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RESEARCH ARTICLE

Surface Plasmon Resonance in the Development of TGF-β Activators for Cosmeceutical Use

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ABSTRACT

Research on new compounds that can improve the skin's condition is no longer focused exclusively on drugs, but is also adequate for cosmeceuticals – cosmetic products with thoroughly tested and scientifically proven biological activity. One of the most important stimulators of collagen biosynthesis is the so-called Transforming Growth Factor-β (TGF-β). Measuring the activation of latent TGF-β and quantification of its efficacy using the Surface Plasmon Resonance (SPR) technique is a great alternative to the currently used Enzyme-linked Immunosorbent Assay (ELISA) method. In this article, the complex process of TGF-β activation and the methods of its quantification are described. SPR was investigated as a relevant method for the TGF-β activity detection. Optimization of measurement conditions is presented, comparing results of antibody immobilization efficacy in different buffers. Two types of antibodies were immobilized onto the SPR chip, and after choosing one of them the selectivity of capturing between active and inactive TGF-β was confirmed. SPR is discussed as a technique with great potential in cosmeceutical design, in comparison to currently applied quantification methods.

Keywords: Surface Plasmon Resonance, TGF-beta, Cosmeceuticals

1 Introduction

In recent years, outward appearance and healthy and young-looking skin have drawn attention from a number of scientific studies. Research on new compounds that can improve the skin's condition is no longer required only for drugs, but also for cosmeceuticals. Compared to conventional cosmetics found in drugstores, cosmeceuticals are more efficient and of superior quality due to their biological activity, which has been scientifically demonstrated and extensively investigated. Recently, the growth of the skin-care products market is visible and thus the requirement for new cosmeceutical agents becomes significant as well [1]. The substantial part of active ingredients found in commercially available products stands for peptides and their conjugates [2]. Peptides are a key component of the cosmeceutical industry because of their diverse range of potential structural modifications, limited possible toxic side effects, and ease of synthesis. [3]. However, peptides and mini-proteins, due to their similarity to *in vitro* occurring large proteins, may be highly susceptible to enzymatic degradation and provide impediments in bioavailability and stability [4]. To overcome these obstacles, manifold techniques and synthetic approaches have been described in the literature [5]. A proper balance between structural protein synthesis and degradation is necessary to control the skin's biological processes. Among diverse proteins in the human body, collagen is one of the

most essential and most abundant ones. Collagen type III acts as the linchpin of type I fibers and supports skin elasticity [6], [7], [8]. Considering this, collagen turnover regulation has a potential interest as a target during the design of new compounds relevant in the cosmeceutical area. Consequently, a wide area for promising new research is provided by the acceleration of collagen biosynthesis as well as the downregulation of its degradation. [9]. Figure 1 presents the collagen turnover in cells.

