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RESEARCH ARTICLE

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# Covalent Biofunctionalization of Cellulose Acetate with Thermostable Chimeric Avidin

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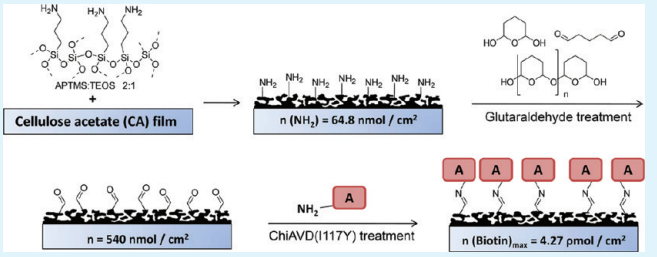
**ABSTRACT:** A stable, bioactive cellulose acetate (CA) surface was developed by functionalizing the surface with highly thermostable avidin form. The CA films were first functionalized with a mixture of 3-aminopropyltrimethoxysilane and tetraethoxysilane to introduce free amino groups onto the surface of CA films. Free amino groups were functionalized with glutaraldehyde to obtain an activated surface for covalent biomolecule immobilization. A genetically engineered, high-affinity biotin-binding protein chimeric avidin, ChiAVD(I117Y), was used for biofunctionalization of the surface. The chimeric avidin protein has an increased stability in chemically harsh conditions and at high temperature when compared to wt (strept)avidin. The biological activity, i.e., biotin-binding capacity, of the immobilized protein was probed by [$^3$H]-biotin. The activity of the chimeric avidin on functionalized CA films was fully retained over the three months' study period. The biotin-binding capacity of the immobilized chimeric avidin was compared to that of immobilized streptavidin, chicken avidin, and rhizavidin and significant differences between proteins were detected. The developed material offers a valuable platform for the development of inexpensive in vitro diagnostics and also supports biosensing applications that are required to operate under demanding conditions.




**KEYWORDS:** cellulose diacetate, sol−gel functionalization, glutaraldehyde, chimeric avidin, protein immobilization, thermostable protein, biomaterials, biotin

## 1. INTRODUCTION

The interest in protein immobilization techniques for biochips and biosensors has grown rapidly. A number of reviews have been published on the immobilization of proteins.[1−7] However, despite extensive studies, it is still challenging to immobilize proteins onto surfaces in such a way that their three-dimensional structure, functionality, and binding sites are well retained. One way to overcome this challenge is to use a tetrameric avidin containing four identical binding sites for biotin as an adaptor protein. The avidin-functionalized surface is used in many life science applications as a bioaffinity capturing tool for controlled immobilization of biotinylated protein.[8] This is based on avidins ultratight affinity toward the H-vitamin, also called as biotin ($K_d \approx 1 \times 10^{-15}$ M).[9] Avidin belongs to the chicken avidin family that also contains several related proteins including avidin related protein 4 (AVR4). AVR4 is one of the most thermally stable proteins among the protein family and in our previous study the stabilizing structural elements of AVR4 were transferred to wt avidin. The resulting chimeric avidin mutant ChiAVD(I117Y) (hereinafter referred to as chimeric avidin) was found to possess significantly increased thermal and chemical stabilities as compared to those of wt (strept)avidin.[10,11] Another valuable property of this protein is that it can be inexpensively produced in *E. coli* in a functionally active form. Because of these favorable characteristics the use of chimeric avidin for the immobilization may enable cost-effective manufacturing of biochips and biosensors that remain stable during long periods of storage or under demanding production conditions.

