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Polysialic acid immobilized on silanized glass surfaces: a test case for its use as a biomaterial for nerve regeneration

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Abstract The immobilization of polysialic acid (polySia) on glass substrates has been investigated with regard to the applicability of this polysaccharide as a novel, biocompatible and bioresorbable material for tissue engineering, especially with regard to its use in nerve regeneration. PolySia, a homopolymer of α-2,8-linked sialic acid, is involved in post-translational modification of the neural cell adhesion molecule (NCAM). The degradation of polySia can be controlled which makes it an interesting material for coating and for scaffold construction in tissue engineering. Here, we describe the immobilization of polySia on glass surfaces via an epoxysilane linker. Whereas glass surfaces will not actually be used in nerve regeneration scaffolds, they provide a simple and efficient means for testing various methods for the investigation of immobilized polySia. The modified surfaces were investigated with contact angle measurements and the quantity of immobilized polySia was examined by the thiobarbituric acid assay and a specific polySia-ELISA. The interactions between the polySia-modified surface and immortalized Schwann cells were evaluated via cell adhesion and cell viability assays. The results show that polySia can be immobilized on glass surfaces via the epoxysilane linker and that surface-bound polySia has no toxic effects on Schwann cells. Therefore, as a key substance in the development of vertebrates and as a favourable substrate for the cultivation of Schwann cells, it offers interesting features for the use in nerve guidance tubes for treatment of peripheral nerve injuries.

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1 Introduction

Injuries of peripheral nerves result in loss of neural functions accompanied by partial or total loss of motor, sensory, and autonomic functions in those parts of the body which are not supplied by nerves anymore. Regeneration of nerve function can only occur if surviving neurons are present because mature neurons have lost their ability for cell division. Under the right conditions, regeneration over a short gap is possible by axon extensions across the gap. Over larger gaps (>10 mm), the regenerating axons have to be guided to reconnect the proximal with the distal end [1].

The gold standard of clinical nerve repair includes suture techniques. In larger gaps there is no possibility for a tension-free suture, so they are bridged with grafts. These grafts are commonly autologous, but also artificial nerve grafts are used. The purpose of introducing a graft between the two ends of a transacted nerve is to offer mechanical guidance as well as an optimal environment for the advancing axonal sprouts. Autografts are segments of nerves of lesser functional importance (e.g. the sural nerve) which are removed from a second operation site of the patient. Disadvantages of autografts include the need for a second surgical procedure including the risk of operation trauma, low availability of adequate donor nerves, denervation on the donor site, limitation in length and thickness of the nerve graft, and size and quality (sensory versus

motor nerves) mismatch with the host nerve [2, 3]. Also mixed grafts from veins and muscles are used with good regeneration rates but with a high technical expenditure [4]. The use of allografts is accompanied by the need of immunosuppression and poor success rates [5].

Artificial nerve guidance tubes are alternatives with a high potential for the regeneration of nerves, avoiding the low availability of autologous nerve grafts and immunosuppression of allogene grafts. This method consists of suturing the nerve stumps into a tubular guide which limits the ingrowth of fibroblasts into the repair site and thus avoids excessive collagen and scar formation. In addition, it provides directional guidance to the regenerating axons and prevents axonal escape into the surrounding tissue. Such artificial nerve guidance channels are mostly made of polymers like collagen [6], poly-L-lactide (PLLA), poly-L-lactide-ε-caprolactone [7], trimethylene carbonate-caprolacton block copolymer (TMC-CL) [8] or silicone [9]. Artificial nerve grafts made of non-degradable polymers like silicone have the advantage of inertness and elasticity, however they are not biodegradable so that a second operation to remove the silicone graft is necessary in order to avoid chronic compression of the regenerated nerve [10, 11]. The action as a nerve guidance channel can be considerably improved when the tubes are filled with scaffolds which provide a favourable medium for nerve growth. For this purpose, the polymer tubes are filled, for example, with neurotrophic factors, laminin or autologous Schwann cells for improved regeneration [12–14].