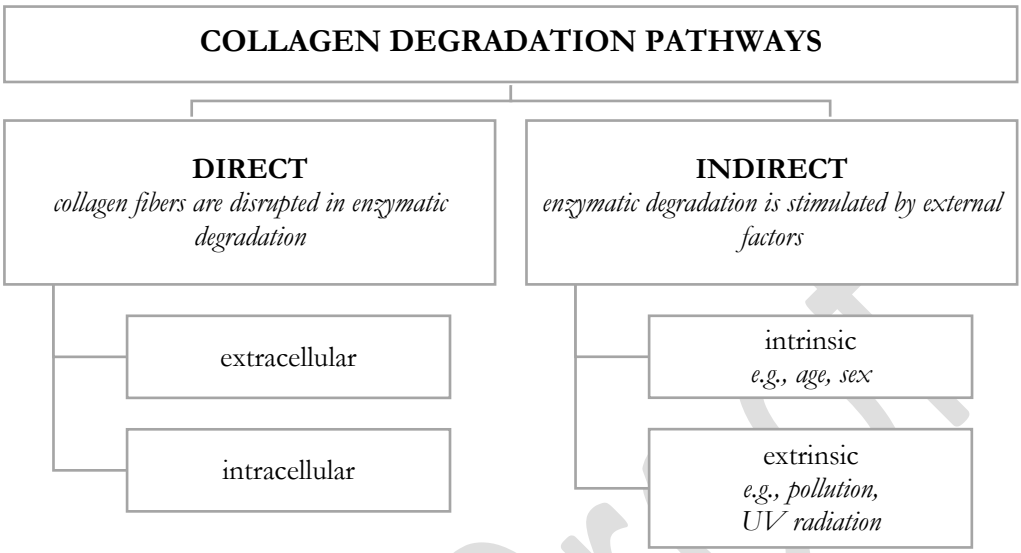

Figure 1: *Direct and indirect collagen degradation pathways with a noteworthy influence on skin aging.*

The group of transforming growth factors β (TGFs-β) consists of five isoforms, namely TGF-$\beta_{1\text{-}5}$ [10]. TGFs-β function is regulated by their activation, as they exist in active or latent form (LAP-TGF-β). TGFs-β are activated by a number of intracellular processes that occur prior to the secretion by the cell. Since LAP-TGFs are secreted in the latent form and are unable to interact with the receptor, they are biologically inert. This dimer protein's C-terminal segment is known as mature TGF-β (112 amino acids long), and its N-terminal portion is known as the latency-associated peptide (LAP, 390 amino acids long). Noncovalently bound LAP can be removed *in vitro* e.g., by radical pH or temperature changes. In addition to these, a variety of physiological substances, including plasmin, proteases, cathepsins, calpain, and the glycoprotein thrombospondin-1 (TSP-1), are known to activate the latent form of TGFs. [10], [11]. Figure 2 illustrates the LAP-TGF-β activation process involving TSP-1 [12]. TGFs-β were found to play a pivotal role in the natural wound healing and remodeling process [13]. Through complicated signaling pathways in fibroblasts, TGF-β can stimulate the synthesis of fibrillar collagens I and III. Furthermore, TGF-β inhibits the function of matrix metalloproteinases (MMPs), which are crucial in the breakdown of collagen fibers [9], [13]. TSP-1 was found to be the major TGF-β_1 activator *in vivo* [14]. It is a homotrimeric protein secreted by many cell types. In the 1990s, Schultz-Cherry *et al.* investigated and described in a detailed way the mechanism of TGF-β_1 activation by TSP-1 [15], [16], [17], [18]. It was shown that TSP activates the LAP-TGF-β_1 *in vitro* and *in vivo*, and thus can influence the collagen biosynthesis process [19], [20].

