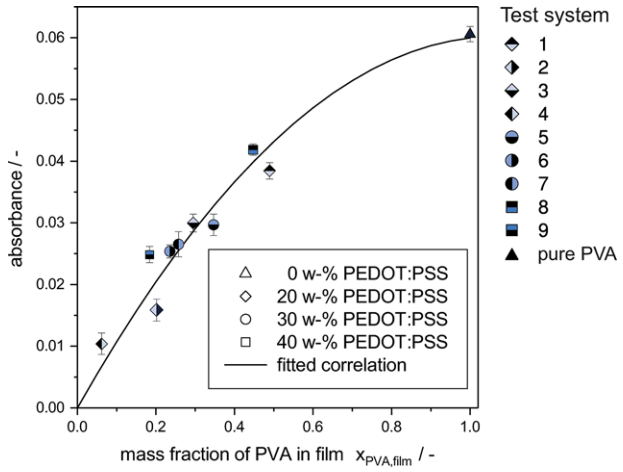

FIGURE 2 Evaluation of the applicability of the BCA assay—investigation of interference between BCA reagents and analysis solution of reference films. The measured absorbance difference between the analysis solution of reference films and the BCA blank (symbols) is plotted as function of the mass fraction of PVA in dry film corresponding to each investigated TSs. The diamond symbols correspond to TSs with 20 w% PEDOT:PSS, the circles with 30 w% PEDOT:PSS, and the square symbols with 40 w% PEDOT:PSS in the dry film ($n = 6$–9, $p = 0.95$). All reference films are prepared without enzyme by keeping solid content and ratio of the residual components constant. Additionally, film analysis of films, consisting of PVA only, is conducted and evaluated as well (triangle). A fit function (solid line), is calculated by least squares method and approximates the observed trend of slight rise of absorbance with increased PVA content in the film

Beyond that, and even more importantly, the BCA assay provides results with a tolerable deviation between reference and blank as demonstrated by the assay comparison (cf. Figure 1A). Thus, it is mandatory to validate its accurate operation in the presence of all components of the analysis solution. For this purpose, a known amount of dissolved enzyme was titrated in various concentrations to the analysis solution of reference film 3 and to the phosphate buffer (PB) illustrated in Figure 1B as diamonds and circles, respectively. Additionally, an independently determined calibration curve is plotted as well (cp. solid line in Figure 1B). The comparison of the shown data proves the accuracy of the used quantification method since the detected maximal deviations between titration and validation as well as titration and calibration are below 1.8% and 3.5%, respectively. This is a reasonable variance when taking the reproducibility and repeatability into account.

Considering these new insights, the discrepancies between reference and blank are supposed to be incorporated into reagent blank to further optimize the accuracy of the protein quantification. Therefore, reference films of all investigated TS were prepared and their deviation to the blank assessed. Figure 2 illustrates the observed interferences between the analysis solution of reference films and the BCA reagents: the

TABLE 1 Parameters of adjusted correlation used to describe the rise of absorbance of reference films with increasing PVA content in the solid film continuously

	$y = (a \pm \Delta a)x^2 + (b \pm \Delta b)x$					
Fit function	**a/–**	**Δa/–**	**b/–**	**Δb/–**	**Valid range**	R^2
References	−0.053	0.008	0.113	0.007	0.00–1.00	0.988

The function characterizes the slight interaction of PVA and BCA reagent where y is the optical density and x is the mass fraction of PVA in the film of the TS. Its parameters are determined by least square method.

TABLE 2 Parameters of the calibration curve for the BCA assay where y is the optical density and x the concentration of enzyme in μM

	$y = (a \pm \Delta a)x + (b \pm \Delta b)$					
Assay	**a/1/μM**	**Δa/1/μM**	**b/–**	**Δb/–**	**Linear range/μM**	R^2
BCA	2.053	0.019	0.027	0.003	0.022–0.350	0.998

Parameters are determined by least square method.

measured absorbance difference between both samples is plotted for each TS. Note that the symbols correspond to the TS that were introduced in the respective article (cf. Table 1 [1]). Additionally, films consisting only of PVA and having the same solid content as the TS films were prepared and analyzed as well (cp. triangle in Figure 2).