Another interesting substance which could have favourable properties on nerve regeneration is polysialic acid (polySia). This is a negatively charged carbohydrate polymer consisting of α-2,8-linked sialic acid [15]. It occurs in embryonic developmental stages as a post-translational modification of the neural cell adhesion molecule (NCAM) and appears to play a crucial role in the motility of neurites during development and regeneration [16]. PolySia enhances migration of neural stem cells and promotes neurite outgrowth [17, 18]. It plays a crucial role in synaptic processes in learning and memory [19, 20]. Removal of polySia from NCAM by endoN inhibits cell migration in wild type animals and is therefore expected to play a crucial role in nerve branching during development and nerve regeneration [21]. As a substance which occurs in the body, polySia should be a perfectly biocompatible and non-immunogenic polysaccharide. Furthermore, it can be degraded in a controlled way by the polySia-specific enzyme endoneuraminidase (endoN) [22, 23]. This enzyme was isolated from certain bacteriophages, e.g. *Escherichia coli* K1. These infect bacteria which use polySia as a capsule [24, 25].

In the present study we report on the immobilization of polySia on different glass substrates as a first step with regard to its further application as a scaffold for nerve regeneration. We chose glass surfaces for this initial study because the different physical, chemical and biological characterization techniques can easily be applied to such samples, although glass will not actually be used in nerve regeneration devices. For immobilization, different glass substrates were silanized with an epoxysilane and then polySia was covalently attached to the modified surfaces. (3-Glycidyloxypropyl)trimethoxysilane (GOPTMS) has already been used to couple oligonucleotides to glass surfaces [26] and should preferentially react with primary amino groups of deacetylated polySia. The quality of the polySia-modified glass substrates was tested with contact angle measurements and polySia-specific ELISA. In cell cultures, we also investigated the influence of the polySia-modification on immortalized Schwann cells in cell viability and adhesion tests. It is of interest to note that polySia bound to glass surfaces in a certain way resembles its physiological form where it occurs attached to membrane-standing lipids (as in the capsules of bacteria) or membrane-standing proteins as NCAM (in the nervous system of vertebrates).

2 Materials and methods

2.1 Preparation of epoxysilane-coated glass surfaces

(3-Glycidyloxypropyl)trimethoxysilane (GOPTMS) (Fluka, Germany) was coupled to microscopic slides (Elka, Sondheim, Germany), cover slips (diameter: 10 mm; Menzel-GmbH & Co KG, Braunschweig, Germany) or glass frits (porosities 3, 4 and 5; ROBU-Glas, Germany) via a dip-coating procedure. Before surface modification the substrates were cleaned in Piranha solution (H_2SO_4 : H_2O_2 6:5) for 15 min, thoroughly washed with water and dried with compressed air. The substrates were then immediately transferred into a solution of GOPTMS (0.5–25 mass%). After 10 min the substrates were slowly pulled out and incubated for 5 min above the solution. The substrates were dried for 1 h at 60°C, then rinsed with acetone to remove excessive GOPTMS and dried with compressed air.

Glass frits were broken into smaller pieces, with masses between 200 and 400 mg, for the dip-coating procedure. Absorbance values observed on such samples using different characterization methods (see below) were scaled to a unit mass of 1 g of glass frit material to simplify comparison between the different samples.

2.2 Coupling of polysialic acid to epoxysilane-modified substrates

Polysialic acid was purchased as colominic acid from Nacalai Tesque, INC. (Kyoto, Japan). Before coupling,

polysialic acid was deacetylated. After adding 60 mL of ethanol (abs., Merck, Germany) to a solution of 2.4 g sodium hydroxide (Acros Organics, Belgium) in 3.9 ml of water, 1 g of polysialic acid was given to the solution. The mixture was heated to 80° over night, then dialyzed against water (pH 9) and completely dried. The epoxysilane-modified substrates were put in a solution of the deacetylated polysialic acid (0.0005–0.05 M) in a slide box. After the reaction the substrates were rinsed with water and dried with compressed air.

2.3 Contact angle measurements

Static contact angle measurements were performed using the Surftens universal contact angle goniometer (OEG, Frankfurt/Oder, Germany). All reported values were an average of at least eight measurements, the standard deviation did not exceed 3° in most cases.