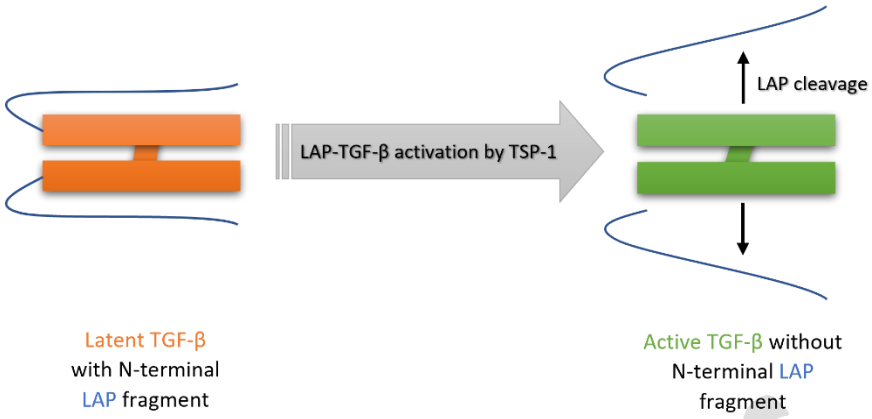

Figure 2: *Latent TGF-β (LAP-TGF-β) activation process by TSP-1.*

2 Up-to-now used techniques for quantification of LAP-TGF-β activation

The first attempts to determine the efficacy of LAP-TGF-β activation are described in papers by Schultz-Cherry and Murphy-Urlich in their diverse papers from the 1990s [15], [16], [17]. Hereby the Authors mention the use of Normal Rat Kidney (NRK) colony formation as an indicator of TGF-β_1 presence in the sample. Briefly, various concentrations of stripped TSP (sTSP; free of associated TGF-β) were incubated with latent TGF-β_1 in PBS. HCl was used as a positive control of the activation. Samples were then examined for TGF-β_1 activity by evaluating NRK colony formation in soft agar. Together with the NRK colony formation technique, Enzyme-linked Immunosorbent Assay (ELISA) was introduced as another method for the evaluation of TGF-β_1 presence. Commercially available KIT (a set of reagents with analytical protocol provided by the supplier) was used in this investigation. Currently, there are other commercially available KITs based on ELISA for active human TGF-β_1 quantification. Other methods not involving KITs for ELISA can be found in the literature. Kropf *et al.* in 1997 published the procedure of ELISA-based assay for TGF-β_1 quantification, compared with four different commercially available (at the moment) KITs [21]. It included the use of recombinant human TGF-β_1, chicken anti-TGF-β_1, and monoclonal specific mouse anti-TGF-$\beta_{1\text{-}3}$ antibodies. At the end, a biotinylated anti-mouse IgG antibody was added, and the color was developed using a peroxidase substrate solution. Another two different attempts were published in 2012. While Areström and co-authors based again on more complex ELISA [22], in the paper by Khan *et al.*, mink lung epithelial cells (MLEC) were used [23]. The bioactive TGF-β level was represented by luciferase activity. In the solid organs, such as the kidney, liver, and heart, the levels of both total and active TGF-β were evaluated.

In this fervent environment of new LAP-TGF-β activation modulators for cosmeceutical use, reliable quantification of the activation process is mandatory. Up to now, it is mainly performed via ELISA, however, this method is relatively simple to complete but time-consuming, as it requires several washing-binding cycles and cannot be easily automated [24]. In the current literature, there is no description of the Surface Plasmon Resonance (SPR) technique used for quantification of the bioactive TGF-β in the sample. While this method is already known for various biosensor applications [25], there are no scientific reports regarding the use of SPR for cosmeceuticals development. For this reason, we decided to evaluate the potential applicability of SPR in LAP-TGF-β activation measurements. In this article, we would like to spotlight this innovative technique as a potential tool to measure the activation of latent TGF-β. Using different buffers and antibodies immobilized on the chip surface, we have put an effort into optimizing the measurement conditions and demonstrating the method's applicability in the quantification of active TGF-β.

3 Surface Plasmon Resonance (SPR) technique

In simple words, SPR is an optical method that permits the observation of changes in the refractive index values in the proximity of the gold surface [26]. When this factor fluctuates the resonance angle shifts,

making SPR suitable for monitoring interactions in real-time recording results in the *sensogram*. To perform the SPR experiments, firstly the ligand (a capturing element) must be immobilized, permanently or temporarily, on the sensor surface. Each cycle of experiments starts with sensor conditioning with a buffer solution. Afterward, the solution containing the analyte (a target component in the solution) is injected, and the capturing by the ligand occurs. A non-specific binding may also take place. To get rid of unspecifically bonded molecules, a reference channel is also recorded and used as blank. After analyte injection, the buffer is flowed again to flush non-covalently bonded molecules during the dissociation phase. Finally, a regeneration solution (e.g., a low-pH buffer) is injected and the specific binding between the analyte and the ligand is broken. If the immobilization of the ligand was correctly performed, during the regeneration solution flushing only the bonded analyte is removed, without destabilizing the immobilized ligand. After the regeneration step, the cycle is completed and the chip with immobilized ligand can be reused. Due to its automatization and repeatability, the SPR technique has a broad spectrum of applications. SPR-coupled bioimaging in cells, viruses, and bacteria has been recently developed [27], [28]. This technique can be used e.g., to observe protein-protein [29], [30] or protein-carbohydrate interactions [31]. In recent years, SPR was applied as a tool to detect antibiotics [32], cardiac [33], [34], or cancer biomarkers [35], and for COVID-19 disease diagnostic purposes [36], [37]. Although Surface Plasmon Resonance is an innovative technique and shows a variety of applications, it also has limitations. For example, high system stability is required but not always possible to achieve; non-specific adsorption can also occur [38]. Description of currently used instruments, chips, and immobilization strategies were broadly described in the recent review [39].