A slight interaction of PVA and the BCA reagent can be found with a rising PVA mass fraction in the solid film which is the highest for the pure PVA film (triangle in Figure 2). When taking a closer look at the absolute values, the detected absorbance is rather low. However, the absorbance of the reference films on average equals 15% of the actual signal measured with the protein present. Because of this, the evaluation method is adjusted by including the absorbance of the reference films into the blank. The proven linearity of the interferences with the protein measurement (cf. Figure 1B) justifies the proposed approach.

In addition to the incorporation of the assessed deviation for each TS individually, it is also possible to fit a correlation to the measured data and to continuously describe the interaction as a function of the PVA content in the film, independent from the investigated TS of the reviewed article. The obtained fit function is plotted as a solid line in Figure 2 and its determined parameters are listed in Table 1.

The calibration of the BCA assay itself was performed according to the standard test tube protocol proposed by its manufacturer Thermo Fisher [17,20] for two PB systems (pH 7.0 and either 50 mM or 100 mM PB). Since no dependency to the used buffer was found, only one calibration curve for enzyme concentrations between 22–350 nM was determined in the linear part. The calculated parameters are listed in Table 2.

With these new insights in mind, the experiments were reproduced as a whole according to the instructions in the article in question [1] with minor adjustments: the final enzyme

concentration at which the activity assay was performed was increased to 1 nM instead of 0.2 and 0.5 nM. Thorough analysis revealed stronger and more stable signals with greater reproducibility and reliability of the detected kinetics.

It should be mentioned that a direct comparison between the new findings derived from the reproduced experiments and the published results in the article in question is not possible. The new evaluation method leads to entirely new conclusions but still the necessary condition of a pH level of the coating solution greater than six has to be met in order to retain the catalytic activity of the enzyme. This strong correlation is shown in Figure 3A which plots the residual enzyme activity of each investigated TSs as a function of their pH values for the redone experiments. Following the instructions of the article (cp. 2.2 methods [1]), at least three films of each TS were manufactured. However, this time the results of the film analyses of all films were not averaged for each TS and shown as a single data point with error bars but rather individually determined for each film of each TS. This leads to at least three data points per TS and demonstrates the reproducibility more clearly. Additionally, the calculated error bars of each film describe the expected maximal deviation of the absorbance which is caused by the integration of the reference films into the blank. The variation of the signal of the reference film itself was determined by a worst-case estimation and considers the potential error of this additional input parameter.

In accordance with the initial postulation in the article [1], an acid environment (pH 2–5) inactivates the enzyme, which is a finding that is valid for TSs 5/8/9. In this context, the relation of salt and PEDOT:PSS is considered as a decisive factor that determines the pH value of the coating solution. As a result, similar amounts of both components in the dry film are required to provide the appropriate pH level.

However, the findings for the remaining TSs clearly differ from the original results. An increase of the residual enzymatic activity with a rising pH value can no longer be observed. Instead, the determined limit of a pH level greater than 6 has to be considered as a threshold below which the enzyme unfolds and already degrades in solution before coating the films.

What is more interesting, however, is that a new trend is found when considering the TSs whose pH value are above 6– these include all except for TSs 5/8/9: in most cases, it seems that the higher the PVA content in the dry film, the higher the residual enzymatic activity, as indicated in Figure 3B. The plot illustrates the dependency between the determined enzyme activity of the revised experiments and the amount of PVA in the dry film for each TS. TSs with good film forming properties is highlighted with red diamonds.

Unlike the original hypothesis, claiming an increasing catalytic activity with rising enzyme content, the polymer PVA appears to play an important role. On the one hand, a clear increase of enzymatic activity is observed from TS2 to TS6, then TS3, and finally the highest for TS1. On the other hand, a closer look at Figure 3B suggests that some exceptions might be found at the first sight:

TS4, e.g., exhibits a high enzymatic activity although the amount of PVA is rather low with 6 w%. In this context, it