The main objective of this research was the development of a method for covalent immobilization of chimeric avidin on a cellulose acetate (CA) surface. CA, also known as zyl or zylonite, is valuable material utilized widely in manufacturing of films and fibers. A sol−gel-based method was chosen to first introduce free amino groups onto CA film using mixture of 3-aminopropyltrimethoxysilane (APTMS) and tetraethoxysilane (TEOS). This amino-functionalized CA film was then reacted with glutaraldehyde to form an activated CA film, which could then be used directly for covalent protein immobilization. The biotin-binding capacity of immobilized chimeric avidin was determined using tritium-labeled biotin ([$^3$H]-biotin). We also studied the durability of the surfaces functionalized with different methods over a three-month period. Finally, a comparative study was performed using other biotin-binding proteins chicken avidin, streptavidin and rhizavidin as linker proteins.[11]

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Cellulose diacetate film (CA) was obtained from Clarifoil (P27, thickness 150 $\mu$m). 3-Aminopropyltrimethoxysilane (APTMS), tetraethoxysilane (TEOS), glutaraldehyde (25% (w/v) in

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$H_2O$) and bovine serum albumin (BSA) were purchased from Aldrich and used without further purification.

Chimeric avidin and rhizavidin core were produced in BL21-AI *E. coli* cells by using pilot-scale fermentor and purified with 2-iminobiotin affinity chromatography as described previously.[12] Chicken avidin was purchased from Belovo (Bastogne, Belgium) and streptavidin from Calbiochem (189730−10MG). D-biotin was purchased from Sigma (B-4501) and D-[8,9-$^3$H]-biotin from Amersham (Buckinghamshire, England).

**2.2. Functionalization and Characterization of CA Films.** *Amino-Functionalization of CA Film.* To prepare a sol−gel stock solution for CA film amino-functionalization, we dissolved TEOS (0.75 mL) in ethanol (4 mL) and 10 $\mu$L of 4 M hydrochloric acid was added. In a separate flask, APTMS (1.5 mL) was dissolved in ethanol (4 mL) and 4 mL of water was added. Both solutions were stirred for 30 min after TEOS solution was added to the APTMS solution. This mixture was stirred for 15 min and then diluted 5-fold by adding 48 mL of ethanol. The obtained stock solution was stored at fridge (+4 °C). After the dilution of the stock solution by ethanol (1:5) CA films were dip-coated by immersing the film to the sol−gel solution for 2 min. The functionalized CA films were dried at room temperature (RT, 21−23 °C) and stored in an desiccator prior the use.

*Glutaraldehyde Functionalization.* Amino-functionalized CA slides were placed in a glutaraldehyde solution (10% in $H_2O$) for 1 h at RT. After glutaraldehyde treatment, the CA slides were washed with water, dried, and stored in an desiccator at RT prior the use.

*CA Film Characterization.* Gas chromatography was used to measure the density of free amino groups on the amino functionalized CA film and amino-reactive groups on the glutaraldehyde functionalized CA film.[13] The amino and amino-reactive groups on the surface of functionalized films were reacted with benzaldehyde and aniline analytes, respectively, to form imine bonds when refluxed in anhydrous toluene solution for 4 h. Anisole was used as a standard in the GC measurements to calculate the amount of reacted analyte with the functionalized surface. The amounts of amino and amino-reactive groups were calculated per area of 1 cm$^2$. Scanning electron microscope (SEM) images of prepared CA films were analyzed using field-emission scanning electron microscope Zeiss ULTRA plus.

**2.3. Immobilization of Avidins onto the CA Films.** Films were cut to 1 cm × 1 cm size and placed into the wells of 24-well plate. A 200 $\mu$L aliquot of avidin solution (0.1 mg/mL in PBS: 0.137 M sodium chloride, 2.8 mM potassium chloride, 11.9 mM Na-phosphate buffer, pH 7.4) was added to each well and films were incubated in the avidin solution for 1 h at 37 °C. The films were washed three times with 300 $\mu$L of PBST buffer (PBS with 0.05% of TWEEN 20) and blocked with 3% BSA solution in PBS for 1 h. The films were finally washed five times with 300 $\mu$L of PBST and dried at RT. The avidin functionalized films were either stored at RT or in a fridge at +4 °C prior the biotin-binding capacity measurements. Following abbreviations are used for the films studied: 1 = unmodified CA film, 2 = amino-functionalized CA film, 3 = glutaraldehyde functionalized CA film, A = chimeric avidin coated film, wtA = wt avidin coated film, RA = rhizavidin coated film, SA = streptavidin coated film, and R = control films without avidin coating.