4 Materials and Methods

ELISA assays were performed with NUNC® Maxisorp™ microplates. SPR measurements were performed with Biacore™ X100 system. Sensor chips CM5-type, amine coupling kit, glycine 10 mM pH 2.5, and running buffer HBS-EP+ 10× (0.1 mol/L HEPES, 1.5 mol/L NaCl, 30 mmol/L EDTA, 0.5 % v/v surfactant P20) were purchased by Cytiva (Uppsala, Sweden). Sodium acetate was purchased by Carlo Erba (Milano). Sodium hydroxide was purchased by Honeywell-Riedel deHaen (Seelze, Germany). Sodium chloride was purchased by VWR International (Radnor, PE, US). The activation of the chip surface was performed using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/*N*-Hydroxysuccinimide (NHS) solutions following the standard Amide Coupling protocol. Amide Coupling Kit, running buffer (HBS-EP+ Buffer), regeneration buffer, and Sensor Chip CM5 (a matrix of carboxymethylated dextran covalently bonded to a gold surface) were purchased from Cytiva. The appropriate activity of the anti-TGF-$\beta_{1\text{-}3}$ antibody (MAb Clone 1D11, BioTechne) was verified by ELISA assays. In order to do this, 100 ng/mL of active TGF-β_1 protein was coated on the NUNC® microplate. Following a saline solution wash, wells were blocked for 1.5 hours at room temperature using a 5% Bovine Serum Albumin (BSA) in phosphate-buffered saline (PBS) solution. Subsequently, a 5 µg/mL anti-TGF-$\beta_{1\text{-}3}$ antibody solution was added to the 2.5% BSA buffer, and the mixture was incubated for 2.5 hours with continuous agitation. The secondary antibody (anti-mouse IgG-AP, Jackson) was added and incubated for 2 hours under agitation, following three washing cycles with saline solution. The microplate was developed with a substrate solution containing 1 mg/mL of *p*-nitrophenylphosphate (*p*-NPP) in buffer carbonate at pH 9.8 in order to assess the binding of anti-TGF-$\beta_{1\text{-}3}$ to the protein. The absorbance in each well was measured at 405 nm. In the SPR measurements, the standard amine coupling strategy has been chosen for the immobilization of anti-TGF-$\beta_{1\text{-}3}$ antibody. Sodium acetate buffer has been selected as an immobilization buffer using the *pH scouting* protocol to adjust the optimal pH conditions for the immobilization. This depends on the isoelectric point (pI) of the antigen that must be immobilized. Before the immobilization, the chip was activated with NHS:EDC (50:50) injection for 480 s. Then, the activated chip was treated with a solution of anti-TGF-$\beta_{1\text{-}3}$ antibody (C_M=10 µg/mL in the selected immobilization buffer 10 mM acetate pH 4.5), by flowing it over the sensor surface for 420 s at a flow rate of 10 µL/min. After immobilization two 60 sec pulses of ethanolamine solution were flowed over the sensor chip to deactivate non-reacted sites on the surface. A standard amine coupling strategy has been selected also for the LAP (TGF-β_1) Affinity Purified Polyclonal

Ab immobilization. The chip activation was performed as described above using acetate buffer (10 mM, different concentrations).

5 Results and Discussion

Considering the lack of any innovative analytical methods in the field of LAP-TGF-β activation measurement, SPR appears as an interesting alternative to classic ELISA. To check whether the SPR method is suitable for such experiments, varied factors were investigated. To evaluate the feasibility of antibody immobilization onto the SPR chip, the conditions of the experiment were optimized. Two different antibodies were used in this measurement: anti-TGF-$\beta_{1\text{-}3}$ and anti-hLAP-TGF-β_1. To check the difference between their immobilization efficacy on the chip, different buffers, and various pH values were evaluated. After the efficient and stable immobilization of one of the antibodies, their selectivity had to be confirmed to define whether this system could be used to detect the active form of TGF-β. Therefore, the binding experiment was performed using both active and latent forms of the protein. The ELISA experiments were performed to confirm the activity of the commercial anti-TGF-$\beta_{1,2,3}$ Ab also in the currently used technique, besides the SPR. The results of ELISA analyses are presented in Figure 3. Among all tested samples, in the presence and absence of LAP TGF-beta, anti-TGF-$\beta_{1,2,3}$ antibody addition showed higher signals when TGF-β_1 is coated on the well. It confirmed the proper activity of the commercial antibodies.