2.4 polySia-ELISA

The polysialic acid modified cover slips were blocked with 1% bovine serum albumine (BSA) in phosphate buffered saline (PBS) for 30 min. The cover slides were washed three times with PBS and incubated with primary anti-polysialic acid antibody (5 µg/ml in PBS + 1% BSA) for 1 h at room temperature. After washing the cover slips three times with PBS, the secondary goat-anti-mouse horseradish peroxidase (goat-anti-HRPO) conjugated antibody was added (1:3000 in PBS + 1% BSA). After 1 h the cover slips were washed three times with PBS. The ELISA was developed via the ABTS [2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] reaction. The absorption was measured on a micro-plate reader (Bio-Rad, München, Germany) using a 405 nm filter. As a negative control untreated cover slips were used.

2.5 Cell experiments

Immortalized Schwann cells (ISC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FCS (fetal calf serum), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and sodium pyruvate (1 mM) at 37°C (5% CO_2). Before cell adhesion and cell viability experiments glass frits were disinfected with isopropanol (70%) over night and subsequently incubated in the respective cultivation medium for another night. After removing the medium each glass frit was inoculated with 3,000 cells in a total volume of 40 µl. The glass frits were covered with culture medium 2 h after incubation. The medium was changed every third day.

2.6 Cell adhesion

Cell adhesion of ISC on the glass frits modified with polysialic acid was determined by DAPI (4′,6-Diamino-2-phenylindole) staining two weeks after incubation. DAPI is a fluorescent molecule which binds to the DNA. After washing the glass frits with PBS, the cell nuclei were stained with 200 µl DAPI solution using a standard protocol [27]. The glass frits were incubated at 37°C for 20 min in the dark and then washed three times with PBS. Subsequently the glass frits were monitored with UV light using a fluorescence microscope (Olympus, Hamburg, Germany).

2.7 Scanning electron microscopy of cell-seeded glass frits

ISC cells were seeded on the modified glass frits for two weeks, afterwards washed three times with PBS and fixed by using Karnovsky fixative at 4°C over night. After washing with cacodylate buffer (0.2 M ddH_2O, pH 7.3) and PBS, the solutions were stepwise covered with solutions with increasing concentrations of acetone. The incubation time was 15 min at 4°C at each step, the last step was repeated three times. After removal of the acetone the glass frits were dried. The scanning electron microscopy (SEM) images were taken with the Philips 30 XL ESEM.

2.8 Cell viability

The viability of cells can be measured via the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] assay (Sigma–Aldrich, Steinheim, Germany). The MTT assay measures the mitochondrial activity of living cells. Mitochondrial dehydrogenase enzymes hydrolyse the tetrazolium ring of the MTT to the insoluble blue formazan [28, 29].

ISC were cultured on polySia-modified glass frits for 14 days. 300 µl medium [DMEM + 10% FCS + 1% sodium pyruvate + 1% L-glutamine + 1% antibiotics (Penicillin/Streptomycin)] and 30 µl of MTT solution were added to each well and incubated 4 h at 37°C (5% CO_2). After sterile filtration 300 µl sodium dodecyl sulphate solution (1 g/10 ml 0.01 M HCl) were added and incubated for 18 h. The absorption was measured at 570 nm using a microplate reader (Bio-Rad, München, Germany).

2.9 TBA assay

The thiobarbituric (TBA) assay is the most widely used assay for determining sialic acids [30–32]. The free reducing ends of polySia are cleaved by acidic periodate,

the emerging formylpyruvate reacts with TBA to form a pink coloured chromophore.

PolySia-modified glass frits were treated with a solution of 25 µl H_3PO_4 (0.1 M) and 225 µl H_2O for 18 h. After neutralizing with 100 µl NaOH (0.025 M) the solution was treated with 100 µl acidic solution of periodic acid for 30 min at 37°C. The excess of periodate was reduced with 500 µl sodium arsenite (0.4 M in 0.2 M H_2SO_4). As soon as the yellow colour disappears 500 µl of TBA (3% in NaOH) were added and incubated for 13 min at 95°C. After cooling the coloured solutions in a water bath, 400 µl were mixed with 700 µl cyclohexanone. The intensities in the organic layer were measured at 549 nm with multiplate reader (Multiskan spectrum, Thermo labsystems, Waltham, USA).

