Intrarenal drug delivery from a hydrogel carrier implanted under the kidney capsule is an innovative way to induce kidney tissue regeneration and/or prevent kidney inflammation or fibrosis. We report here on the development of supramolecular hydrogels for this application. We have synthesized two types of supramolecular hydrogelators by connecting the hydrogen bonding moieties to poly(ethylene glycols) in two different ways in order to obtain hydrogels with different physico-chemical properties. Chain-extended hydrogelators containing hydrogen bonding units in the main chain, and bifunctional hydrogelators end-functionalized with hydrogen bonding moieties, were made. The influence of these hydrogels on the renal cortex when implanted under the kidney capsule was studied. The overall tissue response to these hydrogels was found to be mild, and minimal damage to the cortex was observed, using the infiltration of macrophages, formation of myofibroblasts, and the deposition of collagen III as relevant read-out parameters. Differences in tissue response to these hydrogels could be related to the different physico-chemical properties of the three hydrogels. The strong, flexible and slow eroding chain-extended hydrogels are proposed to be suitable for long-term intrarenal delivery of organic drugs, while the weaker, soft and fast eroding bifunctional hydrogel is eminently suitable for short-term, fast delivery of protein drugs to the kidney cortex. The favourable biological behaviour of the supramolecular hydrogels makes them exquisite candidates for subcapsular drug delivery, and paves the way to various opportunities for intrarenal therapy.
6.1 INTRODUCTION

The worldwide increasing incidence of end-stage renal disease raises the need for innovative therapeutic approaches that promote functional kidney regeneration, thereby preventing chronic renal failure. Current therapies include the systemic, i.e. intravenous or intraperitoneal, administration of anti-inflammatory, immune-suppressive and anti-fibrotic drugs. However, these strategies are accompanied by unwanted side effects due to lack of control over drug localization. Major progress has been made in the research of targeted drug delivery to for example proximal tubular epithelial cells. In this work we show a new approach consisting of the implantation of a drug delivery system under the kidney capsule. As carrier systems we use hydrogels that due to their physical properties are proposed to be able to adjust to the subcapsular space.

Many hydrogels have been used for regenerative medicine and drug delivery purposes, frequently by implantation or injection at various sites in the body. Detailed studies on the tissue response to different hydrogels mainly focus on subcutaneous implantation while studies on subcapsular implantation remain limited. The kidney capsule as implantation site is primarily investigated for pancreatic cell or islets transplantation in diabetic animal models. These cells have for example been encapsulated in microcapsules and their survival and function have been studied under the kidney capsule. However, studies on the tissue response to subcapsularly implanted hydrogel systems, that are designed for intrarenal drug delivery, are to our knowledge not present.

In this study we designed and synthesized three supramolecular hydrogels for use as intrarenal drug delivery systems and investigated the renal tissue response after subcapsular implantation. We use supramolecular hydrogels, i.e. hydrogels in which the network is formed by directed non-covalent interactions, so-called transient supramolecular networks. These supramolecular systems are proposed to have physical properties which allow for easy implantation between and adjustment to the renal capsule and the soft cortical kidney tissue. Recently, we have shown that supramolecular building blocks based on ureido-pyrimidinone (UPy) units are excellent elastomeric biomaterials. Also, bioactive supramolecular materials could be made by simple mixing of UPy-modified prepolymers and UPy-functionalized peptides. In the past, the tissue response to these UPy-modified biomaterials have been studied subcutaneously. Here, we apply this supramolecular and modular principle to design hydrogels by introduction of UPy-moieties on poly(ethylene glycol) (PEG) prepolymers. Recently we have published an extensive study on the properties of different telechelic UPy-modified PEGs. Here, we show the difference between two types of PEG hydrogels, containing UPy-moieties in the main chain and at the chain ends (i.e. telechelic UPy-PEGs) (Fig. 1A). After discussing the synthesis, formulation procedure and rheological behaviour of these hydrogels, we report on their intrarenal behaviour and tissue response, and propose possible therapeutic applications in relation to their physical properties.

