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Abstract

The aim of this work was to develop a nanolayered pH-sensitive coating method whereby proteins are coated at a suitable pH on the surface of chemically modified biomedical/bioanalytical microdevices and protein release is triggered by a pH-shift upon contact with physiological environment. In this work such a coating was developed and was applied onto microneedles. First, the surface of microneedle arrays was modified with basic groups with a surface $pK_a$ below physiological pH. This modification was a multistep procedure: first the surface was hydroxylated in a piranha mixture, then 3-aminopropyl-triethoxysilane was coupled (yielding a “pH-independent” surface with a positive charge over a broad pH range), next 4-pyridinecarboxaldehyde was coupled to the obtained surface amine groups and finally the imine bond was reduced by sodium cyanoborohydride. The obtained pH-sensitive pyridine-modified microneedles were coated with ovalbumin at surface $pK_a > pH > pI$ of the protein, thus the surface of the microneedles is positively charged and the protein is negatively charged. The coating efficiency of ovalbumin was 95% for the amine-modified (pH-independent) and the pyridine-modified (pH-sensitive) surfaces, whereas a non-modified surface had a coating efficiency of only 2%. After the protein-coated microneedle arrays were pierced into the skin, having a pH $> surface pK_a$ of the microneedle arrays, 70% of the protein was released within 1 minute, whereas the protein release from pH-independent microneedle arrays was only 5%. In conclusion, we developed a procedure to efficiently coat microneedle arrays with proteins that are released upon piercing into human skin.
1. Introduction

Chemical surface modifications by self-assembled layers are applied in a broad range of applications, including biomedical and bioanalytical microdevices for drug delivery and the analysis of biological samples. Here we present a novel coating procedure for microdevices: a nanolayered pH-sensitive coating applied onto microdevices and the subsequent coating thereof with a model protein. The concept of pH-sensitive coating and release is based upon electrostatic interactions between the protein and the surface of the microdevice, whereby a protein is adsorbed to the surface at a suitable pH where the surface of the microdevice is charged. Subsequently, upon contact with physiological environment (pH 7.4) the surface of the microdevice becomes uncharged, causing the electrostatic bond to break and releasing the adsorbed protein.

We apply the pH-sensitive coating technique to microneedles. Microneedles are needle like structures with a size in the micrometer range that are used to deliver drugs via the skin. The skin is an attractive organ for drug administration since the skin is an easily accessible organ and drug delivery via the skin is potentially pain free. However, the skin barrier located in the uppermost layer of the skin, the stratum corneum, limits drug delivery via this route [1, 2]. Therefore, microneedles should be long enough to penetrate the 10-20 µm thick stratum corneum layer, but short enough to avoid pain sensation [1, 3].

Protein delivery into the skin via microneedles has been achieved via various approaches [1, 3, 4]. Among those microneedles that are used to penetrate the stratum corneum and contain and deliver the drug as a single delivery system are currently of most interest. There are three methods to use microneedles as a single drug delivery system: 1) by using porous microneedles which contain a drug in solution inside the pores, 2) by using dissolving microneedles wherein the drug is encapsulated, and 3) by using drug-coated microneedles [1]. Dissolving microneedles degrade after hydration inside the skin and coated microneedles dissolve their coated layer upon hydration inside the skin. In this paper we focus on the development of coated microneedles.

The major benefit of using coated microneedles is that the coating process can be performed at ambient conditions in an aqueous environment, and that a dry coating may be more stable than a liquid formulation [5-7]. However, most coating procedures require complex coating devices which often do not allow the coating of multiple microneedle arrays simultaneously [6, 8, 9]. Furthermore, coating of microneedles mostly requires a selected set of excipients which 1) reduce the surface tension between the microneedle surface and the coating solution, and 2) increase the viscosity of the coating solution [5, 6, 10]. Another disadvantage of current coating procedures is that there is a relatively high premature drug loss [11]. Finally, most coating procedures require multiple dip-coating steps, often generating thick coatings which negatively influence the sharpness of the needle tip diameter and thereby compromise the penetration ability of these microneedle arrays [8, 9].

Here, a nanolayered pH-sensitive coating was developed and applied onto the surface of microneedle arrays to adsorb a model protein, ovalbumin, which is released into the skin
upon piercing. First, the surface properties of silicon surfaces modified with a weak base, and the isoelectric point of ovalbumin were determined to define the pH range in which ovalbumin can be coated on the surface. Subsequently, the binding and release of ovalbumin from the chemically modified silicon surface and the antigenicity of ovalbumin after release were determined. Finally, after the chemical coating was applied onto the surface of in-plane silicon microneedles with a length of 300 µm, which were coated with ovalbumin at optimum pH, the microneedles were assessed for morphology and release of fluorescent- and radioactively labeled ovalbumin in human in vitro skin.