3 Results

3.1 Immobilization of polysialic acid and contact angle measurements

Epoxysilane films on glass slides were prepared by reaction of GOPTMS from aqueous solutions with 0.5–25 mass% of the epoxysilane compound (Fig. 1). In a second step these epoxysilane films were incubated in a solution of deacetylated polysialic acid (0.02 M). Table 1 presents the apparent water contact angles for the films prepared for different concentrations of the epoxysilane and different incubation times in polySia solution. Generally, the contact angle of pure epoxysilane films is higher for higher concentrations of GOPTMS. The contact angle increases gradually with epoxysilane concentration from 40 to 71°. The presence of polySia on the surface leads to smaller contact angles which is expected because of the high hydrophilicity of polySia when compared with the more hydrophobic epoxysilane. For a 1%-epoxysilane film the contact angle decreases from 40° for the pure film to 33° after 3 days of deposition in a solution of polySia. After 3 days of storage of a 10% film of GOPTMS in polySia, the contact angle is 37° and thus similar to the films prepared from 1 and 5% epoxysilane concentration. When other concentrations of polySia are used, slight differences can be seen after 4 h, with contact angles of 37° (0.02 M) and 43° (0.0005 M), but after 3 days the contact angle for the lower concentration equals the one for the higher concentration.

Table 1 Contact angles of water (in °) on silanized glass surfaces incubated for different times in polySia solution (0.02 M) and in pure water

Mass% GOPMTS	No polySia	4 h polySia	3 days polySia	1 day H_2O
1	40	38	33	40
5	47	37/43[a]	31	46
10	57	53	37	57
15	70	50/54[a]	–	70
20	71	56	–	70

[a] c(polySia) = 0.0005 M

The epoxysilane films are completely stable in water, there is no change in the contact angle between the freshly prepared films and those which were incubated for 1 day in pure water. If the pH value of the water is raised to 10 and thus equals the pH of the polySia solution, the contact angle of a 10% epoxysilane films decreases from 57 to 40° after 4 h and to 18° after 1 day. Therefore, the contact angle measurements indicate that pure epoxysilane films are decomposed at higher pH values whereas they are stable at a neutral pH. When the epoxysilane films have been in contact with the polySia solution at the same pH value, there appears to be no decomposition of the epoxysilane films, as the contact angle does not decrease below 31°. Increasing the incubation time of GOPTMS films in polySia solutions produces more strongly hydrophilic surfaces, but 31° is the minimum contact angle we have observed in a variety of experiments with different exposition times and different concentrations of GOPTMS and polySia solutions.

3.2 TBA assay

The thiobarbituric acid assay detects the free reducing ends of sialic acids and quantifies their amount. Figure 2 shows

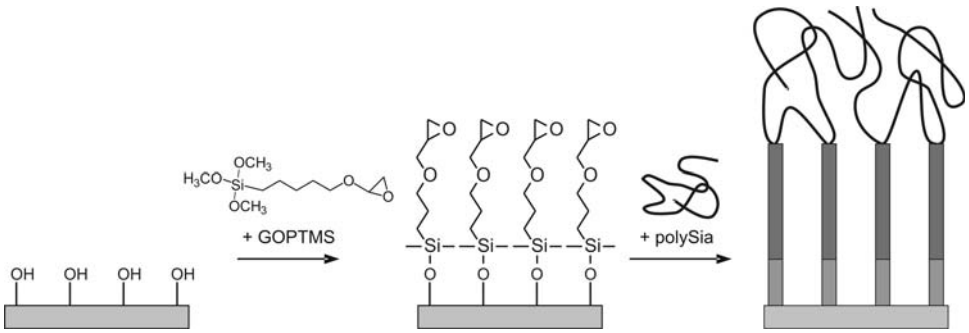

Fig. 1 Scheme for the surface attachment of polysialic acid using the GOPTMS linker