6.2 RESULTS

SYNTHESIS

The three hydrogelators 1-3 (Scheme 1A) were synthesized as followed (Scheme 2): telechelic hydroxy-terminated prepolymers PEG3000 a, PEG6000 b, PCL1250 c, and PEG20000 d
were activated with 1,1-carbonyldiimidazole (CDI). The resulting CDI-activated prepolymers were reacted with 1,6-hexyldiamine for 4a-c, or with 1,12-dodecyldiamine in the case of 4d, yielding diamine-terminated polymers 5a-d. Reaction of diamine-terminated 5a and 5b in 1:1 w/w% ratio with 1 equivalent IPDI-UPy-IPDI synthon\textsuperscript{11} yielded 1 as a white, semi-crystalline, elastic, tough material. This chain-extended hydrogelator 1 contained PEG3000, PEG6000 and a high number of UPy-moieties per polymer chain. Chain-extended hydrogelator 2 was synthesized in a similar way from 5b, 5c and IPDI-UPy-IPDI, which resulted in a white, semi-crystalline, elastic, tough UPy-co-polymeric material of PEG6000 and PCL1250 in 9:1 w/w% ratio. Via reaction of the diamine-terminated PEG20000 5d, with the UPy-hexyl-isocyanate synthon\textsuperscript{11}, bifunctional hydrogelator 3 was obtained as a white powder.

\textbf{SCHEME 1. CHEMICAL STRUCTURES OF THE UPy-MODIFIED HYDROGELATORS, GEL PREPARATION, AND PROPOSED DEGRADATION BEHAVIOUR.} \textit{A.} Two chain-extended UPy-modified hydrogelators, 1 and 2, were used; 1 based on either PEG with M\textsubscript{n}=3000 and M\textsubscript{n}=6000 g/mol in a ratio of 1:1 w/w%, and 2 based on both PEG with M\textsubscript{n}=6000 g/mol and PCL with M\textsubscript{n}=1250 g/mol with a ratio of 9:1 w/w%. One end-functionalized UPy-hydrogelator 3 was investigated, based on PEG with M\textsubscript{n}=20,000 g/mol. \textit{B.} Gelation procedure of UPy-hydrogelators 1-3: \textit{i.} Prepolymers 1 or 2 were dissolved in a mixture of 1:1 THF:0.9% NaCl aqueous solution by stirring at 21 °C. \textit{ii.} THF is removed in vacuo to obtain 5 w/w% gels. \textit{iii.} After removal of THF the gels were formed. \textit{iv.} Prepolymer 3 was dissolved in water by stirring at 60 °C. \textit{v.} The solution was cooled to 21 °C to gelate, and to obtain a 10 w/w% gel. \textit{C.} Schematic representation of the proposed degradation behaviour: conventional hydrolysis must occur for gels 1 and 2 to degrade, and gel 3 is able to erode via a supramolecular degradation mechanism.
CHAPTER 6

SCHEME 2. SYNTHESIS OF THE UPY-HYDROGELATORS 1-3. ia-d. Telechelic hydroxy-terminated prepolymers a-d were reacted with CDI in chloroform at 21 °C for 18 h (a-c) or for 8 h (d). iia-c. CDI-terminated 4a-4c were reacted with 1,6-hexyldiamine in DCM at 21 °C for 1.5 h. iid. CDI-terminated 4d was reacted with 1,12-dodecyldiamine in DCM at 60 °C for 4 h. iii. The IPDI-UPy-IPDI synthon was reacted with diamine-terminated PEG 5a and PEG 5b in chloroform at 40 °C for 18 h to obtain chain-extended hydrogelator 1. iv. The IPDI-UPy-IPDI synthon was reacted with diamine-terminated PEG 5b and PCL 5c in chloroform at 40 °C for 18 h to obtain chain-extended hydrogelator 2. v. The UPy-hexyl-isocyanate synthon was reacted with diamine-terminated PEG 5d in chloroform at 70 °C for 3 h to obtain chain-extended hydrogelator 3.

GELATION PROCEDURES AND DEGRADATION BEHAVIOUR

The gelation procedure for the chain-extended and the bifunctional hydrogelators differed. Whereas organic solvent was required to form the chain-extended hydrogels, for gelation of the bifunctional hydrogelator application of only an aqueous solution was adequate (Scheme 1B). The chain-extended hydrogelators were dissolved in THF, after which a physiological salt solution was added. THF was removed under reduced pressure, and soft and elastic 5 w/w% hydrogels were formed. Relatively, hydrogel 2 was more rigid and shape-persistent than hydrogel 1. Hydrogelator 3 was dissolved in a physiological salt solution at 60 °C, and subsequently allowed to set at 21 °C yielding a soft hydrogel. Short incubation, i.e. for 18 h, of 0.05-0.2 g of the hydrogels 1-3 at 37 °C in 1 mL water, buffer or cell culture medium showed that hydrogel 1 and 2 did not undergo visible changes, while gel 3 was completely dissolved. Apparently, the chain-extended hydrogelators cannot be molecularly dissolved in water because of the higher molecular weight of these macromolecules and the multiple UPy-units per chain. Therefore, an organic solvent is needed which provides an environment in which these hydrogelators can (partially) dissolve. The gelation process takes place after addition of water and removal of the organic solvent. In contrast, the bifunctional hydrogelator dissolves in water at elevated temperatures, and gelates after cooling to room temperature.