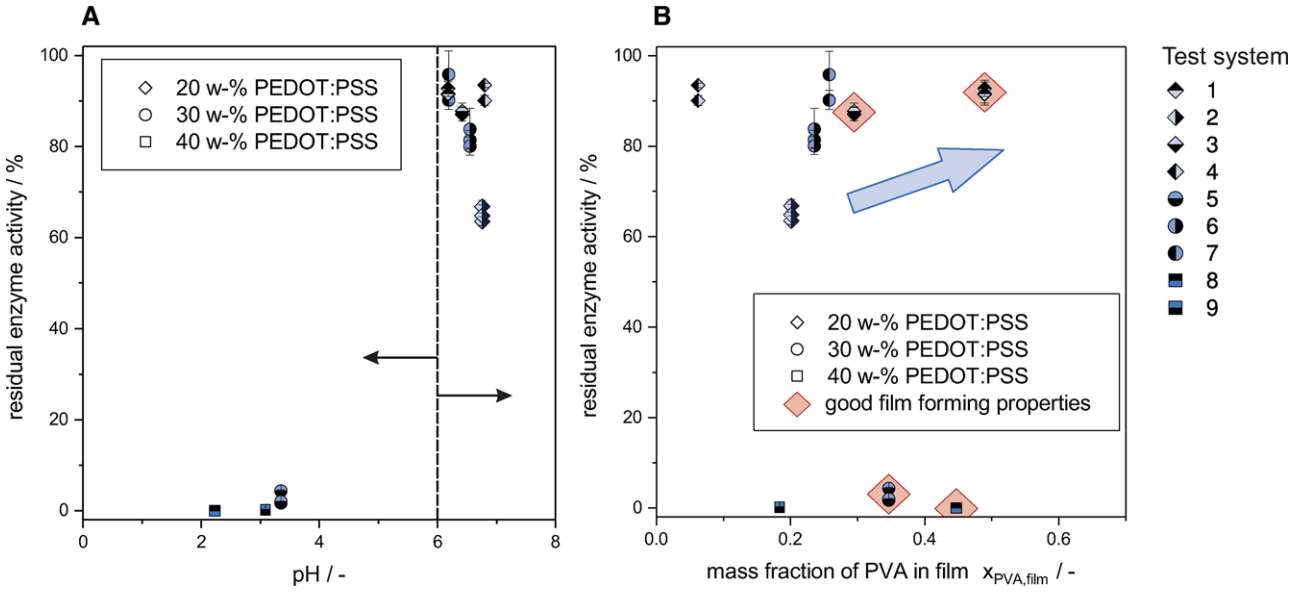

FIGURE 3 Residual activity of the enzyme immobilized into films of the investigated TSs as function of the pH value of the coating solution (A) and of the mass fraction of PVA in dry film (B). The diamond symbols correspond to TSs with 20 w% PEDOT:PSS, the circles with 30 w% PEDOT:PSS, and the square symbols with 40 w% PEDOT:PSS in the dry film. Error bars result from a worst-case estimation caused by incorporating the absorbance of the reference films into the blank. In B, TSs with good film forming properties are highlighted with red diamonds

should be kept in mind that, with a total polymer content of 26 w%, TS4 is featured by the lowest amount of polymeric components of all nine investigated systems. Thus, the prepared films with the composition of this TS cannot be considered as a polymer film since inhomogeneous films with rough surfaces and many crystals were obtained, as explained in the images in Section 3.1 of the respective article [1] (see also additional images [A, C, and E] in the appendix Figure A1). Since a certain limit in the ratio of salt and PVA must not be exceeded in order to provide good films, TS4 does not meet the aspired goal of immobilizing the enzyme in polymeric composite films with a ratio $x_{Salt}^{dry}/x_{PVA}^{dry}$ of 7.4 since there simply is no polymer matrix. As enzyme and salt are the main components, this system provides an environment that is quite close to the native and stable surrounding of the enzyme which is a reasonable explanation for the high residual activity. However, it still is not a suitable system to accomplish the aim of the respective article.