2. Experimental
2.1 Materials
Deionized water with a resistivity of 18 MΩ·cm, produced by a Millipore water purification system (MQ water), was used for the preparation of solutions and the cleaning of the silicon surfaces. Sulfate-modified polystyrene fluorescent orange (520 nm/540 nm, ex/em) nanoparticles of 100 nm, human serum from human male AB plasma, (3-aminopropyl)triethoxysilane (APTES), ethylenediaminetetraacetic acid (EDTA), NaCl, CaCl₂, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrogen peroxide (30%), 4-pyridinecarboxaldehyde, acetic acid, sodium cyanoborohydride, Triton X-100, Tween-20, and Tween-80 were purchased from Sigma Aldrich. Sulfuric acid 96-98% and acetone were obtained from BOOM lab equipment, and sterile phosphate buffered saline (PBS) was obtained from Braun. Alexa-Fluor® 488 carboxylic acid succinimidyl ester, chicken egg ovalbumin-Alexa-Fluor® 488, and stabilized chromochen 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Invitrogen, and chicken egg ovalbumin grade VII was purchased from CALBIOCHEM®. Flat bottomed black 96-well plates and MICROLON® ELISA-plates were obtained from Greiner Bio-One, and polystyrene cuvettes (10x4x45 mm) were from Sarstedt. Molecular sieves 4Å 8 to 12 mesh were purchased from Acros Organics, acetonitrile HPLC Far UV was from LAB-SCAN analytical sciences, and methanol HPLC grade, 2-propanol AR, and toluene AR were purchased from BIOSOLVE. Silicon wafers <110> dsp of 0.38 mm thickness, cut in pieces of either 1 by 1 cm or 1 by 2.5 cm, and silicon microneedle arrays with either 9 or 17 microneedles per array with a surface area of 0.3 mm² and a length of 300 µm per microneedle were a kind gift of U-needle B.V. Bovine serum albumin (BSA) fraction V was obtained from Roche. Polyclonal mouse anti-ovalbumin antibody was obtained from LifeSpan Biosciences, Inc., Pierce® Iodination beads and Pierce® polyclonal rabbit anti-ovalbumin antibodies conjugated with horseradish peroxidise (HRP) were obtained from Thermo Scientific. Iodine-125 radionuclide, 2 mCi (74 MBq), Specific Activity: ~17 Ci (629 GBq)/mg, 10⁻⁵ M NaOH (pH 8-11) (Reductant Free), Concentration: 100 mCi/mL was obtained from Perkin-Elmer. Amicon® ultra centrifugal filter units with a molecular weight cut off of 30 kDa were obtained from Millipore™.
2.2 Methods

2.2.1 Generation of a physiologically relevant surface pKa

The modification of silicon surfaces with pyridine is a multistep procedure. The hydroxylation and the generation of amine groups on the surface were performed as previously described [12], in brief: the surface, either of silicon microneedles or silicon slides, was cleaned with acetone and methanol and was subsequently hydroxylated with a freshly prepared piranha mixture (30 v% H$_2$O$_2$ and 70 v% H$_2$SO$_4$) for 1 h at 80 °C. Then, amine-modified surfaces were formed by incubating the hydroxylated silicon surfaces in 2% (v/v) APTES in toluene for 24 h at room temperature.

The pyridine-modified surfaces were formed in a two-step procedure. First, an imine bond was formed by incubating the amine-modified surface for 16 h in 100 mM 4-pyridinecarboxaldehyde in anhydrous isopropanol with 1% (v/v) acetic acid at room temperature. Subsequently, the pyridine-modified surface was stabilized by reducing the imine bond to a secondary amine in 50 mM NaBH$_3$CN in isopropanol for 2 h at room temperature. Finally, the modified silicon surfaces were washed with isopropanol (2x) and with methanol (3x), and dried in a vacuum oven at 50°C for 30 minutes. The modified silicon surfaces were stored under argon until used.

2.2.2 Reactivity of modified silicon surfaces with a fluorescent dye

To determine whether pyridine-modified surfaces were successfully synthesized, a fluorescent dye was used which specifically reacts with primary amines. The non-modified, the amine-modified, and the pyridine-modified silicon slides of 1 by 2.5 cm were incubated for 1 h at room temperature in 5 mL 2 μg/mL Alexa Fluor® 488 carboxylic acid succinimidyl ester in acetonitrile. Subsequently, the non-reacted fluorescent dye was removed by washing the silicon slides with acetonitrile (2x) and with methanol (5x). Finally, the silicon slides were dried in a vacuum oven at 50°C for 30 minutes and visualized under a fluorescence microscope (Nikon Eclipse E600) with a mercury light source, a magnification of 100x, a GFP filter set, and imaged with a 10 s exposure time.

The pictures were analyzed in ImageJ (available from rsbweb.nih.gov/ij/) with the plugin ‘interactive 3D surface plot’, where the pixel intensity was plotted against the pixel position on the surface. The following settings were used: grid size 128, smoothing 3.0, max. 50%, and min. 4%. The derivatization efficiency of the amine-modified surface into a pyridine-modified surface was determined by equation 1:

$$\text{Derivatization efficiency} = 100 - \left\{\frac{(I_m - I_o)}{(I_a - I_o)}\right\} \times 100$$

(Equation 1)

Here $I_m$ represents the average pixel intensity of the pyridine-modified surface, $I_o$ represents the average pixel intensity of the non-modified surface, and $I_a$ represents the average pixel intensity of the amine-modified surface [12].
2.2.3 Surface analysis by atomic force microscopy

In order to analyze the surface roughness of the coating, non-modified surfaces, pyridine-modified surfaces, and pyridine-modified surfaces coated with ovalbumin were analyzed by atomic force microscopy (AFM) on a Veeco Nanoscope IIIa Multimode AFM in tapping mode with scan size of 1 µm², a scan rate of 1 Hz, and an Si cantilever at 75 kHz. The data was analyzed in Gwyddion software version 2.25 (available from: http://gwyddion.net/).

