Hydrazone-based hydrogel hydrolytically degradable in acidic environment

Miroslav Vetrík*, Martin Přádný, Martin Hrubý, Jiří Michálek
Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovský Sq 2, Prague 6, Czech Republic

1. Introduction

Biodegradable hydrogels are extensively studied in connection with, e.g., tissue engineering [1–4], gastroenterostomy stents [5,6] or fixation of hip prostheses during arthroplasty [7]. Biodegradability is based on the presence of hydrolytically or enzymatically cleavable bonds in the polymer chain or the polymer is crosslinked with a hydrolytically or enzymatically cleavable crosslinker.

For some applications it is advantageous to use “smart” materials which are specifically degraded only in certain pH region or in the presence of certain enzymes. For instance, hydrogels crosslinked with N,N-dimethacryloyl hydroxylamine [3,8] are stable in acidic environment, but are hydrolytically degraded at physiological pH (7.4), or 4,4′-bis(methacryloylamo)azo benzene-based crosslinkers are reductively cleaved by azoreductases in the colon [9]. Oesophageal implants, which are to be used to maintain oesophageal trafficking even after obstruction by gradually growing unresectable tumour, are the case where the pH-dependent degradation rate is highly advantageous. The stent, made from metal rings connected with hydrogel, should maintain its mechanical properties in relatively neutral environment of the oesophagus. If accidentally part of the implant is released into stomach, it should disintegrate quickly in the strongly acidic (pH ca 2) stomach content to avoid the necessity of urgent surgery. Although there are described non-degradable metal-based [10] or polymer based stents [11,12] for this application, a biodegradable stent would be certainly more advantageous. Hydrozone bond is known to be hydrolytically labile in acidic environment and relatively stable in neutral and basic environment. This was especially studied for the drug delivery of cancerostatics, because the solid tumour tissue is more acidic (pH ≈ 6.5) than other tissues, in which the pH is maintained to 7.4. The content of endosomes is also more acidic (pH ca 5 in late endosomes) than surrounding milieu, which further facilitates drug release after endocytosis [13,14]. This is why we have chosen this bond as a principle of biodegradability. We describe in this paper a hydrolytically degradable hydrogel based on acid-labile crosslinker N,N’-3-[2-(4-{2-[3-(methacryloylamino)propanoyl]hydrazono}cyclohexyliden)hydrazino]-3-oxopropyl]-2-methylacrylamide (MACH) intended for the construction of oesophageal stents.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich Ltd., Prague. Dichloromethane was dried with sodium sulphate and distilled, dimethylformamide was dried with calcium hydride and distilled with ninhydrin, methacryloyl chloride was distilled and all other

Abbreviations: HPMA, N-[2-hydroxypropyl]methacrylamide; MACH, N,N’-3-[2-(4-{2-[3-(methacryloylamino)propanoyl]hydrazono}cyclohexyliden)hydrazino]-3-oxopropyl]-2-methylacrylamide; HCEMA, N’-[2-(hydrazinocarbonyl)ethyl]methacrylamide.

* Corresponding author. Tel.: +420 296 809 218; fax: +420 296 809 410.
E-mail address: vetrix@seznam.cz (M. Vetrík).
2.2. Methods

2.2.1. Synthesis of MACH

Crosslinker MACH was prepared by three step synthesis in analogy to [16] (see Fig. 1 for reaction numbering):  

Step 1. The solution of β-alanine methyl ester hydrochloride (8.00 g, 57.3 mmol) and hydroquinone (30 mg) in dichloromethane (80 mL) was cooled to 10–15 °C in an ice bath. Anhydrous sodium carbonate (15.0 g, 142 mmol) was suspended in this solution and methacryloyl chloride (5.55 mL, 57.3 mmol) in dichloromethane (30 mL) was added dropwise with vigorous stirring to keep the temperature below 15 °C. The reaction mixture was stirred for 1 h and filtered. The filtrate was evaporated in vacuo.

Step 2. The solid residue was dissolved in methanol (50 mL) with hydrazine monohydrate (0.98 g, 8.76 mmol) and acetic acid (75 mL) was refluxed for 5 h. After cooling to room temperature, triethylamine (0.6 mL) was added. Reaction mixture was stirred for 5 h. Then, in one-day intervals the weight (m) was determined. The degree of degradation δ was calculated according to Equation (2):

\[ \delta = \left( \frac{m - m_0}{m_0} \right) \]

Step 3. The mixture of HCEMA (3.00 g, 17.5 mmol), 1,4-cyclohexanediol (2-hydroxypropyl)methacrylamide (HPMA, 1.00 g) and MACH (2.91 wt.%; 0.09 g, c = 8.26 wt.%) with the same amount of azobis(isobutyronitrile) (0.030 g) as an initiator in poly(ethylene glycol), (M_w = 400, 100 g) as solvent. Polymerization was done in a temperature-controlled block [17] made of hard-aluminium flow forms with an area of 5 × 5 cm, fitted with reinforced polypropylene foils and firmly closed using screw clamps. The thickness of the unswollen original samples corresponded to the thickness of the silicone seals used (1 mm). The polymerization proceeded for a period of 16 h at 80 °C. After polymerization, hydrogels were washed by phosphate buffered saline, pH 7.4 (phosphate buffered saline, PBS; five times for five days). For quantitative measure of the solvation the hydrogels were characterized by volume fraction of dry polymer content in equilibrium-swollen hydrogel Z_v according to Eq. (1):

\[ Z_v = \frac{m_S}{(m_S + D \rho_S)} \]

where m_S and m_D are the weights of swollen and dry hydrogel (xerogel), respectively, ρ_S is the density of the dry gel (xerogel: ρ_S = 1.2 g cm^{-3}), density of the swollen gel is assumed to be the same as the density of water, i.e. 1.0 g cm^{-3} due to high water content.

2.2.2. Synthesis of hydrogels

The hydrogels were prepared by radical polymerization of N-(2-hydroxypropyl)methacrylamide (HPMA, 1.00 g) and MACH (0.030 g, c = 2.91 wt.%; 0.06 g, c = 5.66 wt.%; 0.09 g, c = 8.26 wt.%) with the same amount of azobis(isobutyronitrile) (0.030 g) as an initiator in polyethylene glycol, (M_w = 400, 100 g) as solvent. Hydrogels were stored by phosphate buffered saline, pH 7.4 (phosphate buffered saline, PBS; five times for five days). The thickness of the unswollen original samples corresponds to the thickness of the silicone seals used (1 mm). The polymerization proceeded for a period of 16 h at 80 °C. After polymerization, hydrogels were washed by phosphate buffered saline, pH 7.4 (phosphate buffered saline, PBS; five times for five days). For quantitative measure of the solvation the hydrogels characterized by volume fraction of dry polymer content in equilibrium-swollen hydrogel Z_v according to Eq. (1):

\[ Z_v = \frac{m_S}{(m_S + D \rho_S)} \]

where m_S and m_D are the weights of swollen and dry hydrogel (xerogel), respectively, ρ_S is the density of the dry gel (xerogel: ρ_S = 1.2 g cm^{-3}), density of the swollen gel is assumed to be the same as the density of water, i.e. 1.0 g cm^{-3} due to high water content.

Degradation of low-molecular crosslinker MACH: MACH (2 mg) was dissolved in water (2.0 mL) with 50 μL of 36% hydrochloric acid and the mixture was kept for 30 min at 37 °C. The degradation mixture was then analyzed with a Chromolith Performance RP-18e C8 silica column (100 × 4.6 mm i.d., Merck, Darmstadt, Germany) using the following gradient method: flow 1 mL min^{-1} mobile phase, A: 0.3 M acetic acid buffer pH 6.5, mobile phase B: acetonitrile, program 0–2 min 0% B, 2–8 min gradient to 30% B, 8–16 min gradient to 50% B, 16–18 min gradient to 0% B, 18–22 min 100% A (column re-equilibration). Analysis was performed on a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with refractive index, fluorescence and PDA (diode array) UV–vis (scanned range 200–400 nm) detectors. The MACH crosslinker has retention time (t_R = 11.68 min), wavelength of maximum absorbance (λ_{max} = 254 nm), 1.4-cyclohexanediol has t_R = 15.04 min (λ_{max} = 242 nm) and HCEMA has t_R = 6.12 min (λ_{max} = 244 nm).
3. Results and discussion

3.1. Synthesis and model degradation of the crosslinker

The crosslinker MACH was synthesized by a straightforward methacryloylation of β-alanine methyl ester hydrochloride, hydrazinolysis of ester to hydrazide HCEMA and its condensation (Fig. 1) with 1,4-cyclohexanedione in acceptable yield after purification.

Hydrazinolysis of hydrazone bond back to ketone and hydrazide (Fig. 2), the principle of degradability of the crosslinker, is acid-catalysed (protonation is the first step), so it proceeds faster in acidic environment. To confirm that the crosslinker MACH is hydrolyzed in the suggested way, we tested its degradation in acidic environment. The crosslinker smoothly and quantitatively without side reactions degraded back into HCEMA and 1,4-cyclohexanedione within the 30 min incubation time.

3.2. Degradation of hydrogels

Given that the intended application of the described degradable hydrogel is the construction of oesophageal stents, a kinetic degradation study was performed at physiological ionic strength at 37 °C. The degradation rate of the hydrogels is expressed in Fig. 3 as the relative weight change of the samples (change in hydrogel weight relative to initial weight). The degradation has two stages (similarly as degradation of basically splitting hydrogel [3]). In the first stage, when the crosslinks between chains break, the density decreases, the hydrogel swells and its weight increases. At the moment when the network density is so low that the copolymer becomes soluble in water, the kinetic curve shows a maximum, the moment when the network density is so low that the copolymer dissolves and its relative weight decreases. At the moment when δ = −1, the whole volume of the hydrogel is dissolved. Degradation rate of a hydrogel increases with decreasing pH, while at physiological pH = 7.4 hydrogel is stable, its cleavage was not observed after 5 months (δ = −0.02).

We carried on another degradation study of hydrogel, where three different concentration of crosslinker (MACH) in the hydrogel were used (2.91 wt.%, 5.66 wt.% and 8.26 wt.%). The degradation study was carried on in the same degradation solution (pH = 3), see Fig. 4. The lower was the crosslinker content, the faster was the degradation rate, because less hydrazone bonds are to be broken before the polymer dissolves for the less crosslinked hydrogels with lower crosslink density. This degradation study demonstrates that the degradation time of hydrogel can be fine-tuned to the desired rate.

ZV were measured for all three samples in dependence on content of MACH present in hydrogel 0.09 g, 0.006 g, 0.06 g (see Section 2.2.2). ZV 0.09 = 0.050, ZV 0.06 = 0.044, ZV 0.03 = 0.037.

This pH-dependence of degradation rate is consistent with non-crosslinked hydrazone-based systems, such as thermoresponsive methacrylamide-based polymers [16], which are also faster degraded in acidic milieu than at neutral pH. However, in comparison with such hydrazone systems, degradation of our hydrogel is significantly slower (two weeks compared to 8 h at pH ca 5, see Fig. 3 and [16]) despite similar chemical surrounding of the hydrazone bond and the hydrogel is not degraded in neutral pH, unlikely the non-crosslinked system. This is also in agreement with injectable gels formed in situ from polymer hydrazides and polymer aldehydes for cell encapsulation, which are also reported to be stable at least for several days at pH 7.4 [18].