**2.4. Determination of Biotin-Binding Capacity with [$^3$H]-Biotin.** The biotin-binding capacity of surface-immobilized avidin layer was measured using D-[8,9-$^3$H]biotin thereafter referred to as [$^3$H]-biotin. Nonspecific binding of the biotin was taken into account by measuring the bound [$^3$H]-biotin after blocking the films with an excess of D-biotin. These results were subtracted from the results without D-biotin blocking to calculate specific biotin-binding capacity. All biotin-binding capacity results are calculated as a bound biotin per area of the film [mol/cm$^2$].

*General [$^3$H]-Biotin Assay Protocol.* The CA films (1 × 1 cm) were incubated for 2 h at RT in a 1 mL sodium phosphate solution (50 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl, pH 7.0) to wash the films. The solution was removed and 1 mL of 12.8 nM [$^3$H]-biotin solution (50 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl, 10 $\mu$g/mL BSA, pH 7.0) was added and incubated for 1 h at RT. A sample (90 $\mu$L) was taken from the solution and added to 4 mL of Opti-Phase scintillation solution (PerkinElmer). The scintillations were counted with LKB Wallac 1217 Rackbeta liquid scintillation counter (Turku, Finland). The amount of bound [$^3$H]-biotin was calculated based on counts measured from free [$^3$H]-biotin solution before used for the experiment.

*[$^3$H]-Biotin Assay Protocol with D-Biotin Blocking.* The CA films were washed first with 1 mL of sodium phosphate -solution for 1 h. The solution was removed and 1 mL of D-biotin solution (50 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl, pH 7.0 with 150 $\mu$M D-biotin) was added and the films were incubated in this solution for 1 h. The D-biotin solution was removed and [$^3$H]-biotin assay was then carried out as described above.

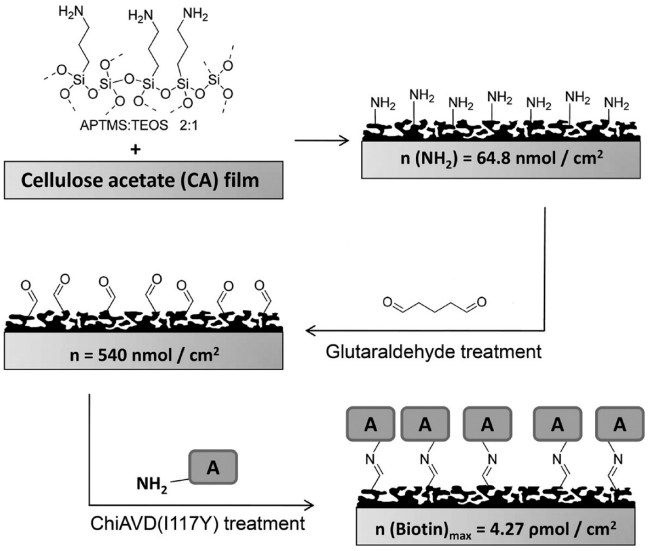


**Figure 1.** Schematic representation of functionalization and protein immobilization on the CA surface. For clarity, the chemistry of glutaraldehyde on the surfaces of CA films is shown as a monomeric dialdehyde.

## 3. RESULTS AND DISCUSSION

**3.1. Functionalization of CA Films.** The functionalization was done in two steps (Figure 1). During the first step, the CA films were treated with a sol−gel solution made from APTMS and TEOS in ethanol to functionalize CA films with free amino groups. A ratio of 2:1 between APTMS and TEOS were found to yield stable amino-functionalized CA film surface.[14] In the second step, a glutaraldehyde treatment was done by stirring the amino-functionalized CA films in a 10% glutaraldehyde solution in water for 1 h. During the glutaraldehyde treatment, the CA slides turned slightly red, indicating a reaction between glutaraldehyde and amino groups.