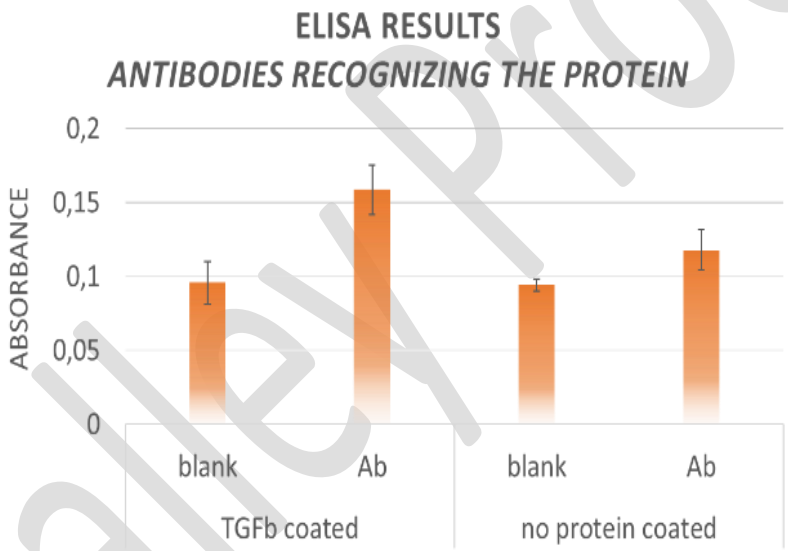

Figure 3: *Results of ELISA assay confirming the anti-TGF-$\beta_{1\text{-}3}$ activity. The bar with increased absorbance corresponds to TGF-β_1-coated wells treated with anti-TGF-$\beta_{1\text{-}3}$ antibodies.*

Using the pH scouting protocol we optimized the conditions for antibodies immobilization onto the chip. Anti-TGF-β1-3 and anti-hLAP-TGF-β1 were immobilized separately. The former captures only the active form of TGF-β1-3, while the latter interacts only with free LAP fragments, dissociated from inactive protein upon its activation. Both TGF-β1-3 and free hLAP-TGF-β1 are present in the sample if the protein activation took place. For anti-TGF-β1-3, optimal conditions were reached at 50 mM acetate buffer, pH=5.0 (Figure 4). For anti-hLAP-TGF-β1, the acetate buffer 10 mM at pH 4.5 was found to be the more suitable for the immobilization protocol (Figure 5).