2.2.4 Surface pKa determination by the fluorescent nanoparticle adhesion assay

The surface pKₐ of pyridine-modified silicon surfaces was determined by the fluorescent nanoparticle adhesion assay [12]. This method determines the surface pKₐ by using the fraction of nanoparticles that is bound to the surface and the fraction of nanoparticles that is in solution as a function of the pH. Because pyridine is a weak base and gets positively charged when reducing the pH, negatively charged nanoparticles were used.

First, 20 nanoparticle suspensions were prepared, each with a different pH in a range of 2-12, in a 1 mM EDTA buffer with 1 µL of 2.5% sulfate-modified fluorescent orange nanoparticles per mL. Subsequently, two aliquots of 0.75 mL nanoparticle suspension were transferred to two 1.5 mL cuvettes for each pH value, wherefrom one cuvette was used as a negative control and the second cuvette was used to be incubated with a 1x1 cm pyridine-modified silicon slide. After the pyridine-modified silicon slides were incubated for 4 hour on a shaking device, two times 200 µL of each sample was transferred to a black 96-well plate and the emission at 540 nm was measured with an excitation wavelength of 520 nm on a Tecan Infinite® M1000. Subsequently, the relative fluorescence was calculated for each pH value by dividing the fluorescence of the nanoparticle suspension incubated with a pyridine-modified silicon slide though the fluorescence intensity of a non-incubated sample at the same pH. The binding (100 - relative fluorescence) was plotted as a function of the pH and was fitted in Prism 5 for Windows according to the Henderson-Hasselbalch equation, from which the surface pKₐ was determined [12].

2.2.5 Determination of the isoelectric point of ovalbumin

The net charge of a protein plays an important role in its binding to the pyridine-modified silicon surface. Therefore, the isoelectric point (pI) of ovalbumin was determined by measuring the ζ-potential as a function of the pH by laser Doppler electrophoresis on a Zetasizer Nano (Malvern Instruments). The ζ-potential of ovalbumin was determined in a 10 mM phosphate buffer with a pH in the range between 3-6 and an ovalbumin concentration of 1 mg/mL.

2.2.6 Ovalbumin binding to pyridine-modified surfaces

To determine whether proteins are able to bind to the pyridine-modified silicon surfaces, the binding was investigated by using ovalbumin. Therefore, pyridine-modified silicon slides of 1 by 2.5 cm were incubated in 1.5 mL 1 mM EDTA buffer, pH 5.8, with different doses of
CHAPTER 4.2: Coated microneedles
Silicon microneedles with nanolayered ionizable surface groups

Silicon microneedles with nanolayered ionizable surface groups coated with ovalbumin (6.25 µg, 12.5 µg, 25 µg, 50 µg, 100 µg, or 250 µg) in 2 mL cuvettes. For all ovalbumin doses, the same amount of ovalbumin conjugated with Alexa Fluor® 488 (12.5 µg) was mixed with non-fluorescent ovalbumin, except the 6.25 µg and 12.5 µg dose, which were solely fluorescent ovalbumin. As a negative control, 25 µg (12.5 µg fluorescently labeled + 12.5 µg native) ovalbumin was incubated with a piranha treated surface in 1.5 mL 1 mM EDTA buffer. The binding of ovalbumin to the pyridine-modified silicon surface was determined by measuring the concentration of non-bound ovalbumin in solution at different time points (1-480 minutes, n=3, each dose on a different day) by fluorescence on a Tecan Infinite® M1000 plate reader with an excitation wavelength of 488 nm and an emission wavelength of 522 nm.

2.2.7 Ovalbumin release from pyridine-modified surfaces

In order to examine whether ovalbumin is able to detach from the chemically modified silicon slides different release media were used. Therefore, APTES and pyridine-modified silicon slides of 1 by 2.5 cm were first coated with 25 µg fluorescently labeled ovalbumin and were quantified for ovalbumin adsorption as described above. Then, the silicon slides were transferred into 1.5 mL release media, and the released amount of ovalbumin was determined by fluorescence at time points between 1 and 60 minutes. The amount of released ovalbumin was divided by the adsorbed ovalbumin to calculate the release percentage. The release from the pyridine-modified silicon slides was assessed in the following release media: 1 mM EDTA pH 5.5 with 1 M NaCl; 5 mM EDTA pH 7.4; 5 mM EDTA pH 9; 1 mM EDTA pH 10; PBS; human serum; 1 mM HEPES pH 5.5 with 1.3 mM CaCl_2; 0.1 M HCl; 1 mM EDTA pH 5.5 with 1% Triton X-100. The release of ovalbumin from an amine-modified surface was assessed in 5 mM EDTA buffer at pH 7.4 and 9.0 as a control for a “pH-independent” surface.