Moving on, a comparison of TS4 (diamond half left) to TS2 (diamond half right) shows a drop of enzymatic activity from 91.8% of the first to 65% of the latter. Both systems have the same pH value, meaning that the PEDOT:PSS and the salt content, with 20 w% and 46 w% respectively, are the same. They differ in the amount of enzyme (28 w% for TS4 to 14 w% for TS2) and PVA (6 w% for TS4 to 20 w% for TS2). The increase of PVA (from TS4 to TS2) leads to a lower salt to PVA ratio; however, with $x_{Salt}^{dry}/\,x_{PVA}^{dry} = 2.3$, it is still too high to obtain good films as previously claimed (cp. additional images [B, D, and F] in the Appendix Figure A1). Conversely, this means that the PVA amount is still too low to provide sufficient protection of the enzyme against external impacts. Thus, when reducing the enzyme content without sufficiently increasing the PVA amount, the enzyme is less stabilized by its natural surrounding and is not adequately shielded due to a missing intact polymeric matrix.

When having a look at TS3 and TS6, their residual enzymatic activity rises from 81.9% for TS6 (circle half right) to 87.3% for TS3 (diamond half bottom). Both systems have the same salt to PEDOT:PSS ratio of 1.2 in common, resulting in quite similar pH levels (6.6 for TS6 compared to 6.4 for TS3). Since TS6 is featured with 30 w% of PEDOT:PSS in the dry film and TS3 with 20 w%, the absolute amount of salt in the films is different (35 w% for TS6 and 23 w% for TS3). Furthermore, TS3 exhibits the greater amount of PVA with 29 w% compared to 24 w% for TS6. Consequently, the resulting enzyme content measures 28 w% for TS3 and only 11 w% for TS6. Although TS3 consists of only 5 w% more PVA and TS6 has the higher total polymer content (54 w% for TS6 and 49 w% for TS3), the differences in salt and PVA content lead to good and homogeneous films for TS3 and rather brittle films with agglomerates already visible on the film surface by eye observation for TS6. Thus, this example validates the importance of the film former PVA which is confirmed in literature as well [21,22]. The greater amount of the latter component contributes to a better immobilization of a functional composite film and protects the enzyme more efficiently against the external impacts. Salt is only needed for the adjustment of a suitable pH level.

The discussed tendency of PVA not only being a good film former but also promoting the stabilization of the enzyme within the composite films [23–25] is continued when looking at TS1 (diamond half up). This system consists of the highest amount of PVA with 49 w%, which means that good and homogeneous films could be processed (cp. Section 3.1 of the respective article [1]) and only a slight inactivation of the enzyme would take place. With 20 w% of PEDOT:PSS in the dry film, TS1 exhibits the highest residual enzymatic activity of 91.9 % (compared to TSs2-4) and the lowest contents of enzyme (12 w%) and salt (19 w%), resulting in a pH value of 6.2 which is the lowest among the 20 w% of PEDOT:PSS systems. This example emphasizes the pH level of 6.0 as a threshold that has to be exceeded. In comparison to TS3, the deviation of composition in enzyme and PVA content are the main differences. Both systems perform comparatively well in context of a good processability, excellent film homogeneity, and high enzymatic activity. With respect to TS1, the additional amount of PVA needed when decreasing the enzyme content at the same time is not at the expense of less active catalytic component. Furthermore, it implies that a high amount of enzyme in the film is not demanded to maintain its catalytic activity, in contrast to the hypothesis in the original manuscript [1]. As for TS3, the enzyme is almost entirely stabilized in the polymeric matrix and can efficiently be protected against the impacts during coating and drying. However, the desirable quantity of enzyme required still has to be explored since it is out of scope of this commentary. It strongly depends on the performance of the proposed pairing of materials in a final biosensor. Additional parameters, such as the film thickness, diffusion or reaction kinetics, have to be considered as well and determine the optimal amount of enzyme for each setup of biosensor as reported in literature [26–28]. The presented investigations of the article rather describe the state of the enzyme while being immobilized in the film surrounded by residual components, and give answer to the suitability of applied formulations by evaluating the residual enzymatic activity and their film-forming properties.