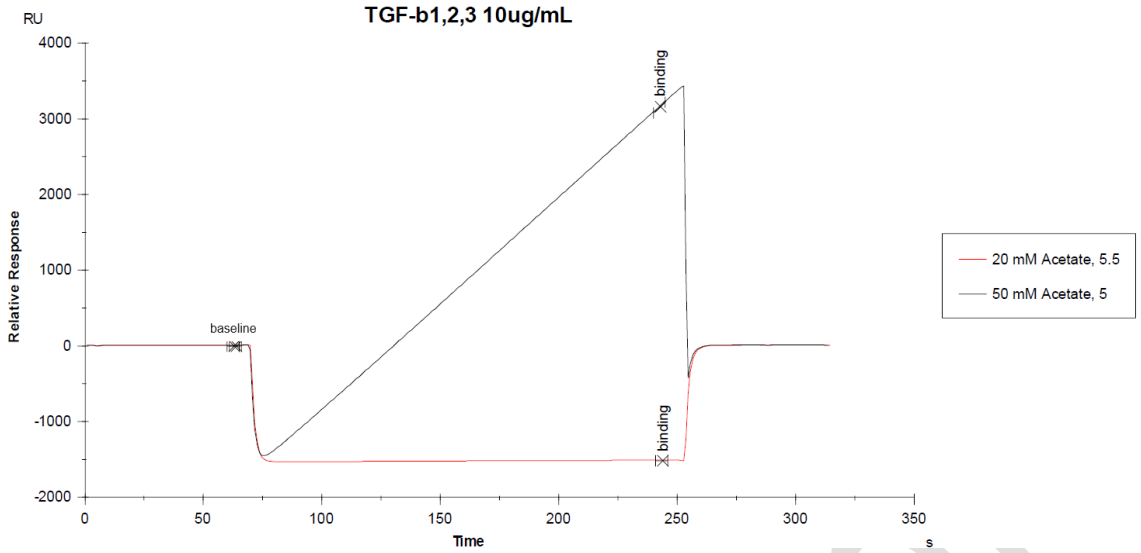

Figure 4: *pH scouting of anti-TGF-$\beta_{1,2,3}$ antibody onto the chip in two immobilization acetate buffers: 20 mM, pH=5.5 and 50 mM, pH=5.0. Relative response in resonance or response unit (RU).*

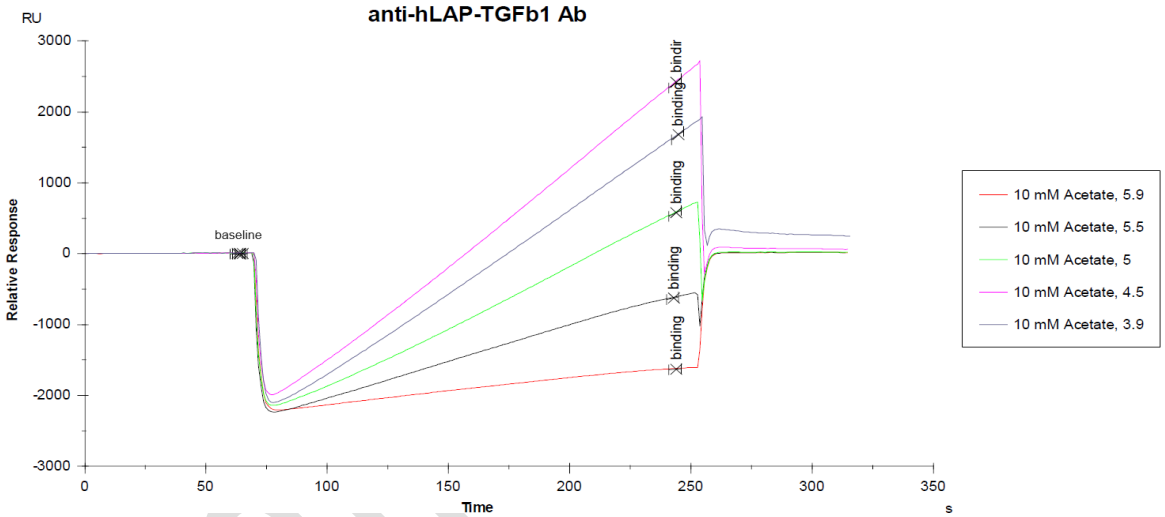

Figure 5: *pH scouting of anti-hLAP-TGF-β_1 antibody onto the chip in various immobilization acetate buffers: 10 mM, pH ranges from 3.9 to 5.9. Relative response in resonance or response unit (RU).*

Both types of antibodies (anti-TGF-$\beta_{1,2,3}$ and hLAP-TGF-β_1) were successfully immobilized to flow channel 2 of two different CM5 sensor chips, while flow channel 1 was used as a reference channel. Considering the observation that binding efficacy was higher for the anti-TGF-$\beta_{1,2,3}$ Ab (2800 vs. 3800 RU), we decided to proceed with this type of antibody. Once the anti-TGF-$\beta_{1,2,3}$ antibody was immobilized onto the sensor chip of Biacore™ X100, solutions of the latent and active form of TGF-β_1 were flown through the system to prove the selectivity of the chosen antibody. Both proteins were tested initially at the concentration of 50 ng/mL. The signals obtained for LAP-TGF-$\beta_{1,2,3}$ and active TGF-β_1 are presented in Figure 6. Buffer and the inactive protein did not provide any significant signal; thus, we confirmed that unspecific interactions between immobilized antibody and inactive TGF-β_1 do not occur. In the case of active TGF-β_1, we tested it initially at 50 ng/mL and the binding to immobilized antibody was observed. To check if the binding will increase with increasing the concentration of the protein, we performed the same test using the TGF-β_1 at 100 ng/mL. It provided excellent binding efficacy. These results proved the selectivity of the used antibody, confirming that this biosensor system can be used to identify active TGF-β_1. Considering that the increase of concentration of active protein in the sample also provides the increase in observed