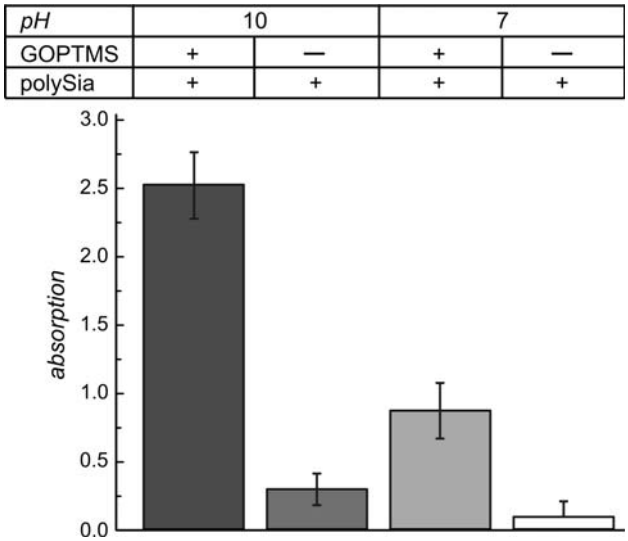

Fig. 2 TBA assay from glass frits with absorbed polySia and polySia immobilized on GOPTMS-modified glass at different pH values. The absorption values refer to 1 g of the original glass frit material

the results of the TBA assay on glass frits modified with GOPTMS and polySia at different pH values. The highest absorption of 2.5 resulted on glass frits modified with the epoxysilane and polySia at a pH value of 10. The second highest absorption, but about 5 times lower, was achieved by coupling the polySia to the epoxysilane at pH 7. When polySia is only physically adsorbed on glass frits, absorptions per mass units between 0.1 and 0.25, depending on the pH value of the polySia solution (7 and 10, respectively) are observed. Consequently, covalent attachment of polySia leads to a much higher amount of immobilized polySia than a simple adsorption, and the binding reaction between the epoxysilane and polysialic acid should be carried out at a pH value of 10.

3.3 polySia-ELISA

To evaluate the amount of polysialic acid immobilized on the prepared materials, a direct ELISA with horseradish peroxidase-conjugated secondary antibody was performed. Figure 3 shows the results of surface modification of cover slips with GOPTMS and polySia. With polySia present on the surface, the absorption is much higher when polySia is bonded to the epoxysilane and not just physically adsorbed on the surface. This indicates that polySia is covalently bound to the epoxysilane and immobilized on the surface. When an unmodified glass surface is exposed to a polySia solution, the absorption is only slightly higher than for those surfaces which have never been exposed to a polySia solution. Physical adsorption of polySia is much more pronounced when glass frits are used instead of cover slips (data not shown), possibly due to the porous nature of the former.

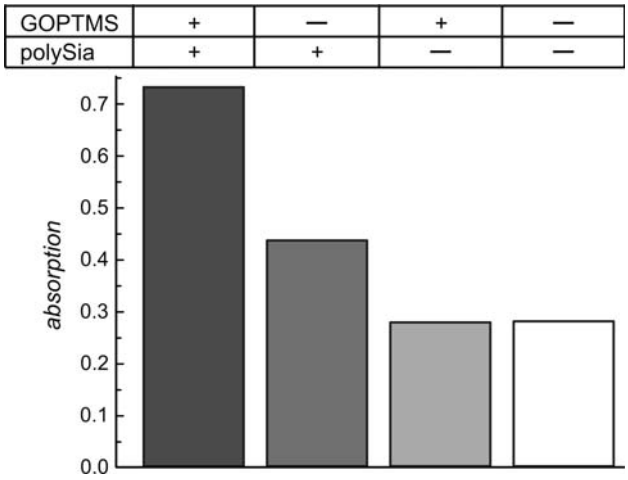

Fig. 3 Results of polySia-specific ELISA tests on modified cover slips

3.4 Cell adhesion

Figure 4 shows the results of cell adhesion tests of ISC on differently modified glass frits. The cells were fixed and stained with DAPI after a culture time of 14 days. There are no significant differences in the numbers of adhering cells on the differently modified surfaces. On unmodified glass frits, the number of cells is as high as on polySia-modified surfaces. Therefore, we state that polySia, when bound to a glass surface via an epoxysilane linker, does not provoke toxic effects and appears as a biocompatible material.

3.5 SEM

After fixation of cells on modified glass frits, scanning electron microscopy images were taken. There are no differences in cell spreading between glass frits, either unmodified or modified with epoxysilane and/or polySia (data not shown). Cutting the glass frits perpendicular to the surface revealed that there has been no cell migration toward the interior of the frits.

3.6 Cell viability

Figure 5 shows the viability of ISC on several differently modified glass frits with different porosities after 14 days. The modification with polySia or with polySia and the epoxysilane shows no toxic effects on the cells as the absorptions observed are similar within errors on all samples. Between the three different porosities of the frits there are minor differences in the absorption but no trend is visible. Therefore, the porosity has no influence on the viability of Schwann cells.