Coming back to the previously mentioned exceptions, Figure 3B suggests that TS7 follows the general tendency that with increasing PVA content in the dry film, the enzymatic activity rises. However, the determined residual activity amounts 93% in average which is, altogether, the highest activity for all considered TSs. A closer look at the individual films and their exact numbers, though, reveals rather big deviations between all prepared films of TS7. The residual activity varies from 90.2 to 95.9% and, with 2.2 to 5.1%, the error bars of this system are rather high compared to the

results of the other TSs (all determined error bars are rather well below 2.7% except for TS6). Analogous to the comparisons of TS2 to TS4 and TS3 to TS6, the pH value of the coating solutions of TS7 and TS1 is, with 6.2, the same due to the same salt to PEDOT:PSS ratio of 0.9 whereas their absolute amount of salt and PEDOT:PSS varies: with 30 w% PEDOT:PSS, TS7 consists of 27 w% salt (and 17 w% enzyme). Since TS1 exhibits 20 w% of PEDOT:PSS, the total amount of salt measures 19 w% as previously mentioned. Thus, the ratio of salt to PVA for TS7 and TS1 is determined to be 1.1 and 0.4, respectively. At the same time, this results in homogeneous and defect free films for TS1 and films with some smaller agglomerates and particles for TS7 since the ratio of the latter slightly exceeds the limit of 0.8. The measured high residual enzymatic activity of TS7 proves the applicability of this system to guarantee protection and stabilization of the enzyme. The individually determined activity of each prepared film still deviates to a greater extent compared to the residual TSs. Furthermore, the determined error bars that are relatively pronounced hint at larger variations in the analysis solution of one film sample when several repetitions of the entire film analysis were conducted. Altogether, this might be an indication that a completely uniform and consistent protection of the enzyme is not equally provided throughout the entire film surface. To sum it up, TS1 and 7 are able to protect the enzyme but the lower PVA content of TS7 is at the expense of its film quality and leads to less consistent results compared to TS1.

Finally, it is obvious that there is a close interconnection between the processability, the resulting film quality, and the residual enzymatic activity which is considerably influenced by the composition of the investigated film formulations. Providing homogeneous films with no particles is a mandatory factor in order to obtain consistent, reproducible, and reliable results regarding not only the manufacturing step but also functional properties such as the catalytic activity. In this context, PVA is found to play a key role: as film former component, the polymer is capable to provide a polymeric composite matrix on the one hand [21,22], and offers shelter to the enzyme against external impacts during processing on the other hand. This compatibility of PVA as a suitable immobilization matrix for enzymes in general is reported in literature as well [23–25]. Therefore, maximizing the PVA content should be the desired objective. Hereto, the ratio of salt to PVA as well as salt to PEDOT:PSS must not be neglected. The first one affects the film quality where a slightly higher content of PVA than salt is demanded. The latter ratio has to be kept in mind since an adequate pH (greater than 6) is supposed to be achieved. PEDOT:PSS which is required to provide a reasonable conductivity of the films [21,29] has a pH level of 2.0. Consequently, similar amounts of salt and PEDOT:PSS are needed as determined by titration (cp. Section 3.2 of the respective article [1]). When considering all these aspects, TS1 and three are revealed as potential candidates exhibiting good processability and high enzymatic activity at a reasonable amount of PEDOT:PSS.

Nevertheless, further investigations should pay attention to the smallest required amount of enzyme in the film and the impact of the solid content. In this study, the solid content was kept constant to sufficiently analyze the influence of the composition of the coating solution at comparable conditions. Furthermore, it might be interesting to see whether the absolute amount of PVA in the film is crucial as not only its presence but also its quantity could contribute to an improved processability, a greater tolerance regarding the enzymatic activity, as well as interfere with the electric conductivity. Last but not least, it should be mentioned that the required amount of PEDOT:PSS in the film is based on literature. Thus, further analysis with regard to the electric conductivity is needed to further optimize the formulation.

To conclude, the presented results give insights into the general requirements that have to be met when aiming at an enhanced throughput of novel biosensors and guide the way towards a novel pairing of materials meant for third generation biosensors.