To measure the aging effect of sol−gel stock solution, a series of functionalized films were prepared where the sol−gel stock solution aging time varied from zero days to ten days. Determination of the free amino groups by gas chromatography revealed that seven days old sol−gel stock solution gave the highest amount of amino groups (64.8 nmol/cm$^2$) after amino-functionalization (Figure 2). However, all films regardless of the aging time clearly had a very high amount of amino groups (26−65 nmol/cm$^2$). In the SEM analysis, a rough surface structure with high surface area was observed (Figure 3). This can explain the high amino content on the R2 films. Similar high amino densities have been reported for amino-functionalized poly(ethylene-*co*-acrylic acid) polymer films, which were functionalized by using amino-modified silica nanoparticles.[15]

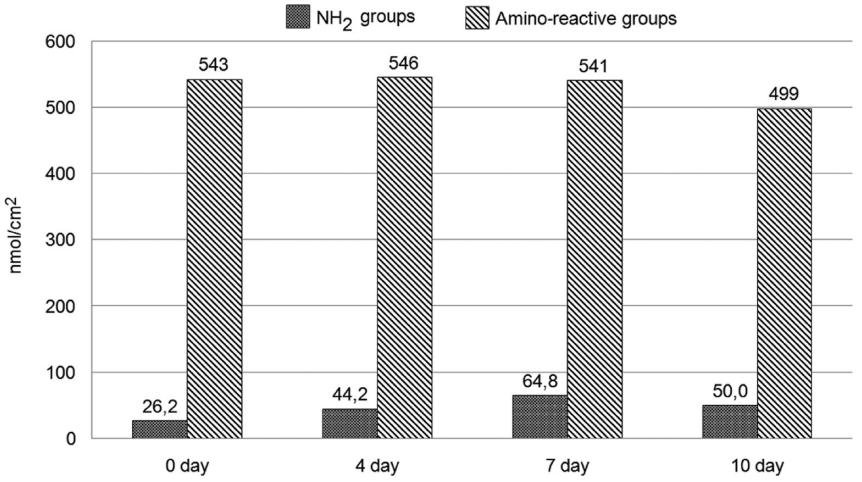


**Figure 2.** Density of free amino groups on R2 surface [nmol/cm$^2$] and amino-reactive groups on R3 surface as a function of the sol−gel aging time.

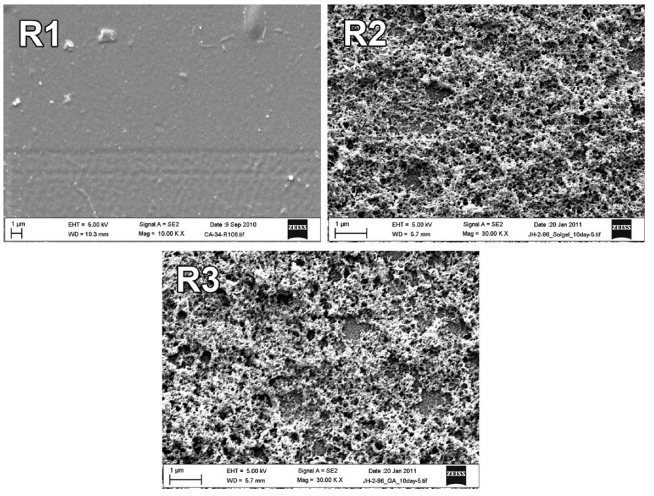


**Figure 3.** Scanning electron microscopy analysis of functionalized CA films. R1 = unmodified CA film, R2 = sol−gel amino-functionalized CA film, R3 = glutaraldehyde functionalized CA film.

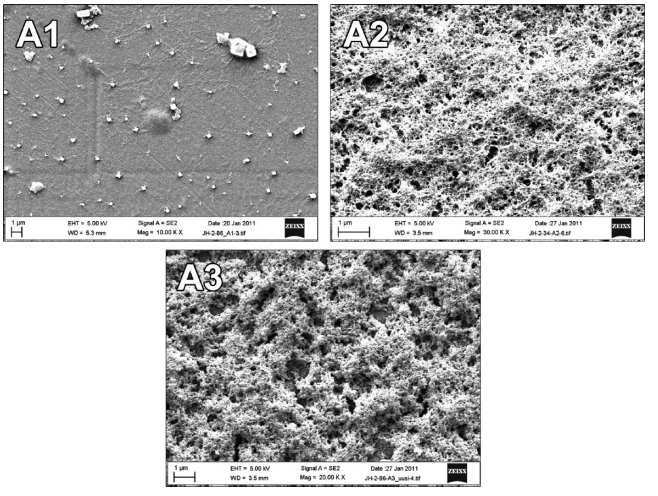


**Figure 4.** Scanning electron microscopy analysis of CA films after incubation with chimeric avidin. A1 = unmodified CA film treated with chimeric avidin, A2 = amino functionalized CA film treated with chimeric avidin, A3 = glutaraldehyde functionalized CA film treated with chimeric avidin.