According to these findings we propose that the chain-extended hydrogels can only degrade by hydrolysis, and not by dissolution of entire polymer chains (Scheme 1C). The bifunctional hydrogelator gelates in situ in an aqueous environment, indicating that a totally different process occurs. It is proposed that in order to form a hydrogel, the UPy-dimers of 3 have to form fibers by π-π interactions, additional hydrogen bonding between the urea-groups,
and accessory hydrophobic interactions of the dodecyl spacers. The bifunctional hydrogels will erode by dissolution of single chains or small polymeric aggregates, i.e. via supramolecular degradation (Fig. 2B). It is expected that similar dissolution behaviour of the hydrogels is manifested in vivo after subcapsular implantation in the kidney; experimental details will be discussed below. The exact mechanism of hydrogel formation and erosion of similar bifunctional UPy-hydrogelators in vitro has been investigated extensively in another study.18

RHEOLOGICAL PROPERTIES

The mechanical properties of the hydrogels were investigated using rheology (Fig. 1). Hydrogel 1 shows pronounced viscoelastic behaviour as the moduli and the viscosity are strongly frequency-dependent. At lower frequencies the material has fluid-like properties; G''>G', i.e. the flow domain. At higher frequencies elastic behaviour is observed; G'>G'', i.e. the rubber plateau. Hydrogel 1 is very elastic but can flow in time showing 'creep' behaviour. This implies that upon sudden stresses, i.e. shorter time scales, the material can elastically be deformed and reformed, while at longer time scales it can adjust its shape to minimize persistent surrounding stresses. The other two hydrogels, 2 and 3, display a rubber plateau over the whole frequency domain measured. Therefore, these materials are more shape persistent. Hydrogel 2 is more rigid than hydrogel 3 because of its higher storage modulus G’ over the whole frequency domain. Hydrogel 2 has a G’ of approximately 1000 Pa, whereas 3 shows a G’ of approximately 100 Pa.

FIGURE 1. DYNAMIC MECHANICAL PROPERTIES. Master curves at 20 °C of the storage modulus G’, loss modulus G’’, dynamic viscosity η*, and the phase angle δ versus the frequency of gel 1, 2 and 3.
Prior to *in vivo* subcapsular implantation, the *in vitro* biocompatibility was assessed as a predictor of *in vivo* behaviour. *In vitro* biocompatibility was investigated using human primary tubular epithelial cells (PTEC) as a model for the renal setting (Fig. 2). The biocompatibility of materials 1-3 was determined by culture of PTEC in the presence of materials 1-3, as dry hydrogelator or hydrogel. Biocompatibility was assessed by morphological examination of the cells, metabolic activity (determined in the resazurin assay) and nucleic acid content (determined by CyQuant assay) (Fig. 2). In all three assays, no differences were found between cells cultured in the presence of materials and cells cultured in pristine culture medium (Fig. 2). In addition, the materials were tested for the presence of bacterial endotoxin, which is known to cause inflammation *in vivo*. The UPy-modified hydrogelators contained approximately 14-23 times less endotoxin than the value permitted by the FDA 0.5 EU/mL (0.036 EU/mL for 5 w/w% 1, 0.022 EU/mL for 5 w/w% 2, 0.026 EU/mL for 10 w/w% 3 in water). These results show that 1-3 are not toxic and are safe for *in vivo* use.