NOMENCLATURE

x_i	$[\frac{\text{mass of component i}}{\text{mass of entire solution}}]$	mass fraction of component i related to entire solution
x_i^{dry}	$[\frac{\text{mass of component i}}{\text{mass of dry film}}]$	mass fraction of component i related to dry film

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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APPENDIX

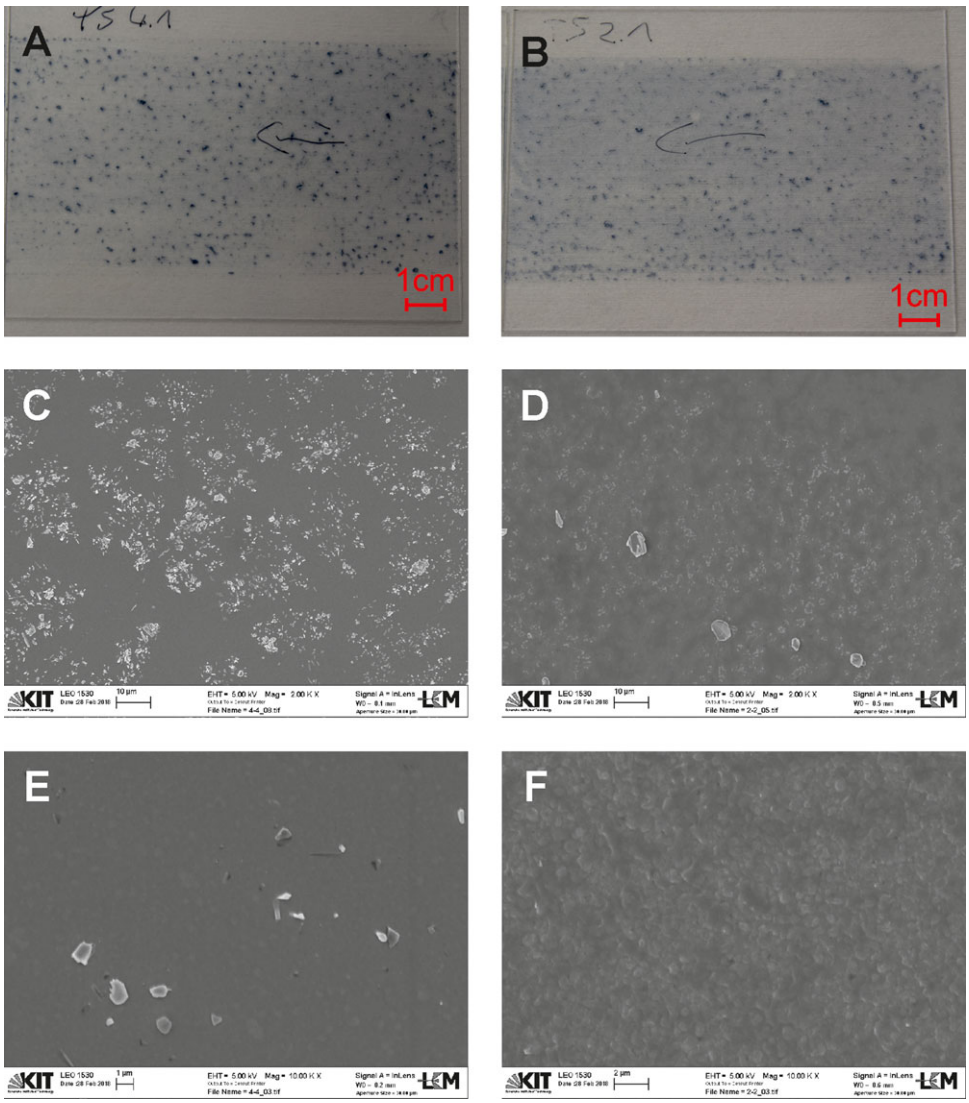

FIGURE A1 Photographies as well SEM images of films of TS4 (A, C, and E) and TS2 (B, D, and F) with varied magnifications. (Composition of TS4: $x^{dry}_{PEDOT:PSS} = 0.2$, $x^{dry}_{PVA} = 0.06$, $x^{dry}_{Enzyme} = 0.28$, $x^{dry}_{Salt} = 0.46$; Composition of TS2: $x^{dry}_{PEDOT:PSS} = 0.2$, $x^{dry}_{PVA} = 0.2$, $x^{dry}_{Enzyme} = 0.14$, $x^{dry}_{Salt} = 0.46$)