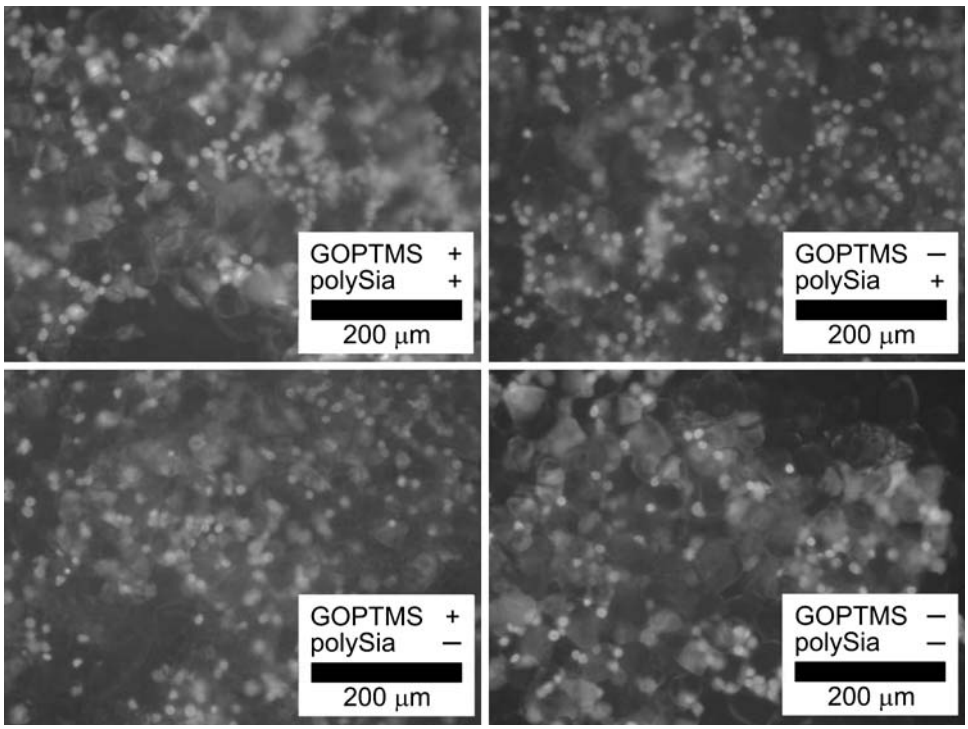

Fig. 4 DAPI-staining of immortalized Schwann cells. Fluorescence images show the results of DAPI-staining after an incubation time of 14 days on differently modified glass frits

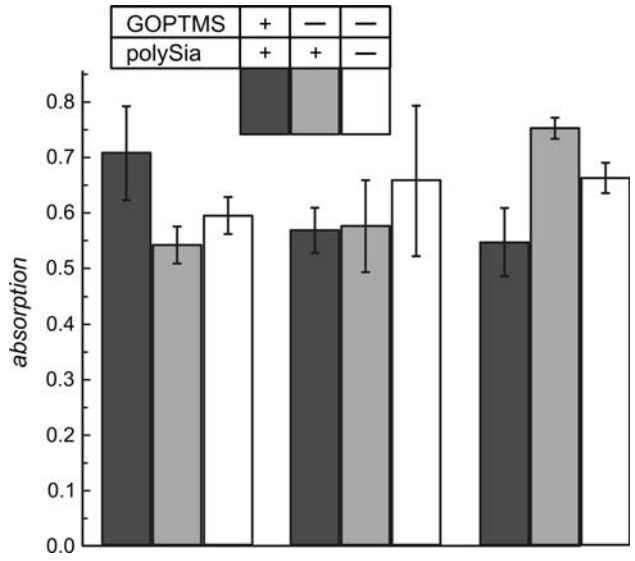

Fig. 5 MTT assay for immortalized Schwann cells. Formazan absorbance after a time period of 14 days as observed on immortalized Schwann cells grown on differently modified glass frits with different porosities. The absorption values refer to 1 g of the original glass frit material