**FIGURE 2. IN-VITRO BIOCOMPATIBILITY.** The morphology of primary tubular epithelial cells (PTEC) incubated without, i.e. control c, or with 1-3 was visualized by optical microscopy. Scale bars represent 100 µm. Metabolic activity of these PTEC cells was determined by the resazurin assay, and nucleic acid content was determined by the CyQuant assay. These biocompatibility tests were performed on **A.** solid hydrogelators and on **B.** gels. No statistical differences with the control c were found.
FOREIGN BODY RESPONSE TOWARDS SUPRAMOLECULAR HYDROGELS UNDER KIDNEY CAPSULE

RENAL IMPLANTATION AND EXPLANTATION

In the clinical setting, the introduction of a drug delivery system under the renal capsule should preferably be non or minimally invasive. Hydrogels 1-3 were implanted under the capsule of rat kidneys by injection, a process that required the generation of a subcapsular pocket by gently detaching the capsule from the renal cortex (Fig. 3A and 3B). To assess the possible effect of subcapsular hydrogel implantation on renal function, plasma creatinine levels were measured at day 3, 7 and 15 after implantation (Fig. 3D). No differences in creatinine levels were found between control animals (that received a subcapsular saline solution) and experimental groups (that received subcapsular hydrogel implants), indicating no deterioration of renal function. The area below the site of implantation was examined histologically via arbitrary scoring and morphometry (the area is depicted with a red box in Fig. 3C; PAS staining is shown in Fig. 4). Upon explantation of the kidneys, hydrogel 1 and 2 were macroscopically detectable at all time points, after 3, 7 and 15 days, whereas hydrogel 3 was not visible at any time point. Enhanced vascularisation around both hydrogel 1 and 2 was detected macroscopically at day 15.

FIGURE 3. RENAL SUBCAPSULAR IMPLANTATION PROCEDURE AND KIDNEY FUNCTION. A. Schematic representation of a kidney sliced in the sagittal plane. The average dimensions of a normal rat kidney are depicted by: x is approx. 1 cm, and z is approx. 1.5 cm. The left kidney was gently lifted through a retroperitoneal, dorsal incision. Then, i. the kidney capsule was loosened from the kidney to introduce a small pocket in which ii. 30 µL of the gels was implanted. 30 µL of saline was introduced in the control kidney c. B. An example of a gel implanted under the kidney capsule. C. A schematic representation shows the area at the site of gel implantation, the kidney cortex and the kidney capsule. In time a fibrous capsule is formed around the hydrogel. A combination of the kidney capsule and the fibrous capsule is depicted with Kc + Fc. The area analyzed for histological examination, i.e. arbitrary scoring and morphometry, is shown depicted with a red box. D. Blood plasma creatinine concentration was determined 3, 7 or 15 days after implantation of the gels. No statistical differences with control animals, which were not operated and did not receive a gel, were found. C = cortex, c = control animals, Fc = fibrous capsule, H = (hydro)gel, Kc = kidney capsule.
FIGURE 4. GENERAL HISTOLOGICAL VIEW ON THE RENAL CAPSULE AND CORTEX AFTER IMPLANTATION OF THE GELS. A. PAS staining was used to visualize histology of the capsule and the cortex under the implantation site after 3, 7 and 15 days. B. Magnification of the exudates present in the explants; which is formed in the capsule for the ctrl. and gel 1, and the exudate which is formed inside gel 2, after 3 days. The double arrows depict the kidney capsule in the contralateral kidneys, the combined kidney capsule and fibrous capsule, i.e. Kc + Fc, in the control kidneys, the fibrous capsule with gel remnants, i.e. Fc + H for the experimental kidneys with gels 1 and 2, and the combined kidney capsule and fibrous capsule with possible gel remnants, i.e. Kc + Fc (+H) for the experimental kidneys with gel 3. All scale bars represent 100 µm. C = cortex, contral. = contralateral kidney, ctrl. = control kidney, Fc = fibrous capsule, G = glomerulus, H = (hydro)gel, Kc = kidney capsule, T = tubulus, e = exudate.
FIGURE 5. MACROPHAGE INFILTRATION IN THE CORTEX AND CAPSULE UNDER THE IMPLANTATION SITE. **Ai, Aii.** Tissue slices were stained for the macrophage marker ED1 3, 7 and 15 days after implantation. The double arrows depict the kidney capsule in the contralateral kidneys; the thickened, fibrous kidney capsule, i.e. Kc + Fc, in the control kidneys; the fibrous capsule for the experimental kidneys with gels 1 and 2; and the fibrous kidney capsule with possible gel remnants, i.e. Kc + Fc (+H) for the experimental kidneys with gel 3. All scale bars represent 100 µm. Contral. = contralateral kidney, ctrl. = control kidneys, C = cortex, Fc = fibrous capsule, H = (hydro)gel, Kc = kidney capsule. Arbitrary scoring of amount of macrophages in **B.** the cortex and **C.** the capsule.
CORTICAL TISSUE RESPONSE

It is conceivable that renal subcapsular implantation of a drug delivery carrier can trigger cortical modifications due to the implantation procedure and/or the tissue reaction to the materials implanted. Ideally, the implantation should not affect cortical integrity, or cause severe adverse reactions such as inflammation or fibrosis. After implantation of hydrogels 1-3 or saline injection, slight cortical modification immediately below the site of implantation was observed, but not at sites distal from the implantation site. Therefore, we selected this site to study the cortical tissue response to hydrogel implantation.