2.2.8 Antigenicity of released ovalbumin

The antigenicity of the released ovalbumin from the pyridine-modified surface was investigated by enzyme-linked immunosorbent assay (ELISA). Polyclonal mouse antibody against chicken ovalbumin was 2000x diluted in 0.05 M carbonate buffer at pH 9.6 and coated overnight at 4°C on a 96-well plate for ELISA (100 µL/well). Subsequently, the plate was washed 4 times with washing buffer (0.1x PBS with 0.05% (v/v) Tween 20) and blocked with 100 µL/well 1% (w/v) BSA in PBS for 1 h at 37°C. Then, 100 µL/well of solution with released ovalbumin and a calibration curve of ovalbumin (0.01-50 µg/mL) were prepared in 0.05% Tween 80, 0.5% BSA, 0.5x PBS and were incubated for 1.5 h at 37°C. Next, the plate was washed and incubated with 100 µL/well 1000x diluted polyclonal anti-ovalbumin rabbit antibody HRP conjugate for 1 h at 37°C. Subsequently, the plate was washed, 100 µL TMB was added to each well, and after 15 minutes the reaction was stopped by adding 100 µL 2 M H_2SO_4 to each well and the absorbance was measured at 450 nm on a Tecan Infinite® M1000 plate reader. The released amount of ovalbumin (R_{ELISA}) was determined from the ovalbumin ELISA calibration curve. Furthermore, the released amount of ovalbumin of the same samples was determined by the intrinsic fluorescence of ovalbumin (R_{intr. fluo}) at an excitation wavelength of 280 nm and
an emission wavelength of 320 nm. Finally, the antigenicity of the released ovalbumin was determined by equation 2:

\[
\text{Antigenicity} = \left( \frac{R_{\text{ELISA}}}{R_{\text{intr. fluo}}} \right) \times 100\% \quad \text{(Equation 2)}
\]

2.2.9 Coating of microneedles

For the assessment of the applicability of microneedles with pH-sensitive surface modifications, the surface of silicon microneedle arrays were modified with APTES or pyridine groups as described above. The in-plane microneedle arrays consist of either 9 or 17 microneedles per array with a surface of 0.3 mm² per microneedle, resulting in a total available surface area of respectively 2.7 mm² and 5.1 mm² per array. For chemical modification of the microneedles surface a highly chemical resistant coating device (figure 1A), made of polychlorotrifluoroethylene (PCTFE), was developed by the Fine Mechanical Department, Faculty of Science of Leiden University. After the chemical coating was applied, the microneedles were transferred to a polyoxyethylene (POM) holder (figure 1B), which were also used as an applicator for applying the microneedles onto the skin. The chemically modified microneedle arrays were coated with either 0.5 µg (9 microneedle array) or 1.0 µg (17 microneedle array) ovalbumin in 145 µL 1 mM EDTA at pH 5.8 for 1 h in a commercially available 96-well plate (figure 1C).

The ability to modify the surface of the silicon microneedle arrays with amine groups was assessed by using 10 µg Alexa Fluor® 488 carboxylic acid succinimidyl ester in 2 mL acetonitrile, to modify the amine groups of APTES-coated microneedle arrays. These microneedles were then photographed under a fluorescence microscope with a magnification of 100x, a GFP filter set, and an exposure time of 400 ms.

2.2.10 SEM of microneedles

To analyze the microneedle sharpness and the coating-thickness before and after the different coating procedures, a thin layer of carbon was coated on the microneedles and subsequently analyzed by scanning electron microscopy (SEM) on a FEI NOVA nanoSEM 200.
2.2.11 Fluorescence microscopy and confocal imaging of ovalbumin delivery into in vitro human skin

The dermal delivery of ovalbumin by the coated microneedle arrays was assessed on in vitro abdominal or mammary human skin, which was obtained from a local hospital within 24 h after cosmetic surgery, and was dermatomed to a thickness of 600 µm using a Padgett Electro Dermatome Model B (Kansas City, MO, USA) after the fat was removed. Pyridine pre-coated microneedle arrays (9 microneedles/array) coated with fluorescently labeled ovalbumin were three times applied on in vitro human skin for either 15, 60, or 300 seconds each time, and subsequently the skin was cleaned with MQ water. The ovalbumin-coated microneedle arrays were photographed by fluorescence microscopy (GFP filter set, 100x magnification, exposure time of 5 s) before and after they had pierced the human skin, and the in vitro human skin was analyzed by fluorescence microscopy (GFP filter set, 40x magnification, exposure time of 500 µs). Besides, to assess the deepness of the ovalbumin delivery inside in vitro human skin confocal laser scanning microscopy (CLSM) was performed with a Bio-Rad Radiance 2100 confocal laser scanning system, equipped with a Nikon Eclipse TE2000-U inverted microscope with either a 4x or 20x Plan Apo lens with an argon laser at 488 nm with a 500 nm long pass emission filter. For controlling the confocal laser scanning system and for image acquisition the Laser Sharp 2000 software (Bio-Rad, Hercules, USA) was used. Images of the conduits created by the microneedles in in vitro skin were taken with an xz scan from 0-600 µm, with a depth resolution of 1.05 µm. Furthermore, xyz-images were taken every 10 µm until a depth of 400 µm was reached.