This is most plausibly caused by the fact that several hydrazone crosslinks must be typically broken to release the polymer chain from hydrogel structure. Since the hydrolysis of hydrazone bond is reversible and the polymer chain is kept after hydrolysis in the neighbourhood of the cleaved-out part by the remaining hydrazone bonds, the apparent local hydrazide concentration is higher and reversible and the polymer chain is kept after hydrolysis in the neighbourhood of the cleaved-out part by the remaining hydrazone bonds, the apparent local hydrazide concentration is higher and equilibrium is entropically shifted more to the hydrazone bond (re) formation.

4. Conclusions

We describe the new pH-sensitive hydrolytically degradable crosslinker N-{3-[2-(4-[2-[3-(methacryloylamino)propanoyl]hydrazono)cyclohexyldien]hydrazino]-3-oxopropyl]-2-methylacrylamide. Poly[N-(2-hydroxypropyl) methacrylamide]-based hydrogels synthesized with this crosslinker are degraded in acidic pH, but are stable
at pH 7.4, so they should be appropriate for the intended application in the construction of oesophageal stents.

Acknowledgements

Financial support of the Grant Agency of the Czech Republic (grants # P108/10/1560, # P207/10/P054 and # MEYS 1M 0538) is gratefully acknowledged.

References


A new type of irreversibly reductively biodegradable hydrogel

Miroslav Vetrik*, Martin Hruby, Martin Pradny, Jiri Michalek

Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic

ARTICLE INFO

Article history:
Received 21 December 2010
Accepted 30 January 2011
Available online 13 February 2011

Keywords:
Biodegradable hydrogel
Disulfide
\(\text{\textit{i}}\)-cysteine
Thiol

ABSTRACT

Reductively biodegradable hydrogels based on poly[N-(2-hydroxypropyl)methacrylamide] crosslinked with N-[3-(methacryloylamino)propyl]-6-[5-[[3-(methacryloylamino)propyl]amino]carbonyl]-2-pyridyldisulfanyl]nicotinamide intended for tissue engineering were synthesized and characterized. The rate of irreversible reductive degradation with thiol-L-cysteine (a model of human body where several thiols are present in extracellular space) was studied using several \(\text{i}\)-cysteine concentrations. The mechanism of the irreversible reductive bond cleavage in the crosslinker structure was confirmed. The hydrogel is stable during storage in phosphate buffered saline and is degraded relatively quickly in a concentration-dependent manner after addition of \(\text{i}\)-cysteine to the surrounding medium.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Extensive research effort is currently devoted to regenerative medicine and tissue repair. Bridging the lesion of the damaged or destroyed tissue and regeneration of the functional tissue is more understood in the last two decades and examined using stem cells, present in the surrounding tissue or supplied externally [1]. The spreading and differentiating cells however require a scaffold resembling the extracellular matrix as a support [2].

Significant benefits may thus be expected from the implantation of biodegradable scaffolds in which the cells can grow, thus bridging the lesions. The scaffolds may eventually serve as cell carriers for the delivery of different population of cells, such as foetal stem cells or even embryonic stem cells, which can effectively repair the injured tissue [3]. After the formation of newly reconstructed tissue, the scaffold should degrade without leaving any artificial deposits. Therefore, scaffolds utilized for cell transfer should be biocompatible and non-immunogenic, should have suitable mechanical properties and controlled architecture and should be biodegradable at a reasonable rate [4], which makes their preparation difficult.

Many authors thus consider biodegradable biomaterials to be superior to non-degradable matrices from the point of view of tissue regeneration. The biodegradability of most supports relies on hydrolytic degradation [5–7], spontaneous or enzyme – catalysed [8–10]. Although hydrothermally biodegradable scaffolds can be prepared from a variety of materials, they still have many important disadvantages, such as problems with their storage in an aqueous environment and the fact that their hydrolysis is accompanied by reactive oxygen species and nitric oxide (NO), which are present in damaged/damaged regions and are considered to rank among inflammation mediators [13]. The exchange reaction with the

Abbreviations: Boc-Cys, N-tert-butyloxycarbonyl cysteine; HPMA, N-(2-Hydroxypropyl)methacrylamide; DMAc, N,N-dimethylacetamide; DMSO, dimethylsulfoxide; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; MA3SS, N-[3-(methacryloylamino)propyl]-6-[5-[[3-(methacryloylamino)propyl]amino]carbonyl]-2-pyridyldisulfanyl]nicotinamide; N-[3-(methacryloylamino)propyl]-6-[5-[[3-(methacryloylamino)propyl]amino]carbonyl]-2-pyridyldisulfanyl]nicotinamide; Pr2SS, N-propyl-6-[5-[[3-(methacryloylamino)carbonyl]-2-pyridyldisulfanyl]nicotinamide.

* Corresponding author. Tel.: +420 296 809 218; fax: +420 296 809 410.
E-mail address: vetrix@seznam.cz (M. Vetrik).

0141-3910/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymdegradstab.2011.01.037
extracellular matrix should lead to the reversible binding of laminin and integrin via disulfide bonds, which should in turn also promote the growth of cells, but may also lead to nonspecific interactions with the cells.

The approach of reductive biodegradability has been mainly described for water-soluble gene delivery systems [14,15], but also in several cases for hydrogel scaffolds made of hyaluronan, poly (γ-glutamic acid) and similar polymers of biological origin [7,11,12]. Such hydrogels are prepared by functionalisation of the biomolecules with thiol-containing agents and subsequent oxidation of the macromolecule-bound thiols to disulfides by air or by diluted hydrogen peroxide (oxidative crosslinking). However, such hydrogels have several drawbacks – poor mechanical properties, difficult functionalisation with specific cell proliferation and differentiation promoting ligands, a chemically and structurally less defined product (since natural polymer is used as starting material) is formed, etc.

The use of synthetic polymers for such purposes offers several additional advantages. The chemical surroundings of the disulfide bond may be fine-tuned to achieve the degradation half-life adjustable over a much wider range, such gels are much more defined, have better mechanical properties, are easier to prepare and functionalize with the use of suitable vinyl comonomers, etc.

Most thiols exchange with disulfides reversibly. However, some disulfides (e.g., 2,2'-dipyridyl disulfide type) are cleaved irreversibly into two separate parts due to the strong tautomeric stabilization of the thiol cleavage product into preferred non-thiol tautomer (2-thiopyridone in this case) [16]. If such disulfide bonds irreversibly cleavable by thiols (see Fig. 2) are employed, it should enable to avoid the side reactions after degradation (re-gelation after exposure of the degraded polymer to air, re-conjugation with thiol-containing proteins such as opsonins, unwanted additional swelling during degradation, etc.). To best of our knowledge, no such gel was described in literature to date. In this paper, we describe the reductively cleavable hydrogel scaffold intended for use in tissue engineering, using crosslinker with irreversibly thiol-cleavable bonds. Easy to prepare, stable in water solutions (PBS), which allow storage our material for a long time and non-toxic products of degradation can be easy released by organism.

2. Materials and methods

2.1. Materials

N-(3-Aminopropyl)methacrylamide hydrochloride was purchased from Polysciences Europe GmbH (Eppelheim, Germany). All other...
chemicals were purchased from Sigma–Aldrich Ltd. (Prague, Czech Republic).

2.2. Methods

2.2.1. Synthesis of monomers and biodegradation models

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized by methacryloylation of 1-amino-2-propanol by methacryloylchloride as described in [17]. The reductively degradable crosslinker by methacryloylation of 1-amino-2-propanol by methacryloyl-2-(methacryloylamino)propyl]-6-\{5-[(3-(methacryloylamino)propyl)amino] carbonyl\}-2-pyridyl\}disulfanyl\}nicotinamide (MA2SS) and the biodegradation model N-propyl-6-\{5-\[(propylamino)carbonyl\]-2-pyridyl\}disulfanyl\}nicotinamide (Pr2SS) were prepared according to the following procedure (see Fig. 3 for scheme):

6,6-Dithiodinicotinic acid (1.00 g, 3.24 mmol), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 1.68 g, 6.81 mmol) and the particular amine \[\text{N}-(3\text{-amino} \text{propyl})\text{methacrylamide}\] hydrochloride (1.16 g, 6.49 mmol) in the case of MA2SS or 1-aminopropane hydrochloride (1.16 g, 6.49 mmol) in the case of Pr2SS or 1-aminopropane hydrochloride (1.16 g, 6.49 mmol) in the case of Pr2SS, respectively] were dissolved in anhydrous \(N, N\)-dimethylacetamide (DMAc, 12 mL) and stirred at room temperature for 24 h. The reaction mixture was then poured into water (70 mL), cooled to 5 °C and filtered. The precipitate was washed with water and dried in vacuum. Yield 1.36 g (81%) for MA2SS and 0.98 g (77%) for Pr2SS.

MA2SS: \(^{1}H\) NMR (DMSO-d6): \(\sigma = 1.67\) (m, 4H), 1.84 (s, 6H), 3.15 (m, 4H), 3.26 (m, 4H), 3.30 (s, 2H), 5.63 (s, 2H), 7.70 (d, 2H), 7.93 (t, 2H), 8.16 (d, 2H), 8.65 (t, 2H), 8.87 (s, 2H). \(^{13}C\) NMR (DMSO-d6): \(\sigma = 18.6\) (2C), 29.0 (2C), 36.6 (2C), 37.1 (2C), 118.9 (2C), 119.2 (2C), 128.1 (2C), 136.8 (2C), 140.0 (2C), 148.5 (2C), 159.8 (2C), 164.0 (2C), 167.5 (2C). Elemental analysis (found/calculated): C 56.26%/56.10%, H 5.63%/5.79%, N 14.84%/15.10%, S 11.60%/11.52%. TLC (silica, acetic acid–ethanol 1:99 v/v): homogenous, \(R_f = 0.69\).

Pr2SS: \(^{1}H\) NMR (DMSO-d6): \(\sigma = 0.88\) (t, 6H), 1.53 (m, 4H), 3.22 (m, 4H), 7.70 (d, 2H), 8.18 (d, 2H), 8.63 (t, 2H), 8.88 (s, 2H). \(^{13}C\) NMR (DMSO-d6): \(\sigma = 11.3\) (2C), 22.2 (2C), 40.9 (2C), 119.1 (2C), 128.2 (2C), 136.7 (2C), 148.4 (2C), 159.7 (2C), 163.9 (2C). Elemental analysis (found/calculated): C 54.99%/55.36%, H 5.26%/5.68%, N 14.47%/14.35%, S 16.87%/16.42%. TLC (silica, acetic acid–ethanol 1:99 v/v): homogenous, \(R_f = 0.88\).