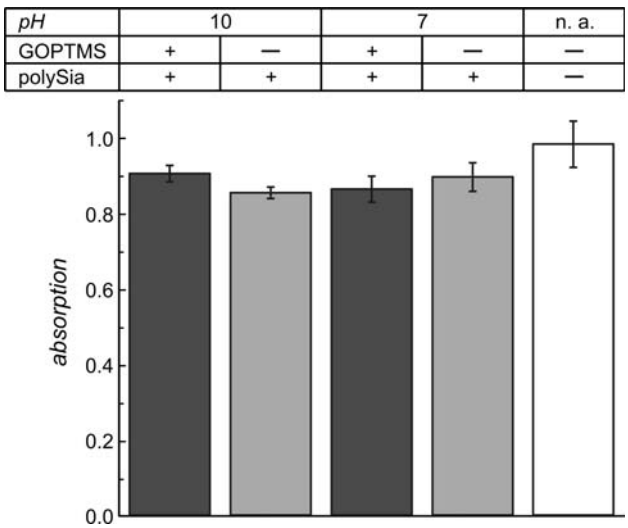

Fig. 6 MTT assay for immortalized Schwann cells. Formazan absorbance after a time period of 14 days as observed on immortalized Schwann cells grown on glass frits which were modified at different pH values. The absorption values refer to 1 g of the original glass frit material

The influence of the pH value of the polySia solution during the reaction with the epoxysilane is shown in Fig. 6. The pH value of the polySia solution was 7 or 10, respectively. Again, there are no differences in cell viability whether the glass surface is unmodified, or whether polySia has been only physically adsorbed or covalently attached, or whether the reaction was carried out at a pH value of 7 or 10.

4 Discussion

The topic of this study was to evaluate the possible use of chemically immobilized polySia as a new biocompatible material, especially with regard to nerve regeneration. For these initial studies, we have chosen glass as substrate, as the different characterization methods can be applied

advantageously on such samples. A simple and effective two-step immobilization procedure has been worked out, consisting in fixing epoxy groups to the surface by silanization and subsequent reaction of deacetylated polySia with the epoxy groups. Contact angle measurements in combination with the ELISA and the TBA assays give evidence for the presence of polySia on the surface of the modified materials. Using immortalized Schwann cells for different cell assays (adhesion, viability) we demonstrate that polySia in combination with the GOPTMS does not have any negative influence on cells. Therefore, surface-immobilized polySia fulfils the basic requirements for the use in bio-implants for tissue engineering: biocompatibility and stability under cell culture conditions. Coupled with increased availability of polySia [33] and the possibility for induced degradation [22, 23], polySia may thus become an important biomaterial.

Schwann cells, the myelin-forming cells of the peripheral nerve, play an important role during nerve regeneration. After the injury they first loose the axonal contact and then proliferate and form the bands of Büngner which guide the regrowing axons with their growth cones towards the denervated targets [34]. Our results which demonstrate that immobilized polySia shows no toxic effects on Schwann cells are of prime importance. Recently, it has been shown that polySia provides a favourable substrate particularly for the culture of Schwann cells and shows no toxic effects for other neuronal components of peripheral nerves [35, 36]. Further studies will address the question if polySia immobilized on silicone tubes as a model system for nerve guidance channels can be used for nerve regeneration in in vivo experiments. Further improvements should also include micro- or nanostructured substrates which are reported to control the alignment of Schwann cells that reorganize the bands of Büngner which guide the regrowing axons within the nerve guides [37, 38]. Another promising approach will include the filling of silicone tubes with genetically modified Schwann cells. This ex vivo gene therapy is based on the delivery of exogenous therapeutic proteins from autogenous cells that have been genetically modified in vitro prior to re-implantation to the lesion site [39].

5 Conclusions

The objective of this study was the chemical immobilization of polySia on glass substrates and the evaluation of such chemically bound polySia as a biomaterial. Our results establish a simple and efficient immobilization procedure for polySia by using an epoxysilane as a linker. The different cell assays (cell adhesion, cell viability) showed no toxic effects on the cells. Therefore, this study establishes surface-bound polySia as a novel biocompatible material for biologized implants with applications in tissue engineering, especially for nerve regeneration. We finally note that polySia attached to a surface resembles the natural state of this polysaccharide as a membrane-attached biopolymer.

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