2.2.12 Delivery of radioactively labeled ovalbumin into in vitro human skin

To quantify the amount of protein that was delivered into in vitro human skin from a “pH-sensitive” and a “pH-independent” surface, we used radioactively labeled ovalbumin to bind to the surface of pyridine-modified and amine-modified microneedles. Therefore, 1 mg ovalbumin in 1 mL PBS was labeled with \(^{125}\text{I}\) by incubating it with 4.6 MBq Iodine-125 in the presence of 4 iodination beads, according to the manufacturer’s protocol. Radio-labeled ovalbumin was purified by washing it three times with PBS in a 30 kDa centrifugal filter unit. Subsequently, microneedle arrays with 17 microneedles were coated for 1 h with 1 µg radio-labeled ovalbumin (dissolved in 1 mM EDTA, pH 5.8), which were subsequently air-dried, and were applied on in vitro human skin for one minute. The γ-counts of the microneedle arrays before and after penetration, and of the microneedle-pierced skin were measured for 5 minutes on a Perkin Elmer Wallac Wizard 1470 Automatic Gamma Counter.

3. Results

3.1 Surface modification and analysis of flat silicon surfaces and ovalbumin charge

To test whether the silicon surface was successfully modified, we used Alexa Fluor® 488 carboxylic acid succinimidyl ester, a fluorescent dye that only reacts with primary amines. The dye was incubated with a non-modified surface, an amine-modified surface, and an
amine-modified surface that was derivatized into a pyridine-modified surface. Subsequently, fluorescence microscopy images of these surfaces were 3D-analyzed by plotting the pixel intensity as a function of the position on the surface, as shown in figure 2. This figure shows that the silicon surfaces were successfully modified with amine groups (2C and 2D vs 2A and 2B) and that these surfaces were subsequently successfully derivatized into pyridine groups (2E and 2F vs 2C and 2D) with an efficiency of 78.5±4.8% (n=3, mean ± SD) as calculated by equation 1.

Subsequently, the surface pKₐ of the pyridine-modified silicon surface and the pI of ovalbumin were determined since both the surface charge of the silicon surface and the charge of the protein as a function of the pH are very important for a protein to bind to a chemically modified ionizable silicon surface. Figure 3A shows the results of a surface pKₐ determination of a pyridine-modified silicon surface by the fluorescent nanoparticle adhesion assay, and figure 3B shows the pH-dependent zeta potential of ovalbumin determined by laser Doppler electrophoresis.
laser Doppler electrophoresis. The surface $pK_a$ of pyridine-modified surfaces determined by the Henderson-Hasselbalch equation was $6.91 \pm 0.30$ (n=3, mean ± SD), thus at a pH < 5.9 more than 90% of the pyridine-modified surface is positively charged. The zeta potential of ovalbumin was gradually decreasing between pH 3 and 7, and had a pI of $4.36 \pm 0.21$ (n=3, mean ± SD), thus having a negative charge above that pH value [13, 14]. As protein solubility and stability is minimal at pH= pI [15], an optimal adsorption of ovalbumin to pyridine-modified surfaces is expected in a pH range of 5.5 to 6.5.

To determine the surface properties of the non-modified and chemically modified surfaces before and after ovalbumin binding, the surfaces were analyzed by atomic force microscopy [13]. Non-modified surfaces were very flat with an arithmetic average of the 3D roughness of $0.332 \pm 0.063$ nm. Furthermore, when the surfaces were modified with pyridine groups and subsequently coated with $5 \mu g/cm^2$ ovalbumin in 1 mM EDTA at pH 5.8 the arithmetic average of the 3D roughness was $0.925 \pm 0.049$ nm (n=3, mean ± SD) and $0.627 \pm 0.147$ nm (n=3, mean ± SD) respectively. This indicates that after chemical modification and after coating with ovalbumin the surface was still very flat and comparable to the 3D roughness of surfaces with similar modifications with APTES described in the literature [12, 13, 16, 17].

3.2 Coating of flat silicon surfaces with ovalbumin

Pyridine-modified surfaces ($5 \text{ cm}^2$) were coated with ovalbumin at pH 5.8, a pH value that assures a high positive charge on the pyridine-modified surface (pyridine groups 93% protonated, as calculated by the Henderson-Hasselbalch equation) and is well above ovalbumin’s pI resulting in a negative charge for ovalbumin. Different doses of ovalbumin, corresponding to surface densities up to $20 \mu g/cm^2$, were coated on the pyridine-modified surfaces, as shown in figure 4A. For each dose of ovalbumin the maximum coating was reached within 2 hours with an average coating efficiency of 95%, which was comparable to ovalbumin coating efficiencies on plain amine-modified silicon surfaces. However, when we incubated a non-modified surface with ovalbumin ($25 \mu g/5 \text{ cm}^2$) at pH 5.8 for 2 hours, the coating efficiency was only $1.85 \pm 0.93\%$ (mean ± SD, n=3). This shows that chemical modification of the flat silicon surfaces drastically increases protein adsorption.