All prepared glutaraldehyde-functionalized R3 films were found to have roughly ten times more amino-reactive groups (500−560 nmol/cm$^2$) compared to free amino groups after amino functionalization. Several studies have shown that commercially available glutaraldehyde−water solution represents multicomponent mixtures including aldehydes, monohydrates, dihydrates, cyclic hemiacetals, and oligomers and polymers of these.[16] All these forms can react with amino groups, which can explain the high density of amino-reactive groups on the surface of R3 films. Also, because the commercial glutaraldehyde can exist as a polymer form, especially when stored at RT, a low density of free amino groups before glutaraldehyde functionalization (26.2 nmol/cm$^2$, 0 day) resulted in the same level of amino-reactive groups after GA functionalization when compared to other films with higher (44−65 nmol/cm$^2$) amino group density.

**3.2. Immobilization of Avidin Proteins.** The immobilization of avidin proteins (chimeric avidin, chicken avidin, rhizavidin, streptavidin) onto the CA films was performed by incubating the CA films in a solution containing 0.1 mg/mL avidin protein for 1 h at 37 °C (Figure 1). Thorough washing with PBST buffer was effective in the removal of the weakly bound avidin proteins from the surface. Measured SEM images of chimeric avidin immobilized films after PBST washing period are shown in Figure 4, indicating the stability of amino and glutaraldehyde functionalization after protein immobilization.

**3.3. Biotin-Binding Capacity.** The specific biotin-binding capacity of the films was determined by comparing the measured total biotin-binding capacity with the results obtained after blocking the surfaces with D-biotin before [$^3$H]-biotin assay. No significant differences were observed between different control films (R1-R3) in their binding capacities.

All chimeric avidin-coated films showed high biotin-binding capacity. Initially (the first day) both nonfunctionalized film (A1) and glutaraldehyde activated film (A3) bound almost the same amount of [$^3$H]-biotin, indicating that the same amount of chimeric avidin was immobilized onto both films. The amino-functionalized film (A2) was found to have one-half less immobilized chimeric avidin compared to A1 and A3 films.