signal, this method can be used for the quantitative determination of LAP-TGF-$\beta_{1,2,3}$ activation efficacy. Even though the discussed SPR method undoubtedly enhances the workflow and decreases the probability of human error on the result, it also has limitations. For example, analytes with conjugated fatty acids may provide a variety of unspecific interactions with the chip and impede the measurement.

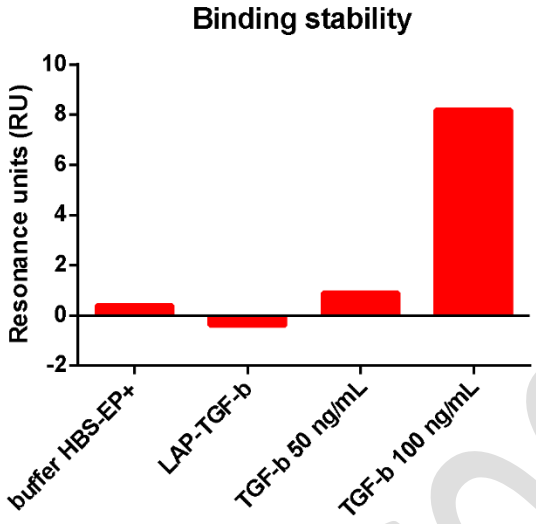

Figure 6: *Binding stability to the anti-TGF-$\beta_{1,2,3}$-immobilized chip was observed for 4 different solutions: buffer, inactive TGF-β_1, and active TGF-β_1 in two concentrations.*

6 Conclusion

Development of new compounds with anti-aging properties, every now and then based on already described products but with additional beneficial features such as enhanced bioavailability, stability, and efficacy, is highly required. For example, to evaluate the efficacy of designed and synthesized therapeutic candidates in the case of collagen biosynthesis activators, it is necessary to check if the activation occurred at a sufficient level. Up to now, mostly ELISA techniques have been applied for this purpose. In this article, we faced the issue of the time-consuming and hard-to-automatize ELISA. Using the broad spectrum of opportunities provided by the SPR technique, we designed a capturing protocol leading to the verification of LAP-TGF-$\beta_{1,2,3}$ activation efficacy. To do so, we compared two diverse types of applicable antibodies, optimized the conditions of antibodies immobilization, proved the activity of antibodies also in classical ELISA, and finally evidenced the selectivity of immobilized anti-TGF-$\beta_{1,2,3}$ antibodies by SPR experiment. It has been shown that inactive (LAP-conjugated) protein is not recognizable, while the signal corresponding to the free form is visible and increases with the protein concentration confirming the stability of the immobilized antibody and the efficacy of the regeneration protocol. The results of the Biacore™ assay were confirmed by the ELISA assay, which showed that the well containing active protein and anti-TGF-$\beta_{1,2,3}$ antibody had the greatest signal strength. Presented research includes potential applications in the development of cosmeceutically relevant compounds. The discussed analytical technique, SPR, has never been used for screening purposes in the field of TGF-β activators development and is a faster alternative for up-to-now used ELISA. Compared to the time-consuming and complex ELISA technique reported previously, the novel protocol for Biacore™ measurements of TGF-β_1 activation will enable the assay to be performed in a robust, fast, and automated way.

7 Declarations

7.1 Acknowledgements

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7.3 Competing Interests

The authors declare that there is no conflict of interest.

7.4 Publisher's Note

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