3.3 Release of ovalbumin from flat silicon surfaces

The release of ovalbumin from a “pH-sensitive” pyridine-modified surface was assessed under various conditions and was compared to that from a “pH-independent” amine-modified surface, as shown in figure 4B and 4C. This images show that at pH 5.8, as expected, no ovalbumin was released from a “pH-sensitive” pyridine-modified surface. However, when the pH was increased to 7.4 or higher, more than 45% of the ovalbumin was released from the pyridine-modified surface. This was 2-3 times as much compared to the release from a “pH-independent” amine-modified surface at the same pH value, indicating that a “pH-sensitive” surface promotes the release of ovalbumin. Despite the partial release, we found that ovalbumin did not lose its antigenicity after it was released from the chemically modified
surface in 5 mM EDTA at pH 7.4, being 103.0±29.8% (mean ± SD, n=3).

Other components in the skin such as lipids, surfactants, salts and calcium ions could induce the release of ovalbumin from pyridine-modified surfaces. Therefore, the effect of these components was assessed at pH 5.8 where the pH-induced release is minimal. In figure 4C it is shown that a high salt concentration (1 M NaCl), a surfactant (Triton X-100), and Ca\(^{2+}\) induced partial release of ovalbumin from the silicon surface (albeit substantially less than at neutral or alkaline pH), indicating that for skin delivery factors other than pH could also play a role. In contradiction to the release of ovalbumin from pyridine-modified surfaces in a 5 mM EDTA buffer at pH 7.4 hardly any ovalbumin was released in PBS at the same pH (2.01±0.07%), which is probably due to crystal formation on the pyridine-modified surfaces when PBS is used (data not shown). Furthermore, when an acidic solution (0.1 M HCl), which has a pH below the pI of ovalbumin, was used to make the pyridine surface as well as ovalbumin positively charged, 20% ovalbumin was released, likely due to repulsive electrostatic forces.

3.4 Application of the chemical modification and coating technology to silicon microneedle arrays

In the previous sections we demonstrated the successful modification and subsequent coating with ovalbumin of flat silicon surfaces. The next step was to apply the pH-sensitive coating on the surface of silicon microneedle arrays. Therefore, we used a specially designed coating device, as shown in figure 1A. To assess the chemical modification of the microneedle
CHAPTER 4.2: Coated microneedles
Silicon microneedles with nanolayered ionizable surface groups

3.5 Release of ovalbumin from microneedles into in vitro human skin

To examine the applicability of the coating technology on microneedles in vitro, we first investigated the effect of wear time (the time that a microneedle array stays inside the skin) and number of applications of one coated pH-sensitive microneedle array on the release of ovalbumin from the microneedles into the skin. Therefore, pH-sensitive microneedle arrays coated with fluorescently labeled ovalbumin (Alexa Fluor® 488) were applied three times with different wear times between 15 and 300 s on in vitro human skin, as shown in figure 5A. We observed that, independent of the wear time, most of the ovalbumin was released after the first application (representative fluorescence microscopy images of a 3×15 s application into in vitro human skin, figure 5B). Furthermore, based on the fluorescence of the microneedles before and after skin application we conclude that most of the fluorescently labeled ovalbumin disappeared from the microneedles after a single application into the skin (figure 5D). However, when pH-independent amine-modified microneedle arrays coated with fluorescently labeled ovalbumin were pierced into the skin, hardly any fluorescence was visible in the skin samples (data not shown). Furthermore, these ovalbumin-coated microneedle arrays had about the same fluorescence intensity by fluorescence microscopy before and after they had penetrated the skin (data not shown). These results were confirmed by doing a similar study with radioactively labeled ovalbumin: with our pH-sensitive coating (single application of 60 seconds) we found that 71.2±10.0% (mean ± SD, n=3) of the ovalbumin was released from the microneedles into in vitro human skin. Regarding the coating and release efficiency of ovalbumin from the pH-sensitive coated microneedle arrays, for dermal vaccination a sufficient dose of coated ovalbumin can be delivered by microneedles. However, when we used a “pH-independent” amine-modified surface coating only 4.73±2.87% (mean ± SD, n=3) of the radioactively labeled ovalbumin was released from the microneedles. These data, combined with the release of ovalbumin from flat silicon chemically modified surfaces, show that pH-triggered release is crucial for effective delivery of ovalbumin into human skin.

Microneedles often require the use of an applicator since microneedles regularly do not penetrate the skin completely upon manual application onto skin [1, 18-20]. Therefore,
Figure 5: (A) Pyridine-modified microneedles coated with 1 µg fluorescently labeled (Alexa Fluor® 488) ovalbumin were applied three times on in vitro human skin with three different wear times (15, 60, and 300 seconds). The bars represent the pixel intensity (mean ± SD, n=3) of the fluorescence of the microneedle holes filled with fluorescent ovalbumin and the control represents the background fluorescence of the skin. (B) Representative example of three successive microneedle piercings (P1, P2, P3) with one coated microneedle array in human skin in vitro, with a magnification of 40x (B1) and 100x (B2). (C) Microneedles to which a fluorescent dye (Alexa Fluor® 488) is covalently coupled to its surface. (D) Ovalbumin-coated microneedle array before skin penetration (D1) and after 3x application for 15 seconds (D2).