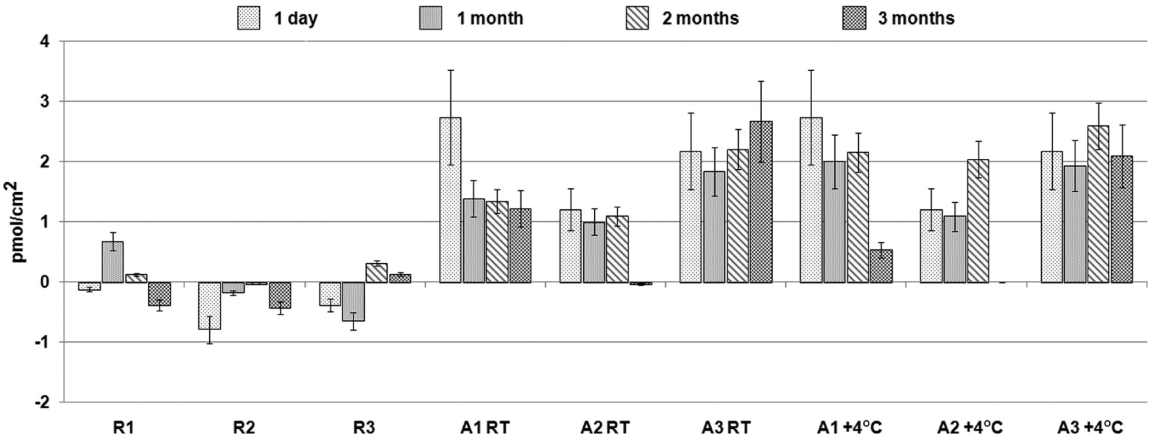


**Figure 5.** Biotin-binding capacity of the CA films. The control films (R1, R2, R3) showed no biotin-binding activity. The most stable film was the sol−gel- and glutaraldehyde-functionalized CA film with chimeric avidin coating (A3), because the binding activity of that film did not decrease during the 3-month storage period. Furthermore, no difference was observed between the different storage conditions in the case of A3. The A1 film stored at RT already showed a significant decrease in binding capacity after one month of storage, whereas the film stored at +4 °C showed slower decay of the binding capacity. (The A2 sample stored at +4 °C for 3 months was not analyzed.).

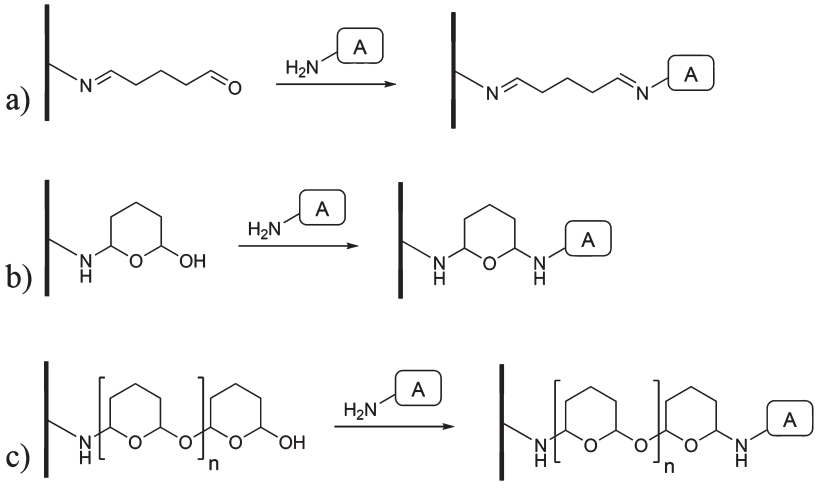


**Figure 6.** Reactions of different glutaraldehyde forms with protein (A) under neutral conditions: (a) glutaraldehyde, (b) cyclic hemiacetal, (c) polymeric hemiacetal.

During the 3 month study period, the A3 film was found to be the most stable: covalently bound avidin remained fully active. Additionally, there were no significant differences in binding capacities between A3 samples stored at different temperatures. The biotin-binding activity of A1 film stored at RT already showed a significant decrease after one month's storage, whereas the decay was slower when the film was stored at +4 °C (Figure 5). The difference between the behavior of A3 and A1 films can be explained by the means of protein attachment to the films. In the case of A1 film, avidin is physisorbed on the film, whereas in the case of A3 film the protein is covalently bound to the surface. It is well-known that glutaraldehyde has preservative properties against biomolecular unfolding, since the covalent multipoint attachment, presumed to occur with glutaraldehyde, prevents the unfolding of proteins.[17] Moreover, the polymeric nature of glutaraldehyde provides a long leash, attaching the protein to the matrix, which may permit greater flexibility for protein conformational changes required for activity. Under the used neutral conditions used for protein immobilization, a reaction between cyclic hemiacetal or its polymeric form and an amino group in protein is reported to form the most stable bond,[18] but several different reactions can proceed simultaneously leading to protein immobilization (Figure 6). Amino-functionalized film (A2) lost biotin-binding capacity completely after three months when stored at room temperature. Unfortunately, we were not able to analyze the sample of A2 stored at +4 °C after 3 months, so the difference between storage conditions can not be evaluated.