2.2.2. Synthesis of hydrogels

The hydrogels were prepared by radical UV polymerization of hydroxypropyl methacrylate (HPMA, 0.250 g, 1.74 mmol) and MA2SS (0.100 g, 0.180 mmol, respectively) with 2-hydroxy-2-methylpropioiphenone (0.014 g, 0.014 g, 85.3.10 \(^{-6}\) mmol) as the UV initiator in different amounts of dimethylsulfoxide (DMSO, 1.00 g, 0.75 g or 0.50 g, respectively) as solvent. Polymerization was done in polymeric blocks equipped with quartz glass on front side [18] made of hard-aluminum flow forms with an area of 5 × 1 cm, fitted with reinforced polypropylene foils and firmly closed using screw clamps. The thickness of the unswollen original samples corresponded to the thickness of the silicone seals (1 mm) used. The polymerization proceeded for a period of 30 min at r.t. using irradiation with wide spectral UV lamp (6 × 125 W; Tesla, Prague, Czech Republic). After polymerization the hydrogels were washed by DMSO (five times for five days) after by H2O (five times for five days) and finally phosphate buffered saline, pH 7.4 (PBS; five times for five days).

The hydrogels were characterized by volume fraction of dry polymer content in equilibrium-swollen hydrogel \(Z_v\) according to Eq. (1):

\[
Z_v = \frac{m_s}{(m_s \rho_D)}
\]

where \(m_s\) and \(m_D\) are the weights of swollen and dry hydrogel (xerogel), respectively, \(\rho_D\) is the density of the dry gel (xerogel; \(\rho_D = 1.2\) g cm \(^{-3}\)), density of the swollen gel is assumed to be the same as the density of water, i.e. 1.0 g cm \(^{-3}\) due to high water content.

Fig. 3. Synthesis of crosslinker MA2SS and model substrate Pr2SS.
2.2.3. Degradation of model compound Pr2SS

The solution of Pr2SS (10 mM in DMAc) and the solution of N-tert-butyloxy carbonyl cysteine (Boc-Cys, 10 mM in DMAc) were mixed at room temperature to obtain the molar ratios of reagents Boc-Cys:Pr2SS 0.25, 0.50, 0.75, 1, 2, 3 and 4, respectively. After incubation at room temperature (5 h for equilibrium experiments, 0 min, 5 min, 20 min, 30 min, 1 h, 2 h and 7 h for the reaction rate experiments), the reaction mixtures were analyzed by both UV–VIS spectrophotometry (equilibrium + reaction rate experiment) and high performance liquid chromatography (HPLC, equilibrium experiments only).

UV–VIS spectrophotometry: The reaction mixture was diluted with methanol (1 + 299 v/v) and absorbance was recorded in a 1 cm optical length quartz cuvette in the range 190–600 nm on a Helios Alpha (Spectronic Analytical Instruments, Leeds, United Kingdom) UV–VIS spectrophotometer. The absorbances were recalculated to the initial concentration of Pr2SS 33 μM.

HPLC: The reaction mixture was diluted with DMAc (1 + 19 v/v) and analyzed on the Chromolith Performance RP-18e C18 silica column (100 × 4.6 mm i.d., Merck, Darmstadt, Germany) using the following gradient method: flow 1 mL min⁻¹, water–acetonitrile–trifluoracetic acid: A: 95:5:0.1 v/v/v, B: 50:95:0.1 v/v/v, program: 0–2 min 100%, A, 2–8 min gradient to 100% B, 8–10 min 100% B, 10–11 min gradient to 100% A, 11–17 min 100% A (column re-equilibration). Analysis was performed on a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with refractive index, fluorescence and PDA (diode array) UV–VIS detectors (scanned range 200–400 nm). The standard of “reduced Pr2SS” (N-propyl-6-thioxo-1,6-dihydro-3-pyridinecarboxamide) was obtained by dissolution of Pr2SS (0.2 mg mL⁻¹) in the 3-mercaptopropionic acid–DMAc (1:9 v/v) mixture and the only UV₃₁₆ nm-active peak (tᵣ = 6.76 min) was attributed to reduced Pr2SS.

Degradation rate was followed by UV–VIS spectrophotometry by mixing the solutions of Boc-Cys and Pr2SS to obtain 0.420, 0.084 and 0.017 mM Boc-Cys and 5:1 Boc-Cys to Pr2SS molar ratio and following absorbance in a 1 cm optical length quartz cuvette at 316 nm during incubation at 37 °C.

2.2.4. In vitro degradation of the hydrogels

The weighted strips of hydrogels (weight m₀, ca 1 × 1 cm) swollen in PBS were immersed in the 50 mL solution of l-cysteine (0 mmol L⁻¹, 0.33 mmol L⁻¹, 4.2 mmol L⁻¹, 12.6 mmol L⁻¹ and 42 mmol L⁻¹, respectively) and tetracycline (0.23 mmol L⁻¹) in PBS and maintained at 37 °C. Every day the hydrogel was weighted (m), re-immersed in fresh l-cysteine solution and returned to the incubator. Then, degree of degradation (δ) was calculated according to Eq. (2):

\[ \delta = \frac{(m - m₀)}{m₀} \]  
(2)

where m is the weight of polymer sample in the observed time and m₀ is weight of polymer sample at the beginning of the degradation study.

3. Results and discussion

The hydrogels were designed to contain poly[N-(2-hydroxypropyl)methacrylamide] (polyHPMA) polymer chains soluble in water crosslinked with biodegradable irreversibly thiol-cleavable disulfide crosslinker MA₂SS. PolyHPMA is a hydrophilic polymer with known biocompatibility [19]. During cleavage of crosslinker network density decreases and the material becomes soluble in water, which should allow its elimination from organism by kidneys.

We first synthesized the crosslinker with 2,2’-dipryridyl disulfide type bonds, MA₂SS, by EEDQ-mediated amino-carboxylic acid condensation in good yield (81%). The hydrogels were synthesized by radical copolymerization of this crosslinker with HPMA as main monomer using 2-hydroxy-2-methylpropionophenol as an UV initiator. Three different concentration of DMSO were used (1.00 g, 0.75 g and 0.5 g per 0.25 g HPMA) to set best condition during degradation study, because in case of in vivo study, long time washing for elimination monomer and other chemical present in hydrogel after the polymerization reaction is necessary and also network density decreases when increasing dilution of the polymerization mixture.

Solvation of polymer chains is important for the polymer degradation rate [20], because all the thiols present in organism that would degrade the implant are very hydrophilic, and also sterically accessibility of the disulfide crosslinks is increased with the hydrogel expansion in water. The solvation of the polymer chains also influences compactness of the gel and thus diffusion rate of biological thiols into it. Quantitative measure of the polymer solvation may be expressed as volume fraction of dry polymer in equilibrium – swollen hydrogel – Zᵥ for all three polymer gels, there were not found any significant differences among the samples (Zᵥ is the same for all samples containing different amount of solvent in polymerization mixture, Zᵥ = 0.2, Zᵥ = 0.2 and Zᵥ = 0.5). However, all these results indicate that the degree of solvation is thus probably influenced only by hydrophilicity of the polymer, which is the same for all samples since they were prepared from the same ratio of hydrophilic HPMA and relatively hydrophobic crosslinker. The mechanical properties (resistance against fragmentation) of the gel are significantly better for the gels with lower solvent content in polymerization mixture in analogy to most hydrogel systems described in literature [21].

The total concentration of all free thiols in plasma is mostly given by the free thiol groups on the Cys 34 position on serum albumin. The average content under normal conditions is 0.7 free thiol (SH) groups per albumin molecule [22]. The content of serum albumin (Mₘ = 67 kDa) in human blood (30–50 g/L) [23] thus corresponds to the pool of SH groups 0.31–0.52 mM. The Cys 34 group is highly reactive [24], however steric considerations especially in the case of the reaction with polymer are to be responded. The content of low-molecular-weight thiols in blood plasma is significantly lower. l-Cysteine is the most abundant low-molecular-weight thiol in blood plasma (9 μmol/L SH) and also some other thiols are present in blood plasma (glutathione and l-cysteinyl glycine aa 1.5 μmol/L SH, l-homocysteine 0.15 μmol/L SH) [25]. However, there are also significantly higher concentrations of disulfides present in blood plasma (l-cysteine 150 μmol/L disulfide groups (SS), glutathione 2 μmol/L SS, l-cysteinyl glycine 11 μmol/L SS, l-homocysteine 7 μmol/L SS) [25], that may also promote degradation due to SH–SS exchange reaction (see Fig. 1), where thiol is a catalyst only. Degradation kinetics may also be accelerated by cell membrane-bound reductases, omnipresent on the surface of mammalian cells, and diffusion of thiols from the surrounding of the implant is also of limited rate [26]. More to that, the commercially available albumin samples are more oxidized than in blood plasma, where the redox state is actively maintained by the cells. On the other hand, maximum degradation rate of the gel is to be expected in the place of maximal metabolic activity (i.e. cell growth), which is favourable.

However, this all makes in vitro simulation of the degradation in human organism very difficult. We have chosen five model l-cysteine concentrations – 0 mmol L⁻¹ (stability against hydrolysis, not present in the Fig. 4), 0.33 mmol L⁻¹ (biologically relevant free thiol concentration if we consider reactivity of albumin and low-molecular-weight thiols comparable) and three higher concentrations due to mechanistic study – 4.2 mmol L⁻¹, 12.6 mmol L⁻¹ and 42 mmol L⁻¹. Lower l-cysteine concentrations comparable to free
low-molecular-weight thiol concentration (9 \( \mu \)mol/L SH) in blood were not studied, because in such case it is not possible to maintain defined concentration in model stock solution (stoichiometry disulfide in gel vs. thiol in external volume of L-cysteine solution is too shifted to complete depletion of external thiol, traces of oxygen that oxidizes free thiols). All the observed degradation rates except that without L-cysteine are thus the upper limit that may be expected in vivo.

The rate of degradation of hydrogels is shown on Fig. 4 and is expressed as time dependence of the degree of degradation (\( \delta \)), only one degradation graph is presented, other two degradation rate have similar slope and can be found in Supplementary materials. The higher is the network density (i.e. the lower is solvation with water expressed as \( Z^V \)), the slower is the degradation rate. This effect is most plausibly caused by the fact that more crosslinks have to be cleaved in more densely crosslinked gel to dissolve it. Also hydrophobic nature of the crosslinker may contribute to this effect (causing the decrease of equilibrium content of water in gel, which leads to less expanded and less thiol-accessible polymer chains). The complete degradation time is ca 60 days and is expected to be significantly longer under in vivo conditions (similarly as for a system with reversibly reductively degradable bonds [5]).

The gels are completely stable in PBS without cysteine (i.e. are stable against hydrolysis) and the higher is the L-cysteine concentration, the faster is the degradation of the gel. It is important that the swelling phase was not observed during degradation in any case. This is most plausibly because the reaction of thiols and thus dissolution of the gel from the surface is faster than diffusion into gel and breaking disulfide bonds inside the gel, making the polymer chains more flexible and able to expand and swell the gel. This is highly advantageous especially for the porous 3D scaffolds, where the swelling phase during degradation may mechanically oppress the growing and differentiating cells.