Figure 6: A) Visualization by confocal laser scanning microscopy of ovalbumin delivery by ovalbumin-coated pyridine-modified microneedle arrays. Ovalbumin was fluorescently labeled with Alexa Fluor® 488. On the xy-plane the surface of in vitro human skin is shown after microneedle penetration (40x magnification, 2980x2384 µm), and on the xz plane an in-depth scan of ovalbumin delivery in the upper row of microneedle penetrations is shown. B) xyz visualization of ovalbumin delivery into a single microchannel in in vitro human skin (200x magnification, 596x477 µm).
the depth of ovalbumin delivery into in vitro human skin was determined by CLSM after the application of ovalbumin-coated pH-sensitive microneedle arrays on human in vitro skin using the manual applicator. Furthermore, CLSM was used to visualize the microchannels generated by the pH-sensitive pyridine-modified microneedle arrays coated with ovalbumin. Figure 6A shows an xy-image of the surface of the skin and at the position of the microchannels an in-depth scan was made from the surface of the skin to a depth of 600 µm. Furthermore, xyz-images were made with a magnification of 200x to visualize the ovalbumin delivery into a single microchannel (figure 6B). Of the confocal microscopy images 3D reconstructions of the ovalbumin delivery into the skin were made (movie available online, http://pubs.rsc.org/en/Content/ArticleLanding/2013/TB/c3tb20786b#!divAbstract). These images show that there was almost no ovalbumin present on the surface of the skin, but that it was rather delivered into the epidermis and dermis. Furthermore, these images show that ovalbumin was delivered up to 300 µm in depth, indicating that the whole microneedle had pierced the skin.

To determine to what extent the coating changed the structure of the microneedles, the microneedles were visualized by SEM before and after the different coating procedures (shown in figure 7). The tip of non-modified microneedles was very sharp (diameter < 10 nm), and therefore the application of these microneedles by using an applicator for manual piercing was sufficient to penetrate the skin. Also, after the surface of the microneedles was chemically modified with amine groups, pyridine groups, and subsequently coated with ovalbumin, the microneedle tip was still very sharp (diameter < 100 nm). After the coated microneedles were pierced into the skin, the tips of the microneedles were still very sharp (diameter < 100 nm), indicating that the needles stay intact after they had pierced the skin.
4. Discussion

Coated microneedle arrays have great potential for pain free administration of potent biologicals, such as therapeutic proteins and vaccines, via the skin [1, 3]. In the design of such devices, it is important to have a high coating efficiency as well as a high release efficiency of the coated drug into the skin in order to minimize the loss of expensive drug. We successfully developed a novel coating procedure which is based upon pH-dependent electrostatic interactions, whereby proteins are efficiently coated onto microneedles and efficiently released from microneedles into the skin.

For initial experiments plain silicon slides were used instead of microneedle arrays, because it is easier and more accurate to measure derivatization and coating efficiencies on a large flat surface than on the small tip of a microneedle.

The coating efficiency of flat silicon surfaces was very high (95%) for coating densities up to 20 µg/cm² (figure 4A). Moreover, ovalbumin was successfully released from the pH-sensitive surface in a pH-dependent manner. However, under none of the incubation conditions (figure 4B) ovalbumin release from flat pyridine modified silicon surfaces was complete. One of the reasons is the presence of residual surface charge on the modified surfaces at physiological pH. Although 79% of the surface amine groups were derivatized into pyridine groups (figure 2), still 21% of the primary amine groups are unmodified. About 97% of these primary amine groups will be protonated at physiological pH [12]. Furthermore, pyridine-modified surfaces have a pKₐ of 6.91 (figure 3B), meaning that 39.4% of the pyridine groups are charged at physiological pH. Combining these two effects one can calculate that at physiological pH 51% of the ionizable (pyridine and APTES) groups will be protonated and thus can still electrostatically interact with the negatively charged ovalbumin (especially because the negative charge of ovalbumin will be higher at pH 7.4 than at the coating pH of 5.8). Furthermore, these data combined with the low release with 1 M NaCl at pH 5.8 indicates that the release mechanism of ovalbumin from pyridine-modified silicon surfaces was predominantly pH-dependent, rather than ionic strength dependent. Besides, this technology is promising for vaccination since the released ovalbumin from pyridine-modified silicon surfaces did not lose its antigenicity as shown by ELISA. This indicates that the native epitopes of ovalbumin, which are recognized by the ovalbumin specific antibodies, are still intact.

The little release of ovalbumin from “pH-independent” amine-modified flat silicon surfaces (figure 4B) can be explained by the fact that APTES surfaces have two pKₐ values (6.55 and 9.94), where the first pKₐ accounts for about 25% of the surface charge [12]. This implies that at pH 7.4, only 22% of the APTES surface is deionized. However, a high pH (9.4) of the release medium did not lead to a higher release of ovalbumin from an amine-modified and a pyridine-modified surface, even though at that pH value respectively 42% and 83% of the ionizable surface groups should be uncharged. This may be caused by an increased negative charge of ovalbumin at elevated pH (figure 3A), conformational changes of ovalbumin in the adsorbed state and/or non-electrostatic (e.g., hydrophobic) interactions contributing to adsorption.
Similar to plain silicon surfaces, the surface of microneedle arrays was successfully chemically modified (figure 5C) and coated with ovalbumin (figure 5D1). As determined by SEM, the coating thickness on flat silicon surfaces, including the cutting planes of the microneedles, was very small (< 20 nm) after chemical modification and after ovalbumin coating (figure 7). In addition, coated silicon surfaces retained a very flat surface, as determined by AFM. These results were similar to comparable modifications reported in the literature [12, 13, 16, 17]. Furthermore, the coating thickness at the cutting edges of the microneedles was larger (100-1000 nm) after APTES coupling, indicating that APTES tends to aggregate at the edges of surfaces. However, ovalbumin coating on the pH-sensitive microneedles was much thinner than most coatings reported in the literature, which are generally 3-15 µm thick [8, 10, 11, 18]. Therefore, this nanolayered pH-sensitive coating retains the geometry of the microneedles without compromising their sharpness.