The binding capacity of chimeric avidin coating can also be evaluated based on molecular dimensions. According to the 3D-structure,[12] chimeric avidin can be described as a box with a dimension of about 5 nm, and a full monolayer of the avidins would result in a protein density of 6.6 pmol/cm$^2$. The measured specific biotin-binding capacity of A3 surface was 2.17 pmol/cm$^2$ (1 day). Therefore, the generated surface had a capacity that is clearly lower than a fully active avidin monolayer, and, by converting the obtained values for molecular density, there were 0.33 biotin-binding sites per surface area of an avidin tetramer. Based on the crystal structure, (chimeric) avidin is quite symmetric. Therefore, the number of available binding sites on

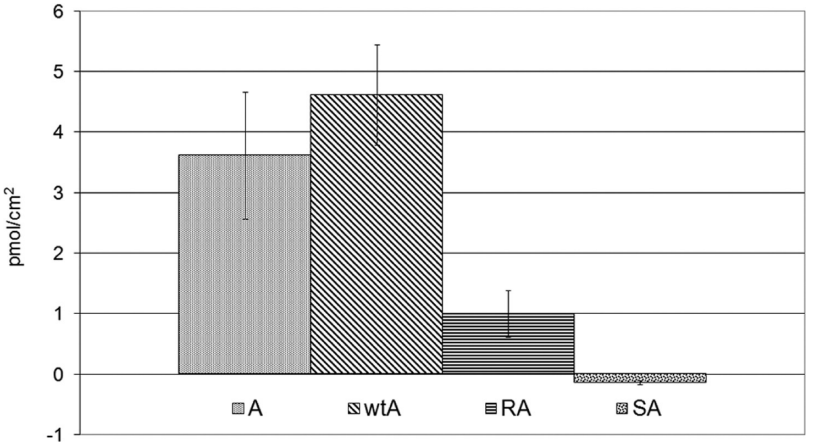


**Figure 7.** Comparison of biotin-binding capacity of the activated CA surfaces coated with various different avidins. Chimeric avidin (A) was found to behave similarly to chicken avidin (wtA), whereas in the case of rhizavidin (RA) and streptavidin (SA), a low binding capacity was observed.

surface-immobilized avidin would be two or less, and only one binding site per immobilized protein would be a fair assumption. Therefore, in the case of A3 films, the determined specific binding capacity suggests an average coverage of approximately 33% or less of the surface with functional chimeric avidins. In reality, because of the roughness of the surface (Figures 3 and 4), the coverage may be significantly smaller.

We also analyzed films coated with chicken avidin, streptavidin and rhizavidin. Interestingly, chimeric avidin and chicken avidin showed similar high biotin-binding capacities, whereas films coated with streptavidin and rhizavidin had low biotin-binding capacities (Figure 7). This may reflect the chemical reactivity of the proteins with the activated surface. Other possible explanations are the physicochemical differences between proteins. Rhizavidin is dimeric avidin form, which has significantly lower thermal stability when compared to chicken avidin.[11] Streptavidin in turn, has thermal stability similar to chicken avidin, but appears to be more sensitive to treatment with different chemicals, such as methanol.[12] Therefore, it is possible that the result reflects the chemical stress applied to the immobilized protein because of the adjacent surface. Chimeric avidin was selected for the experiment instead of wt avidin, due to its more stable structure in harsh conditions. Also, the efficient and scalable expression in *E. coli* supported the use of chimeric avidin in the study. However, the long-term stability of wt avidin on the CA films should be studied more closely in the future.

## 4. CONCLUSION

A new method for biofunctionalization of cellulose acetate films by a simple two-step protocol was developed. A sol−gel solution containing 3-aminopropyltrimethoxysilane and tetraethoxysilane was first used to introduce free amino groups onto the surface of cellulose acetate (CA) film. The amino groups were then reacted with glutaraldehyde to obtain a reactive glutaraldehyde layer on the surface of CA film. The protein immobilization was studied with highly thermostable chimeric avidin, which is a genetically engineered version of the high-affinity biotin-binding protein avidin. The activity of CA-immobilized chimeric avidin retained completely for three months when stored at RT or at +4 °C when assayed by a tritium-labeled biotin assay. Immobilization of other avidins (streptavidin, chicken avidin, and rhizavidin) was also analyzed and chimeric avidin and avidin were found to behave similarly. In contrast, streptavidin and rhizavidin yielded notably weaker biotin-binding capacities. The method developed in the study opens up a great potential for the use of avidin-coated CA-films as universal base for various applications, for example, in the development of an inexpensive and sensitive diagnostic tools to be used in personalized medicine platforms.

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