This hypothesis was tested by a reaction on low-molecular-weight model, where the steric considerations and diffusion play much lower role and the reaction progress may be followed easily. For the in vitro model experiments with the S–S bond cleavage with thiol, the dipropylamide derivative of 6,6'-dithiodinicotinic acid (Pr2SS) was synthesized. Pr2SS has the same chemical surrounding of the disulfide bond as MA2SS has (6,6'-dithiodinicotinic acid aliphatic diamide), but unlike MA2SS it does not contain activated double bond, that may complicate the model experiments with Michael-type thiol additions. Due to solubility considerations, the reaction was carried on in organic solvent and Boc-Cys was used as model thiol.

As assayed by UV–VIS spectrophotometry, substituted 2-thiopyridone formation during the disulfide bond cleavage leads to dramatic difference in the UV–VIS spectra. Namely, 2-thiopyridone “reduced Pr2SS” absorbs at 316 nm, where the original
disulfide is practically transparent (see Supplementary material). The reaction extent may thus be followed by UV–VIS spectrophotometry. The first experiment was done with various disulfide–thiol ratios and the reaction was allowed to reach equilibrium before measurement. The data reveal (see Fig. 5), that the equilibrium is nearly completely shifted towards complete consumption of the aliphatic thiol, the same molar amount of the substituted 2-thiopyridone is released as is the amount of the aliphatic thiol added. The dependence is linear (linear regression $R^2 = 0.995$) up to the stoichiometry 1:2, in accordance with theory (the two thiols are consumed to completely reduce one disulfide bond, see Fig. 1). The data from UV–VIS spectrophotometry well match with the analysis of the substituted 2-thiopyridone concentration determined by HPLC (see Fig. 5, correlation coefficient 0.997).

Reaction rate of the disulfide bond degradation on the low-molecular-weight model was followed using the same concentrations of thiols as in the case of the gel degradation and the thiol–disulfide molar ratio 5:1. The degradation of the low-molecular-weight model follows the first order kinetics ($R^2 = 0.998$, 0.997 and 0.977 for the Boc-Cys concentrations 0.420, 0.084 and 0.017 mM, respectively) with the corresponding half-lives $T_{1/2}$ 41.2 ± 2.7 min, 56.6 ± 4.5 min and 90.8 ± 18.8 min for the Boc-Cys concentrations 0.420, 0.084 and 0.017 mM, respectively. The degradation of the low-molecular-weight model is thus much faster than the degradation of the gel under comparable thiol concentration, which means that the degradation rate and missing of the swelling phase is diffusion-driven as expected.

4. Conclusions

Irreversibly reductively biodegradable hydrogels polyHPMA crosslinked with disulfide crosslinker intended for tissue engineering were synthesized and characterized. The rate of reductive degradation with thiol L-cysteine (a model of the human organism where several thiols are present in the extracellular space) was studied using several L-cysteine concentrations and the mechanism of the irreversible reductive bond cleavage in the crosslinker structure was confirmed on a low-molecular-weight model. The hydrogel is stable during storage in phosphate buffered saline and is degraded relatively quickly after addition of L-cysteine to the surrounding milieu in a concentration-dependent manner.

Acknowledgements

Financial support of the Grant Agency of the Czech Republic (grants #P108/10/1560, #P207/10/P054, #P108/10/1560 and #MEYS1M 0538) is gratefully acknowledged.

Appendix. Supplementary material

Supplementary material related to this article can be found at doi:10.1016/j.polymdegradstab.2011.01.037.

References

Biopolymer-based degradable nanofibres from renewable resources produced by freeze-drying

Miroslav Vetrik, Martin Pradny, Libor Kobera, Miroslav Slouf, Mariia Rabyk, Aneta Pospisilova, Petr Stepanek and Martin Hruby*

We describe a new biopolymer-based nanofibrous material possibly suitable for tissue engineering prepared by an environment-friendly organic solvent-free method. Glycogen, a biodegradable hyperbranched D-glucose polymer, comes from renewable resources and is normally present in man. It forms nanofibres by simple freeze-drying from aqueous solutions with concentration less than 0.5%. However, the architecture of the freeze-dried material depends on the starting biopolymer concentration within the tested range 0.1–5 wt%; in particular higher concentrations produce porous sponge-like structures with communicating pores. Because of the solubility of glycogen in water, nanofibres were modified by solvent-free grafting biodegradable poly(ethyl cyanoacrylate) from vapor phase. Exposing glycogen nanofibres to vapors of ethyl cyanoacrylate only slightly changed the material architecture while producing a water-insoluble biodegradable material with glycogen-to-poly(ethyl cyanoacrylate) ratio depending on the polymerization time. The material was proven to be hydrolytically degradable over the course of several months.

1. Introduction

There is an extensive quest for new suitable biodegradable, biocompatible polymers, which can act as scaffolds in tissue engineering. These polymeric materials may be of natural, synthetic or hybrid origin.1–4 Synthetic polymers offer more possibilities of chemical modification and in many cases are better defined.5 On the other hand natural polymers usually have better biodegradability. Hybrid materials usually combine advantages of the natural and synthetic materials. Some biopolymers such as the polysaccharides hyaluronic acid,6 dextran,7 chitosan8–10 and alginate,11,12 are studied for these purposes, because they possess suitable in vivo behavior and come from renewable resources. The scaffold must resemble an extracellular matrix in all structure, morphology, chemical and chemical properties to support the growing cells, which functionally repair and replace the damaged site. The scaffold should also be nonimmunogenic and biodegradable at a reasonable rate. The degradation rate must be optimum otherwise the implant degrades before the regenerated tissue is formed or prevents full replacement of the implant with the newly formed tissue.13 The main types of materials tested for the construction of scaffolds are hydrogels,14,15 porous materials,16,17 non-woven fibres18,19 and nanofibres.20 Especially, nanofibres are advantageous for this use due to their large specific surface area, defined and appropriate pore size between fibres and the structure of scaffold made from this material has fully communicating pores. Nanofibrous scaffolds have attracted considerable interest,20–25 because their architecture is similar to the naturally occurring protein fibrils, to fibrin26 in the extracellular environment and also they have a variety of applications.27–29 Each individual nanofibre has a high surface to volume and aspect ratio allowing more surface area contact of the scaffold with the cells. The physical and biological properties of the scaffold depend on the material used for electrospinning and on its properties such as surface wettability, mechanical properties and degradation.30

Nanofibres are generally fabricated by electrospinning, which allows to prepare nanofibrous structure in the range from submicrons to microns diameters.30 However, electrospinning in most cases requires the use of organic solvents that are more or less toxic, which does not match current demand on environment-friendly organic solvent-free sustainable technologies where water is the preferred solvent.

Glycogen (GG) is the main animal D-glucose storage form. It is present mainly in liver and muscles. GG is a hyperbranched poly(D-glucose) with D-glucose units connected with α (1 → 4) bonds, branching is via α (1 → 6) bonds.31,32 GG is natural high-molecular-weight fully biodegradable dendrimer structurally related to dextrin, but much more branched.31,32 This is why GG is relatively slowly degraded by amylases (which are omnipresent in the bloodstream), but its intracellular degradation by glycogen debranching enzyme is considerably faster.

Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 162 06 Prague 6, Czech Republic. E-mail: mhruby@centrum.cz; Fax: +420-296 809 410

Cite this: RSC Advances, 2013, 3, 15282-15289

Received 25th March 2013,
Accepted 13th June 2013
DOI: 10.1039/c3ra42647e
www.rsc.org/advances

PAPER

Part AIII
RSC Advances
We describe in this paper a specific property of GG allowing environment-friendly preparation of nanofibrous materials from this renewable resource. It was possible to prepare GG nanofibres by simple one step freeze-drying procedure where no electrospinning technique is necessary. The main advantage of this procedure is, except for its simplicity, the possibility to prepare even bulk layers of nanofibrous homogenous material, which is not so easy to perform by electrospinning, with just water as the only solvent.

Because GG is readily water soluble, the nanofibres must be made insoluble to enable their use in the aqueous milieu. GG was thus used as a shape template and initiator for organic solvent-free polymerization of ethyl cyanoacrylate from the gaseous state. Polymerization of volatile cyanoacrylates from gaseous phase is used, e.g., in forensic chemistry to visualize fingerprints. Cyanoacrylates are used as tissue biocompatible adhesives – surgical glues. They are slowly biodegradable in the course of few months by combined spontaneous and esterase-catalyzed enzymatic hydrolysis. Therefore our product, nanofibrous glycoen-graft-poly(ethyl cyanoacrylate), is also expected to be biodegradable. The rate of biodegradation can be adjusted by changing the alkyl type in the ester group; here ethyl ester was chosen due to its high volatility. This paper reports the synthesis and characterization of these materials with tuneable morphology.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Ltd. (Prague, Czech Republic) and were used as received.

2.2. Fabrication of glycogen nanostructures

Aqueous solutions of glycogen from oyster [concentrations 0.1, 0.2, 0.5, 1, 2 or 5 wt%, respectively] were placed into 30 mL vials (5 mL solution per vial, i.e. forming ca. 5 mm thick layer) and were allowed to freeze at a temperature set to −18 °C in a freezer (freezing rate was ca. 2 °C min⁻¹). The frozen samples were then lyophilized on a ScanVac Coolsafe 110-4 Pro (MERCI Ltd., Brno, Czech Republic) freeze drier, shelf temperature −10 °C, pressure 20 Pa, duration 48 h.

2.3. Synthesis of glycogen-graft-poly(ethyl cyanoacrylate) nanostructures

The GG nanostructures, prepared by freeze-drying from water solution, were exposed to saturated vapors of ethyl cyanoacrylate in argon atmosphere in a closed chamber at 103 kPa and 23 °C for 7 or 30 days, respectively.

2.4. Characterization of nanostructures

The nanostructures were characterized by scanning electron microscopy (SEM), elemental analysis [CHN, on a Perkin-Elmer Series II CHNS/O Analyzer 2400 (PE Systems Ltd., Czech Republic) instrument], Fourier-transform infrared spectroscopy (FT-IR) and solid-state nuclear magnetic resonance (ssNMR).

SEM microscopy was carried out with a microscope Quanta 200 FEG. The samples were sputtered with 4 nm thin platinum layer (Vacuum sputter coater Leica SCD 050) in order to increase contrast and to avoid charging and electron beam damage. All micrographs are secondary electron images, taken in high vacuum at accelerating voltage 30 kV.

The 1D solid-state NMR spectra were measured using a Bruker Avance 500 NMR spectrometer with a 4 mm MAS probe. Magic angle spinning (MAS) frequency of the sample was 11 kHz. In all cases the dried samples were placed into the ZrO₂ rotors and stored under silica to prevent rehydration. Amplitude modulated cross-polarization (CP) with duration 1750 μs was used to obtain ¹³C CP/MAS NMR spectra with 2 s recycle delay. The number of scans for the accumulation of ¹³C CP/MAS NMR spectra was 2048. During detection, a high-power dipolar decoupling was used to eliminate strong heteronuclear dipolar coupling. The isotropic chemical shift of ¹³C scale was calibrated with glycine as external standard (176.03 ppm – low-field carbonyl signal). Taking into account frictional heating of the samples during fast rotation all NMR experiments were performed at 308 K. Temperature calibration was performed on Pb(NO₃)₂ using a procedure described in the literature.