Ovalbumin-coated pH-sensitive microneedles penetrated human skin successfully and delivered the fluorescently labeled ovalbumin up to a depth of 300 µm (figure 6). The major fraction of the fluorescently labeled ovalbumin dose was released from the pH-sensitive coating after a single application for 15 s into human skin (figure 5). This was confirmed by a radioactivity assay demonstrating that the release of ovalbumin from a pH-sensitive microneedle array into in vitro human skin was 71% after a single application of 60 s. This delivery efficiency was high compared to that of other coatings described in the literature, which show a release of the active compound varying between 1-91% mostly within 15 minutes, depending on the coating procedure [5, 7, 10, 11, 21-24]. Moreover, a relative high amount of active compound is lost during these coating procedures, in shrill contrast with our novel coating method.

In contrast to the pH-sensitive coating, the pH-independent coating resulted in only 5% release of the ovalbumin from a microneedle array, which shows the necessity of the pH-sensitive coating for the delivery of ovalbumin. Furthermore, the release efficiency of ovalbumin from the microneedle arrays into in vitro human skin differed from that of flat silicon surfaces into a buffered solution: the release from a pH-independent microneedle was lower than that from a pH-independent flat surface, and the release from a pH-sensitive microneedle was higher than that from a pH-sensitive flat surface. These differences may be caused to some extent by the analytical method (fluorescence vs. radioactivity) but more importantly to the differences in release medium (the complex environment in human skin, containing lipids, calcium, and proteins, vs. a simple buffered solution), suggesting that pH is not the only factor contributing to the release of ovalbumin into human skin.

Despite the protein ovalbumin showed an excellent binding onto and an excellent release from pyridine-modified silicon microneedles, this approach will not be suitable for each protein. In the case of ovalbumin, the protein bound reversibly onto the surface, but proteins may undergo conformational changes in the adsorbed state, aggregate and/or bind irreversibly onto surfaces, as reviewed by Rabe et al. [25]. Furthermore, the packing, loading and the reversibility of protein adsorption is dependent on many factors, including the buffer...
composition, ionic strength, pH, temperature, type of protein (e.g. hard/soft), shape of the protein, and the distribution of charge and isoelectric point of the protein [25-27]. Therefore, for some proteins it might be beneficial to encapsulate them into micro/nano-particles prior to microneedle adhesion.

Drug delivery into the skin via microneedles is minimally-invasive and potentially pain free and can be achieved via several approaches. However, for each approach it is important to minimize the amount of drug loss and to deliver the drug in a reproducible manner and fast (especially for patient compliance in vaccination). Therefore, this novel approach of coating microneedles in a pH-dependent manner has great advantages: the coating and release efficiencies are high, and the major part of the drug is released within 15 s. Furthermore, the pH-sensitive microneedle coating can be combined with a Layer-by-Layer coating, whereby multiple nanolayers are formed by alternate coating of positive and negatively charged polyelectrolytes [28, 29]. Besides, drugs that are encapsulated into micro- or nanoparticles can be coated on the surface of microneedles, thus this pH-sensitive coating procedure has a broad application ability to deliver drugs into the skin by microneedles. Furthermore, the developed microneedle holders for 96-well plates render the coating procedure amenable for high throughput screening of different coating excipients/compounds (figure 1) and the coating procedure can be fully automated.

5. Summary and conclusion

Silicon surfaces were successfully modified with pyridine groups with a high derivatization efficiency of 79%. Because ovalbumin has a pH of 4.4 and the pyridine-modified surfaces were shown to have a surface pH of 6.9, there is a coating window between pH 5.4-6.8. Pyridine-modified silicon surfaces were coated with ovalbumin at pH 5.8 in a 1 mM EDTA buffer without using further excipients. This led to a high coating efficiency of ovalbumin (> 95%) with a dose up to 20 µg/cm² within 2 h coating time. The in vitro release of ovalbumin at physiological pH was 45% within the first minute whereby the released protein retained its antigenicity.

Microneedles modified with the pH-sensitive surface modification and subsequently coated with ovalbumin, released the major fraction of ovalbumin after a single application of 15 s into in vitro human skin. Furthermore, the microneedle coating minimally affected the microneedle’s geometry and sharpness, since the coating on the cutting planes was < 20 nm thick and the tip diameter of the microneedles was < 100 nm after coating.

In conclusion, this nanolayered pH-sensitive coating for microdevices may provide a useful tool for the delivery of potent therapeutic proteins and vaccines.

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CHAPTER 4.2: Coated microneedles
Silicon microneedles with nanolayered ionizable surface groups