Fourier-transform infrared spectroscopy was performed with a Perkin-Elmer PARAGON 1000 PC FT-IR Spectrometer in a potassium bromide tablet. Graphs are normalized to the skeletal vibration band at 1002 cm⁻¹.

2.5. In vitro degradation study

The degradation study was done in phosphate buffered saline (PBS) buffer pH = 7.4 at 37 °C. Three replications were analyzed for each time point. Five milliliters of PBS buffer were added to the vial containing exactly weighed nanofibre sample (ca. 5 mg). At the beginning of the kinetic study all samples were shortly put into vacuum to get the material more in contact with the degradation buffer and to remove all gaseous bubbles from samples. At selected time points (29 days, 58 days, 87 days) the samples were placed on coarse frit (size 4), washed with distilled water for removing all salts present in environment, and dried at 50 °C for 12 h. The samples were weighed and the degree of degradation (i) was calculated according to eqn (1), where m is the weight of sample after degradation for the particular time and m₀ is weight of samples before it was placed in the degradation solution.

\[ i = \frac{(m - m₀)}{m₀} \]  

The molecular weight of glycogen after degradation of glycogen-graft-poly(ethyl cyanoacrylate) was determined by size exclusion chromatography (SEC) in acetate buffer (pH 6.5; 0.3 mol L⁻¹) as a mobile phase on Superose 6 column (Polymer Laboratories Ltd., UK) using HPLC System AKTA Explorer (Amersham Biosciences; Sweden) equipped with RI, UV and multiangle light-scattering DAWN DSP-F (Wyatt, U.S.A) detectors.
3. Results and discussion

We found that lyophilization of aqueous glycogen solution may lead to nanofibres and not a honeycomb-like structure as commonly observed. By this technique it is possible to easily prepare even bulk layers, which are otherwise hard to obtain by electrospinning. The morphology of freeze-dried glycogen depends on the starting concentration of GG in aqueous solution. We examined freeze-dried GG solutions in water with different initial concentration (0.1%, 0.2%, 0.5%, 1%, 2% and 5% by weight, respectively). The morphology was observed by SEM microscopy (Fig. 1). We can see that at higher GG concentration (>1%) no fibers are arising. The material had a sponge-like structure with certain communicating pore fraction. This structure arises by fusion on fibers, what was observed even at GG concentration from 2% to 5% (see Fig. 1).

However, more diluted aqueous solution of freeze-dried GG leads to fibrous structures with fibre thickness from 10^2 nanometers to 10^4 micrometers (see Fig. 1A–D). Higher concentrations (Fig. 1E) lead to structures with the majority of leaflets and the highest concentration (Fig. 1F) lead to sponge-like porous structures with communicating pores. Similar concentration-dependent morphology change of the freeze-dried nanostructures was described for chitosan, sodium carboxymethyl cellulose, lignin, keratin, polydiphenylacetylene or chitin—the fraction of (nano)fibrous morphology increases upon decrease of polymer concentration before freeze-drying. However, for glycogen, a nanofibrous material is obtained even at higher concentrations than described for other polymers, which is probably given by the degree of conformational flexibility and low tendency of glycogen to form crystalline domains given by hyperbranched structure as followed by NMR (see below). This is especially evident when compared to rigid polydiphenylacetylene with a high tendency to crystallize, where a concentration as low as 0.003 wt% was reported to be necessary to obtain nanofibres. In addition to this, even cooling in a freezer is enough to obtain a nanofibrous material without the necessity to use liquid nitrogen (we used a freezer with lower temperature decrease rate to obtain a homogenous material even in thicker layers, which is otherwise hard to obtain by rapid cooling in, e.g., liquid nitrogen). The final temperature after freeze-drying seems to have no significant effect on the morphology, because the material is completely frozen before water sublimation [the solution is salt-free and glycogen has very high molecular weight (see below), so molar depression of the melting point of water is negligible here]. In all cases, there is a high volume fraction of pores (the concentration of GG in starting solution 0.1–5 wt% gives 99.9–95% pore volume fraction in the material, the volume change during freezing and lyophilization can be neglected), which is advantageous for eventual use of such a material as a scaffold for tissue engineering applications, because cells need for their growth high specific surface of scaffold and high porosity. The mechanism of creating fibrous structure is not completely understood yet. One possible explanation is through the formation of ice microcrystals during freezing process and their continuously growth through and create a network of multi-crystalline aggregates of ice. After lyophilization, this process leads to interconnected network of pores left after evaporation of ice, in a similar way as in the case of cryogels and freeze-dried carbohydrate polymer (e.g., starch or cellu-
lose) foams, however with different (preferentially fibrillar in this case) micromorphology. It is unlikely that fibrous structures are present in solution before freezing, because no such structures were observed for glycogen and its conjugates in aqueous solutions.

Due to its solubility in water, GG itself is not appropriate for use in aqueous media, e.g. as a scaffold for tissue engineering. Therefore, it should be modified to become insoluble, which can be reached by exposing GG nanofibres in ethyl cyanoacrylate vapors, where polymerization from gaseous state occurs (Scheme 1).

Polymerization from gaseous state is very gentle to mechanical properties of nanostructured material and does not lead to collapse of the material (it changes the original morphology of GG structure only a little to a leaf-like structure). In addition, it is environment-friendly, because it does not require the use of organic solvents. Polymerization occurs by anion mechanism on alcohol groups on the molecule of GG, so glycogen-\textit{graft}-poly(ethyl cyanoacrylate) is formed. This graft polymer is insoluble in water and both GG and poly(ethyl cyanoacrylate) are biodegradable substructures (see above).

Grafting was performed with two GG samples with different architectures, which were prepared by freeze-drying 0.5% and 5% aqueous GG solutions, respectively. The GG samples were exposed to ethyl cyanoacrylate vapors for 7 days and 30 days, respectively and morphology is shown at Fig. 2.

The nanofibrous samples exposed to ethyl cyanoacrylate vapors for 7 days dissolve in water within several hours, which is too fast for practical use as a scaffold. Therefore samples

![Scheme 1 Polymerization of ethyl cyanoacrylate on glycoeurine macroinitiator.](image)

![Fig. 2 SEM micrographs of glycogen-\textit{graft}-poly(ethyl cyanoacrylate) prepared from nanostructures freeze-dried from different concentration of glycogen and with different subsequent different exposition times to ethyl cyanoacrylate vapors; 1 – 0.5%, 7 days, 2 – 0.5%, 30 days, 3 – 5%, 7 days, 4 – 5%, 30 days; (a) – minimal magnification, (b) – maximal magnification; e.g. (1a) – sample 0.5%, 7 days, minimal magnification.](image)
were left in a cyanoacrylate atmosphere for 30 days, after that the fibrous structure was stable and the polymer did not dissolve in water. The material architecture depends on the cyanoacrylate vapor exposition time; longer exposition times lead to more leaflets, which are similar to icing in shape. Basic architecture and communication of pores however still stay unchanged even after the 30 days exposition and the pores stay interconnected and their basic attributes are preserved.

The polymerization reaction was observed by infrared spectroscopy where alcohol O–H and ester C=O bands were compared. Polymerization reduces the alcohol band at 3400–3300 cm\(^{-1}\) while the C=O ester band at 1750 cm\(^{-1}\) rises up as polymerization proceeds. (see Fig. 3a and 3b). In accord with this, the ester C–O stretching vibration at 1254 cm\(^{-1}\) rises up during polymerization and the C–H stretching band around 2950 cm\(^{-1}\) is somehow suppressed and shifted to shorter wavelengths (higher wavenumbers) due to a change in abundance of various C–H bonds in the course of polymerization.

One can also clearly see on the infrared spectra that the samples synthesized from 0.5% aqueous GG solution contain more poly(ethyl cyanoacrylate) grafts than the samples

---

**Fig. 3** Spectra of polymerization ethyl cyanoacrylate on glycogen nanofibers (0.5% sample – (a), 5% sample – (b)), followed by infrared spectroscopy.
synthesized from 5% aqueous GG (when comparing samples exposed for the same time in ethyl cyanoacrylate vapors). This is consistent with the fact that polymerization probably mostly occurs on the surface of GG.

The results from infrared spectra were confirmed by elemental analysis, where the content of nitrogen in poly(ethyl cyanoacrylate) was determined. GG does not contain nitrogen in the molecule while poly(ethyl cyanoacrylate) contains 11.19% nitrogen. The results are summarized in Table 1 and they fully agree with the infrared spectral data. Further experiments with prolonging exposition time of the sample prepared from 0.5% GG solution to ethyl cyanoacrylate vapors to 60 days led to material with 8.91% N [i.e. 80.16 wt% poly(ethyl cyanoacrylate)].

To get a closer look at the structure of the materials, we used solid state nuclear magnetic resonance (ss-NMR). In native GG (Fig. 4a), the two broad shoulders that appear at $\delta 103$ and 95 ppm are thought to arise from non-crystalline material for C(1) carbons, and the broad resonance at $\delta 82$ ppm arises from non-crystalline material for C(4) carbons. These resonances are very broad due to the distribution of chemical shifts in the non-crystalline regions. Glycogen-graft-poly(ethyl cyanoacrylate) (Fig. 4b) shows amorphous character similar to native GG both in signals from the GG part and in signals from poly(ethyl cyanoacrylate) parts. The amorphous nature is advantageous for biodegradation, making it more homogenous within the sample since eventual crystalline microdomains are likely to be degraded slower than amorphous microdomains.

Surface area is in information relevant to degradation rate; however, unfortunately, the nitrogen adsorption technique available to us [BET, Quantasorb apparatus (Quantachrome, Greenvle, USA)] gave inconsistent results given by the polar polysaccharidic nature of the material resulting in low its affinity to nitrogen, as reported.47

The in vitro hydrolytic degradation study proved that the material gradually degraded and was almost completely dissolved after 87 days (Fig. 5).

Macroscopically, the material only slightly decreased its volume until its degradation was complete, therefore we suppose that the material degrades from the surface of the nanostructures, making them thinner during degradation but keeping the main shape, and only after degradation the structure collapses and gets fragmented. This is consistent

**Table 1** Content of nitrogen in glycogen-graft-poly(ethyl cyanoacrylate) and calculated weight contents of poly(ethyl cyanoacrylate) grafts; nitrogen content in original glycogen was not detectable within experimental error (i.e. less than 0.20 wt%) before exposition to ethyl cyanoacrylate vapors.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original glycogen concentration (wt%)</td>
<td>0.5</td>
<td>0.5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vapor exposition time (days)</td>
<td>7</td>
<td>30</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>Nitrogen content (wt%)</td>
<td>1.29</td>
<td>5.75</td>
<td>0.80</td>
<td>2.33</td>
</tr>
<tr>
<td>Content of grafts (wt%)</td>
<td>11.53</td>
<td>51.39</td>
<td>7.15</td>
<td>20.82</td>
</tr>
</tbody>
</table>

**Fig. 4** The solid state $^{13}$C NMR spectrum of native glycogen (a) and of glycogen-graft-poly(ethyl cyanoacrylate) (b).

**Fig. 5** Weight loss dependence on time, glycogen-graft-cyanoacrylate samples in PBS solution.
with the relatively hydrophobic nature of poly(ethyl cyanoacrylate).

Even after 125 days we can observe small fragments present in degradation solution by light scattering, however these fragments are well under weight measuring range, or more precisely in error aberrance of the analytical weight.

We proved total degradation by faster degradation study where the temperature was increased to 90 °C, where all solid structures dissolved in solution. We assume that these more slowly degradable parts may correspond to poly(ethyl cyanoacrylate)-rich domains. We tested the molecular weight of fragments dissolved into solution in the course of degradation by size exclusion chromatography, the obtained weight-average molecular weight of the fragments (7.89 MDa) was only a little higher than that of native glycogen from oyster (7.29 MDa). According to both refractometric and light scattering detections, all mass was in the peak near the upper exclusion limit of the column corresponding to glycogen (i.e. no low-molecular-weight polymers were detected in solution). Molecular weight of the fraction dissolved in water during degradation experiment [after filtering-off eventual remaining nanofibres with a 0.22 micron poly(vinylidene fluoride) filter] also did not depend on the concentration (dominant fraction of nanofibres at lower concentrations, more leaflets in higher concentrations and finally sponge-like structure with partially communicating pores at the highest concentrations). Polymerization of ethyl cyanoacrylate from the gaseous state on such a template leads to water-insoluble, yet degradable polymer. Both native and poly(ethyl cyanoacrylate)-grafted glycogen are amorphous, which was proven by solid state NMR. A degradation study showed a > 90% weight loss in a three months period. It is thus possible to easily prepare a biopolymer-based nanofibrous material by organic solvent-free green chemistry from glycogen, which is a renewable resource.

4. Conclusions

Glycogen forms nanostructures by freeze-drying of its aqueous solutions with morphology dependent on the original glycogen concentration (dominant fraction of nanofibres at lower concentrations, more leaflets in higher concentrations and finally sponge-like structure with partially communicating pores at the highest concentrations). Polymerization of ethyl cyanoacrylate from the gaseous state on such a template leads to water-insoluble, yet degradable polymer. Both native and poly(ethyl cyanoacrylate)-grafted glycogen are amorphous, which was proven by solid state NMR. A degradation study showed a > 90% weight loss in a three months period. It is thus possible to easily prepare a biopolymer-based nanofibrous material by organic solvent-free green chemistry from glycogen, which is a renewable resource.

Acknowledgements

Financial support of the Grant Agency of the Czech Republic, grants # P108/10/1560 and 202/09/2078, and of the Academy of Sciences of the Czech Republic, grant # M200501201, is gratefully acknowledged.

References

INTRODUCTION

Spinal cord injury (SCI) is one of the most common traumatic injuries with lifelong consequences. No successful treatment has yet been developed. Current surgical techniques and pharmacotherapy help to stabilize the environment but are unable to restore lost function and only minimally alleviate on-going side effects (Fehlings et al. 1976, Pointlart et al. 2000, Kwon et al. 2004). After the primary insult, nonselective massive cell death and on-going secondary processes render the microenvironment unsuitable for endogenous regeneration (Fitch and Silver 2008, Oyinbo 2011).

One of the promising approaches to treatment is the application of stem cell therapy due to the “rescue” and possible “replacement” effect of such cells in the lesion environment (Nandoe Tewarie et al. 2009, Ruff et al. 2012). Currently, among the many stem cell types used in spinal cord injury research, neural stem/progenitor cells appear to display the ability to maintain a potential for both the rescue and the replacement effects.
Moreover, several publications have already proven the effect of transplanted neural stem cells (NSCs) on functional outcome in different types of animal models (Cummings et al. 2005, Karimi-Abdolrezaee et al. 2006, Hooshmand et al. 2009, Nandoe Tewarie et al. 2009). In the search for a stable and less ethically controversial source of neural stem cells, advances in molecular biology now enable the reprogramming of stem cells and the derivation of genetically modified neural precursor cell lines. Conditionally immortalized cell lines can be easily expanded in vitro, and after transplantation, these cells survive and differentiate under in vivo conditions better than primary cultures of NSCs. One of the possibilities is to establish a conditioned cell line by the insertion of the c-myc gene-regulated hormone response element responding to the presence of 4-hydroxy tamoxifen (cMycERTAM technology) (Pollock et al. 2006). This technology has helped to obtain several different fetal neural lines derived from the human striatum, cortex and spinal cord (Jung et al. 2010). One of these cortical cell lines, CTX0E03, has proven its effectiveness and is currently being evaluated as a treatment for stroke in a phase I clinical trial (Pollock et al. 2006, http://www.reneuron.com/the-pisces-clinical-trial-in-disabled-stroke-patients). Based on these results, three cell lines of fetal NSCs derived from fetal human spinal cord – SPC-01, SPC-04 and SPC-06 – were described, and their properties in regards to their potential use in cell therapy for SCI were examined. One of these cell lines, SPC-01, is further explored in the present study. In our previous work, the SPC-01 line was described as a ventral-specific subtype precursor able to create NKX6.1+/LHX3+/CHX10+ V2a interneurons and Isl1+/ChAT+ motoneurons under specified condition in vitro. SPC-01 cells robustly survived in a spinal cord injury environment for a 4 month period and differentiated into relevant neuronal subtypes, including ChAT+ motoneurons in vivo (Price and Cocks 2011, Cocks – unpublished results). Grafted cells in the lesion produced, and stimulated the production of, several trophic factor and thus supported recovery not only by protecting the remaining tissue, but also by facilitating the sprouting of endogenous GAP43+ axons, which led to a significant improvement of locomotor and sensory function recovery (Amemori et al. 2011, Amemori et al. – unpublished results) as early as 2 months after transplantation.

In recent years it has become evident that combination strategies will play an important role in SCI treatment and that a multimodal approach will have a better chance to lead to functional improvement and tissue regeneration, particularly in chronic lesions where posttraumatic cavities are fully developed. When used in combination with biocompatible hydrogels and/or trophic factors, transplanted stem cells have a better chance to survive in the hostile lesion environment (Blesch et al. 2002, Fouad et al. 2005, Sykova et al. 2006). The hydrogel surface can be modified to increase the specific ingrowth of neural elements and facilitate stem cell differentiation (Lieb et al. 2005, Aizawa et al. 2008, Hejcl et al. 2009, Kubinova et al. 2009, 2010, Buzanska et al. 2009, Zychowicz et al. 2011). For hydrogel surface modifications, multifunctional molecules, such as the neurotransmitter serotonin, which can support the attachment of implanted or endogenous cells and positively influence and facilitate neuronal differentiation, can be used (Khurshid et al. 2010, Platel et al. 2010, Peng et al. 2012).

In our study we assessed the effect of treating a spinal cord hemisection in rats with a combination of the conditionally immortalized human neural stem cell line SPC-01, derived from fetal spinal cord, and a poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel modified with serotonin molecules (pHEMA-5-HT). We were particularly looking at the implant’s ability to serve as a cell carrier and 3D matrix for in vitro growth and differentiation and the effect of implantation on tissue reconstruction and sensory-motoric recovery after SCI.

**METHODS**

**Chemicals for hydrogel synthesis**

2-Aminopropyl methacrylamide hydrochloride was purchased from Polysciences Ltd. (Warrington, PA, USA). All other chemicals were purchased from Sigma-Aldrich Ltd. (Prague, Czech Republic).

**Hydrogel synthesis**

The porous hydrogels were synthesized by the radical polymerization of 2-hydroxyethyl methacrylate (835 mg, 6.42 mmol), [2-(methacryloyloxy)ethyl](trimethyl)ammonium chloride (57 mg, 0.274 mmol), ethylene glycol dimethacrylate (10 mg, 0.050 mmol) and 6 mg (0.033 mmol) of 2-aminopropyl methacrylamide
hydrochloride in poly(ethylene glycol) \((M_w=400, 1,900\) g) using azobis(isobutyronitrile) (4 mg) as an initiator and sodium chloride (5.01 g) as a porogen. The polymerization was carried out at 70°C in a sealed steel tube for 16 h and the gel 1 was then washed with water (3 × 24 h). All of the following reactions (see Fig. 1) were carried out at room temperature. Gel 1, containing protonated primary amino groups, was then converted to free base 2 and swollen by immersion into a solution of ethyldiisopropylamine (1 wt. % in methanol, 24 h) and washed with methanol (2 × 24 h). The free base-containing gel 2 was reacted with poly (ethylene glycol) diglycidyl ether \((M_w=526, 10\) wt. % in methanol, 48 h), and the resulting epoxide-containing gel 3 was washed with methanol (2 × 12 h). The epoxides were then reacted with a solution of serotonin hydrogen oxalate (200 mg) and triethylamine (2.00 g) in methanol (200 mL) for 3 days. The resulting serotonin-containing gel 4 was then washed with methanol (3 × 24 h) and phosphate buffered saline, pH 7.4 (PBS, 14 × 24 h). The gel was stored in phosphate-buffered saline (PBS) under argon at +5°C.

**Hydrogel seeding with the SPC-01 cell line**

The SPC-01 line was derived from 8-week-old fetal spinal cord tissue and conditionally immortalised with the 4-hydroxy tamoxifen (4OHT) inducible cMyc (cMycER\textsuperscript{TAM}) using an MMLV type of retrovirus. The product of the cMycER\textsuperscript{TAM} gene in the presence of 4OHT stimulates cell proliferation. In the absence of 4OHT, cell growth arrests, and the cells differentiate into neurons and astrocytes (Price and Cocks 2011, Cocks – unpublished results, Pollock et al. 2006). The SPC-01 cells were cultured in DMEM/F12 supplemented with 3% human albumin, 50 mg/ml apotransferine, 8.1 mg/ml putrescine DiHCL, 10 mg/ml human recombinant insulin, 20 μg/ml progesterone, 200 mM L-glutamine, 20 μg/ml sodium selenit, 10 μg/ml hrFGF, 10 μg/ml hrEGF, 45 penicillin/streptomycin at 50 U/ml (GIBCO) and 1 mM 4OHT as an anti-apoptotic factor. SPC-01 cells were seeded on the HEMA hydrogels. The gels with the cells were cultured for 28 days. The medium was changed every two days, and every seven

Fig. 1. Synthesis of the serotonin-containing hydrogel
days several samples of the gels with cells were chosen for immunohistochemical analysis. After 28 days the gel implants were used for implantation. In some of the in vitro and in vivo experiments, SPC-01_GFP3 cells (transfected with a GFP construct using a lentiviral vector) were used.

Hydrogel implantation

Two-month-old male Wistar rats \( (n=27) \) (Velaz, Prague, Czech Republic) with body weight between 300–330 g underwent a hemisection at the Th8 level. The animals were separated into three groups: A group \( (n=11) \) treated with a pHEMA-5HT hydrogel seeded with SPC-01 cells and two control groups bridged with a pHEMA-5HT hydrogel alone \( (n=7) \) or only hemisected, then left untreated \( (n=9) \). The animals were injected intraperitoneally with pentobarbital for anaesthesia (solution of 1 g/100 ml, 6 ml/1 kg of animal weight), Antibiotics (gentamicin 0.05 ml i.m.), and Atropine (0.2 ml s.c. 1:5) to prevent salivation during the surgery. The surgery was carried out using an operating microscope (Zeiss) at 15–25× under aseptic conditions. A linear 1–2 cm skin incision at the Th8-9 level was performed. The paravertebral muscles were detached from the lamina, and a laminectomy at Th8 was performed. Then, a 2 mm length of the dura mater was separated from the midline to the right edge of the spine, followed by a lateral hemisection. During the hemisection procedure, a cavity 1 mm³ in size was created. The hydrogels with /without SPC-01 cells were properly adjusted to fill the cavity volume in order to avoid mechanical stress and prevent cavity formation. The dura was sutured with Dafilon 10/0 thread (BBraun, Aesculap, Tuttlingen, Germany). From the muscles to the skin the layers were sutured separately (Hejcl et al. 2009).

Following the hemisection procedure, cyclosporin A (10 mg/kg, Sandimmun, Novartis) and MPSS (1.7 mg/kg, solu-medrol, Pfizer) were injected once a day; after one month, the frequency decreased to once every two days. To prevent infection, the animals were housed in pairs with food and water ad libitum in a system of internally ventilated cages (IVC, Tecniplast). This study was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Central Commission for Animal Protection of the Academy of Sciences of the Czech Republic in Prague.

Behavioral assessment

The behavioral part of the experiment included a 12 week period postinjury. The animals were tested by two blind observers 5 days before the injury in order to obtain presurgical control data and then once a week starting 7 days after surgery. Hind limb performance was evaluated using the Basso–Beattie–Bresnahan (BBB) open-field locomotor test (Basso et al. 1995). Sensory sensitivity (Thermal nociception) was examined by measuring the latency of hind limb withdrawal from a thermal stimulus using a plantar test device (Ugo Basile, Italy) as described previously (Urdziková et al. 2006.)

Immunocytochemistry

Cells seeded on hydrogel cubes were washed in PBS (pH 7.2) and fixed with 4% paraformaldehyde in PBS for 30 minutes. Prior to immunostaining, the fixed cells were twice washed in PBS. Permeabilisation and blocking were carried out in a blocking buffer consisting of 0.1% Triton (Chemicon 2170-S), 5% goat serum, and 1mg/ml bovine serum albumin in TRISS buffer for 20 min. To identify SPC-01 cells, antibodies directed against neuronal-cell adhesive molecule (N-CAM Chemicon AB5032), neurofilaments 70 (Chemicon MAB1615), nestin (Chemicon MAB5320), synaptophysin (Chemicon MAB5258), myelin/oligodendrocyte-specific protein (MOSP Millipore MAB328), CNPase (Abcam ab109758) and βIII-tubulin (S-SigmaT-8660) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 (molecular probes, Invitrogen A11029, A11032). The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide.

Immunohistochemistry

Rats were sacrificed one or three months after surgery. Anesthetized animals were perfused with 4% paraformaldehyde in 0.1M PBS (pH 7.4). After perfusion, a 3 cm piece of the spinal cord containing the center of the lesion was removed from the body, and the spinal cord, along with the bone, was left in 4% paraformaldehyde in 0.1 M phosphate buffer overnight. Then, the spinal cord was removed from the bone and post-fixed in the same fixative for 1 week. The fixed spines were immersed in
Fig. 2. SPC-01 human fetal neural stem cells twenty-eight days after seeding on a pHEMA-5HT hydrogel. The HEMA hydrogel, containing serotonin molecules, was grown through to confluence by the SPC-01 cells. The cells showed positivity for the neuroectodermal markers neurofilaments 70 (A) – higher magnification (B), neuronal-cell adhesive molecule (C), nestin (D), synaptophysin (E) and βIII-tubulin (F). Scale bar is 40 µm (A, C, D, E, F) and 20 µm (B).
Fig. 3. SPC-01_GFP3 cells seeded on a pHEMA-5HT hydrogel one month after implantation into a spinal cord hemisection cavity. The cells survived well, mostly at the hydrogel-tissue border and in the peripheral part of the implanted hydrogel. The implanted cells were positive for the neuronal markers nestin (A) and βIII-tubulin (B) as well as for GFAP (C) ChAT (D) and Tau (E). The implanted cells also showed an astrocyte-like morphology (F). Scale bar is 50 µm (C, E), 20 µm (A, B), and 10 µm (D, F).
PBS with 30% sucrose. Frozen spinal sections (40 µm) were cut through the area of interest. To identify SPC-01_GFP3 cells in the spinal cord, antibodies directed against βIII-tubulin (S-Sigma T-8660), glial fibrillary acidic protein (GFAP- Cy3 Sigma C-9205), choline acetyltransferase (ChAT, ab68779 Abcam), mitochondrially encoded cytochrome c oxidase II (MTC02, ab109739 Abcam), nestin (Chemicon MAB5320) and Tau (A0024 Dako Cytomation) were used. To assess the interaction between the implant and the surrounding lesioned tissue, hematoxylin-eosin staining and antibodies directed against GFAP (GFAP- Cy3 Sigma C-9205), neurofilaments 160 (Sigma N5264) and the endothelial cell marker RECA1 (abcam ab9774) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse IgG conjugated with Alexa-Fluor 488 or 594 and goat anti-rabbit IgG conjugated with Alexa-Fluor 594 (molecular probes, Invitrogen, A11029, A11032, and A11012). Since all of the antibodies cross-react with rat proteins and since the cells can lose GFP during their differentiation, for colocalization studies we used not only GFP, but also human-specific markers such as MTC02, which does not cross-react with rat cells. The samples were washed three times with PBS and mounted with vectashield (Vector H-1000) on a glass slide.

**Fluorescence and confocal microscopy**

The samples were examined using a spectral confocal microscope (LSM 5 DUO, Zeiss) equipped with an Ar/HeNe laser or a ZEISS AXIO Observer D1 microscope (Carl Zeiss, Germany). For confocal microscopy, 405 nm (DAPI), 560 nm (gamAF594), and 488 nm (Ar, gamAF488) lasers were used to visualize the neural markers expressed by differentiated SPC-01_GFP3 cells *in vitro* and *in vivo*. The Observer D1 microscope was used to visualize the effects of the cell and hydrogel implant on the microstructural reconstruction of the surrounding damaged tissue and the implant’s *in vivo* ability to support host tissue ingrowth.

**Quantification of microstructural changes**

For evaluating spinal tissue atrophy, the surface areas of the tissue on hematoxylin-eosin slices were...
averaged using image J software; the analysis was performed at 25× magnification.

To evaluate the degree of astrogliosis in the tissue surrounding the hydrogel, an analysis based on the size of the area displaying high fluorescence intensity for GFAP positivity was made at 200× magnification. Five specific locations surrounding the main lesion cavity were measured to determine the extent of GFAP positivity. The measurements were carried out on every 6th slice from the spinal cord of each treated and control animal at the terminal point of the experiment.

To evaluate the ingrowth of axons into the hydrogel and hydrogel revascularization, an analysis based on the size of the area displaying high fluorescence intensity for NF160 or RECA positivity was made at 200× magnification. Three specific locations inside the hydrogel were measured to determine the extent of NF160 or RECA positivity. The measurements were made on every 6th slice from the spinal cord of each treated and control animal at the endpoint of the experiment. All of the fluorescent analyses were carried out using Axiovision 4.8 software (Zeiss).

**Statistical evaluation**

In the immunohistochemical analysis, the F-test was used to determine variability within the groups. For analyzing differences in the histological data among the groups and to compare two different time points in the histological data, Student’s t-test was used [one side, two choices, for the same or different data variability (regarding the F-test results)]. In order to distinguish the effect of treatment on behavioral recovery, one way ANOVA was used at each time point.

Fig. 5. Differentiation of SPC-01_GPF3 cells and their interaction with endogenous neural cells three months after the implantation of a cell-polymer construct. The implanted cells that remained in the vicinity of the hydrogel-tissue border were positive for nestin (A) and ChAT (C). They interacted with host NF160-positive axons (B) and integrated into the GFAP-positive scar (D). Scale bar is 40 µm (A,B), 10 µm (C), and 80 µm (D).
point. The standard error of the mean was calculated in order to better assess the data variability.

**RESULTS**

**SPC-01 culture**

Prior to transplantation, we evaluated the ability of SPC-01 cells to grow and differentiate on pHEMA-5HT hydrogels in vitro. After 28 days of culturing, the populations of SPC-01 as well as SPC-01_GFP3 cells on the pHEMA-5HT hydrogels were nearly confluent. Despite the presence of 4OHT, both cell lines differentiated into a neural phenotype. The cells were positive for several markers of neural differentiation, including neurofilaments 70 (Fig. 2A, in detail B), neuronal-cell adhesive molecule (Fig. 2C), nestin (Fig. 2D), synaptophysin (Fig. 2E) and βIII-tubulin (Fig. 2F). No CNPase- or MOSP-positive cells were detected. These results show that the pHEMA-5HT hydrogel can support the expansion and growth of spinal progenitor cells in a 3D artificial environment. Serotonin molecules can facilitate the neural differentiation of these cells even in the presence of 4OHT, which under standard culture conditions maintains SPC-01 cells in a proliferative and immature state.

**SPC-01 proliferation and differentiation in vivo**

One month after the implantation of the cell-polymer construct, the SPC-01_GFP3 cells survived very well in the lesion area. The majority of them remained in the peripheral part of the hydrogel and at the hydro-

![Fig. 6. Quantification of the effect of treatment on the host tissue. The evaluation of the spared spinal tissue (A) and the extent of astrogliosis (B) in lesioned animals and in animals treated with combined therapy or hydrogel only, one and three month after surgery. The ingrowth of host axons (C) and blood vessels (D) into the hydrogel or cell-polymer implant one and three months after injury. The peripheral (P) and central (C) parts of the implant were evaluated separately.](image-url)
gel tissue border; a few cells were dispersed at a distance from the lesion area. The implanted cells were nestin-positive (Fig. 3A); however, they expressed several markers of advanced neural differentiation including βIII-tubulin (Fig. 3B), GFAP (Fig. 3C), ChAT (Fig. 3D) and Tau (Fig. 3E). Some of the grafted SPC-01 cells showed an astrocyte-like morphology (Fig. 3F).

Three months after the implantation of the cell-polymer construct, the grafted cells still survived well in the tissue (Fig. 4A); however, the majority of them migrated into the spinal cord stump, some of them as far as 4 mm rostrally from the hydrogel-tissue border (Fig. 4A, B). We did not observe such long distance migration towards the caudal end of the spinal cord. Other cells remain in the hydrogel-tissue border leaving the center of the hydrogel populated only sparsely (Fig. 4A, C). The implanted cells were still positive for nestin (Fig. 5A), while other cells were positive for NF 160 (Fig. 5B), ChAT (Fig. 5C) and GFAP (Fig. 5D). The majority of the implanted cells showed interconnectivity with endogenous tissue elements in the spinal cord tissue, and some of them remained primarily in clusters on the edge of the hydrogel. GFAP-positive cells formed part of the glial scar. We did not observe any colocalization of GFP or MTC02 with oligodendrocytic markers, such as CNPase or MOSP.

**Spinal cord tissue atrophy**

To assess the effect of treatment on protecting against tissue atrophy, the extent of spared tissue on the hydrogel border was evaluated. On haematoxylin-eosin stained slices 1 month after surgery, a difference in the area sum of the remaining spinal tissue between the cell-polymer group (5.78 ± 0.18 mm²) and both other groups, control (4.99 ± 0.18 mm², F₃,₄=1.47 P<0.35, tₛ=2.39 P<0.05) and hydrogel-only (4.63 ± 0.14 mm², F₃,₃=2.00 P<0.29, tₛ=3.43 P<0.01), was observed (Fig. 6A). No statistical difference in the amount of remaining tissue was observed among the groups 3 months after surgery.

**Glia scar**

On GFAP-Cy3 stained spinal tissue, a significant decrease in the extent of astrocyte marker positivity (GFAP) was observed in both the SPC-01_GFP3-seeded pHEMA-5HT hydrogel group (F₃,₄=3.80 P<0.15, tₛ=2.07 P<0.05) and the pHEMA-5HT hydrogel group (F₃,₃=28.61 P<0.01, tₛ=2.76 P<0.05) compared to the control animals (Fig. 6B) one month after surgery. No differences between the animals treated with a hydrogel alone and those treated with a hydrogel seeded with cells were detected. No statistically significant difference in astrocyte remodeling of the glial scar was observed among the groups 3 months after surgery.

**Ingrowth of endogenous tissue elements into the hydrogel**

We evaluated the implant’s suitability for endogenous axon and blood vessel ingrowth. The hydrogel was partially populated by connective tissue elements. In hydrogels in which clusters of SPC-01_GFP3 cells were present at the border of the hydrogel, an insignificant trend towards an increase of axonal ingrowth into the peripheral and central zones of the polymer was detected one month after surgery. A significant long term effect of SPC-01_GFP3 cells on axonal infiltration was observed two months later (seeded vs. un-seeded hydrogel, F₄,₄=2.40 P<0.21, tₛ=1.99 P<0.05) (Fig. 6C). A long term effect of SPC-01_GFP3 cells was observed on the vascularization of the peripheral parts of the hydrogel. We observed a gradual increase in blood vessel infiltration (SPC-01 cell-seeded pHEMA-5HT hydrogel 1 m. vs. 3 m. F₄,₃=4.82 P<0.11, tₛ=2.15 P<0.05) (Fig. 6D), and at the end of the experiment a strong trend towards an increased attraction of blood vessels into the hydrogel was observed in the combined therapy group (F₄,₃=3.30 P<0.18, tₛ=1.86 P<0.06).

**Behavioral testing**

No significant differences in the average hindlimb locomotor score or in the average latency of hindlimb withdrawal were observed among any groups over a twelve week period.

**DISCUSSION**

In the present study we used a 01 clone of human fetal neural stem cells (SPC-01) seeded on poly hydroxyethyl methacrylate-based hydrogels modified with serotonin molecules to treat a lateral hemisection of the spinal cord in adult rats. We demonstrated the synergic effect of serotonin-modified pHEMA hydro-
gels on the growth and differentiation of neural stem cells *in vitro* in the presence of 4OHT and the paracrine effect of the implanted cells on the protection of the remaining tissue and the ingrowth of host axons and blood vessels. The polymer itself reduced astrogliosis one month after injury. The SPC-01 cells survived well, proliferated and differentiated into relevant neuronal subtypes *in vivo*. However, we did not observe any long term effect that would lead to tissue reconstruction and the improvement of locomotor and sensory function.

The initial aim of utilizing a pHEMA hydrogel modified on its surface with serotonin molecules was to support the attachment of seeded SPC-01 neural precursors and to accelerate their differentiation process, since serotonin is considered to be a positive regulator of adult neurogenesis in the subventricular zone (Platel et al. 2010) and inhibiting serotonin uptake can promote neurogenesis and protect the viability of neural stem cells (Peng et al. 2012). In our work the serotonin molecules were covalently bound on the hydrogel with only a slight chance of being released into the injured spinal cord.

After 28 days of *in vitro* co-cultivation, the modified pHEMA hydrogels were filled with SPC-01 neural precursors that, due to the presence of the serotonin molecules on the hydrogel surface, started to differentiate despite the presence of 4OHT and the absence of additional differentiating factors in the medium. The cells were positive for several markers of neural differentiation, attached well to the surface of the hydrogel and grew through it within 4 weeks. We performed *in vitro* experiments with SPC-01 cells as well as with SPC-01_GFP3 cells with no difference in cell behavior. The pHEMA-5HT hydrogel appeared to be an ideal material for further *in vivo* study and an excellent 3D cell carrier for transfer into the injured tissue.

The differentiation of implanted SPC-01_GFP3 cells continued *in vivo*, and one month after implantation the cells were positive for relevant markers of neuronal differentiation, including those for neurons (Tau) and motoneurons (ChAT), or for GFAP, a marker of astrocytes. A portion of these cells displayed an astrocyte-like phenotype morphology and showed interconnectivity with endogenous tissue elements. At the end of the experiment the cells remained positive for the same markers (Nestin, GFAP, NF160 or ChAT), but they demonstrated a more mature phenotype. In our previous work we have shown that SPC-01 cells, when transplanted into a balloon-induced spinal cord compression lesion, differentiated and matured very slowly. Two months after grafting they were positive only for nestin and GFAP, and only 4 months after grafting did they express Islet2, Tau, and Chat, more mature neuronal markers. In none of our studies did we observe the expression of oligodendrocytic markers. This might be due to the original p2 and pMN sub-domains from which the SPC-01 cell line was derived, since these sub-domains give rise to two main lineages of interneurons, V2a and V2b, as well as to motoneurons during development (Price and Cocks 2011). In the present study the implanted cells were more mature already one month after surgery, pointing out the effect of the pHEMA-5HT hydrogel as a material accelerating their differentiation. Our previous work had also shown that SPC-01 cells were able to spare the white matter and prevent tissue atrophy due to the expression of neurotrophins while stimulating the production of host neurotrophic factors as a result of their robust survival (17% of grafted cells) in the center of the lesion (Amemori et al. 2011, Amemori et al. – unpublished results). It is well known that the production of NGF, NT3 and BDNF by either implanted or by endogenous cells is crucial for neuronal cell survival, proliferation and tissue reconstruction after SCI (Jakeman et al. 1998, Jones et al. 2001, Blesh et al. 2002). In our experiments, one month after implantation a supportive effect of SPC-01 cells in protecting the remaining tissue was found in the combined therapy group. On the other hand, the presence of the hydrogel itself decreased the extent of astrogliosis one month after injury. A similar effect on glial scar reduction was observed in the case of pHPMA-based hydrogels (Woerly et al. 2004). This effect was not seen 3 months after injury, possibly due to the fact that the SPC-01_GFP3 cells migrated out of the hydrogel and differentiated into GFAP-positive astrocytes, therefore contributing to the GFAP positivity found using immunohistochemistry.

The implanted hydrogels were partially penetrated by endogenous axons and capillaries, and the SPC-01 cells increased the ingrowth of host axons into the implant. All of these results were apparent one month after surgery, i.e. at the time when the majority of the transplanted cells were still present in the center of lesion and along the hydrogel-tissue border. The efficacy of the serotonin molecule as an attractant proved to be sufficient for new tissue ingrowth and the differentiation of the implanted cells. However, for long term cell attachment and survival in the hydrogel, further modification of the hydrogel surface, such as the
addition of primary AA sequences of extracellular matrix proteins attached to the biomaterial, might be beneficial (Lieb et al. 2005). The decreased effectiveness of serotonin from a long-term perspective may also be caused by the eventual enzymatic degradation of serotonin bound to the hydrogel surface, which may require the use of synthetic serotonin analogs-agonists that are resistant to enzymatic degradation.

During the time of the experiment, over a period of 3 months, the majority of the implanted SPC-01 cells migrated up to 4 mm from the center of the lesion, while only a minority remained in the peripheral part of the hydrogel and along the hydrogel-tissue border. This fact prevents the prolongation of the initial effect of paracrine stimulation and thus the functional reconstruction of the injured tissue. Despite the fact that at the end of the experiment the hydrogel material was still supporting the ingrowth of endogenous tissue elements, and despite the synergic effect of SPC-01-derived neural precursors on attracting endogenous blood vessels and axons into the seeded hydrogel, no further reduction in tissue atrophy or astrogliosis was observed.

The possible reasons why the treatment did not affect the behavioral recovery are more complex. The major reason, already described above, is that the initial paracrine effect became less effective due to the inability of the pHEMA-5HT hydrogel to support the proliferation of the implanted cells and their accumulation in the central part of the hydrogel. Those cells dispersed along the spinal cord and thus could not contribute to the cumulative effect described in our previous work (Amemori et al. 2011, Amemori et al. – unpublished results). Further, the hemisection model of SCI is not the ideal model for the assessment of functional outcome due to large intra-group variability, which can be caused by variability in the number of spared axons along the incision line in the central part of the spinal cord lesion.

Most in vivo studies have focused on the initial acute and sub-acute intervals following SCI, up to one month survival, and thus describe mostly the paracrine effect of the treatment without analyzing its effect on long term reconstruction in chronic injury (Bakshi et al. 2004, Piantino et al. 2006, Austin et al. 2012, Moradi et al. 2012, Niapour et al. 2012). In our experiment, a significant effect of the treatment during the acute and subacute phases was found as well. However, instead of a long-term effect on tissue reconstruction within the implant leading to behavioral recovery, we observed vanishing effects as well as the prevalence of typical endogenous repair processes, such as glial scar formation and tissue atrophy. It is therefore obvious that for assessments of spinal cord regeneration, long term experiments are necessary.

**CONCLUSIONS**

We have shown that serotonin-modified pHEMA hydrogels can serve as a supportive environment to stimulate and accelerate the differentiation of implanted SPC-01_GFP3 cells both in vitro and in vivo. SPC-01_GFP3 cells seeded on a pHEMA-5HT hydrogel migrated out from the polymer center and survived well at the cell-hydrogel border as well as in the spinal cord tissue. The treatment resulted in an initial reduction of tissue atrophy and glial scar formation, probably due to a paracrine effect, and long term support of the infiltration of the implanted material by host blood vessels and axons. However, the hydrogel did not provide ideal long term support for the continued growth and differentiation of the cells within the implant, most probably due to the aggressive spinal cord injury environment. Further surface modifications will be necessary to achieve long term tissue reconstruction after spinal cord injury.

**ACKNOWLEDGEMENTS**

We thank Hana Hronova, Lenka Kohoutova, Alena Vesela, and Pavlina Mackova for excellent technical assistance. We thank James Dutt for critical reading of the manuscript.

We acknowledge the support provided by the grants GAUK521712, GA CR P108/10/1560, GA CR P304/11/P633, GA CR 13-00939S and GA AV IAA500390902.

**REFERENCES**