The inflammatory reaction was investigated by macrophage (Mφ) staining using ED-1 as a marker (Fig. 5A,B). No Mφ were present in the tubulointerstitial space of contralateral kidneys at any time point studied. In kidneys injected with saline, a mild tubulointerstitial infiltration was observed at all time points, with the exception of day 7, where a higher infiltration was possibly caused by the implantation procedure. Mφ infiltration in kidneys implanted with 1 was stable over time and comparably mild as in saline-injected kidneys. At day 3, after implantation of 2 Mφ infiltration was comparable to kidneys receiving saline or 1, but increased at day 7, and slightly decreased at day 15. A similar response was observed after implantation of 3. Overall, hydrogel 1 elicited the mildest response of the three hydrogels. However, the strong local restriction of macrophages in the interstitial space immediately below the implantation site and their transient presence indicated that all three gels displayed a virtually negligible cortical inflammatory response.

To determine whether hydrogel implantation elicited the formation of interstitial myofibroblasts, kidneys were stained for α-smooth muscle actin (αSMA) (Fig. 6A,B). No interstitial myofibroblasts were observed in contralateral kidneys at any time point. In kidneys injected with saline, interstitial myofibroblasts accounted for approximately 2% of the cortical area at all time points. Hydrogel 1 elicited virtually no interstitial myofibroblast formation at early time points, whereas at day 15 myofibroblast numbers slightly exceeded those found in saline control kidneys. Hydrogel 2 evoked transient interstitial myofibroblast formation that peaked 7 days after implantation but was comparable to control levels at day 3 and day 15. In kidneys implanted with hydrogel 3, myofibroblast numbers did not exceed control values, although spots of αSMA staining were detected occasionally. Overall, the implantation of all three hydrogels was associated with a relatively negligible and locally restricted presence of myofibroblasts in the renal cortex, with exception of hydrogel 2 in which a transient cortical accumulation of myofibroblasts 7 days after implantation was detected.

Myofibroblasts are key players in fibrosis by producing extracellular matrix (ECM) proteins. One of these ECM proteins is collagen III, a component of tubular basal membranes that is deposited in the renal interstitium during fibrosis. To investigate whether subcapsular hydrogel implantation elicited collagen III deposition in the kidney, collagen III staining was quantified by morphometry (Fig. 7A,B). Comparable amounts of collagen III deposition were found in contralateral and saline control kidneys at all time points. The implantation of hydrogels 1 and 3 was associated with cortical collagen III deposition similar to the control kidneys, irrespective of the time point. Hydrogel 2 elicited a transiently elevated collagen III deposition at day 7, whereas at day 3 and day 15 no differences were observed as compared to the controls. Interestingly, this increase in collagen III paralleled the increase in αSMA staining observed at day 7 after implantation of gel 2.
FIGURE 6. DETECTION OF MYOFIBROBLASTS IN THE KIDNEY CORTEX AND THE CAPSULE UNDER THE IMPLANTATION SITE. **Ai, Aii.** Myofibroblasts were stained for α-smooth muscle actin after 3, 7 and 15 days of implantation. The double arrows depict the kidney capsule in the contralateral kidneys; the fibrous, kidney capsule, i.e. Kc + Fc, in the control kidneys; the fibrous capsule for the experimental kidneys with gels 1 and 2; and the fibrous kidney capsule with possible gel remnants, i.e. Kc + Fc (+H), for the experimental kidneys with gel 3. All scale bars represent 100 µm. Contral. = contralateral kidney, ctrl. = control kidneys, C = cortex, Fc = fibrous capsule, H = (hydro)gel, Kc = kidney capsule, v = blood vessels. The amount of myofibroblasts was quantified by morphometry in B, the cortex and C, the capsule.
FIGURE 7. THE PRESENCE OF INTERSTITIAL COLLAGEN III IN THE CAPSULE AND CORTEX UNDER THE IMPLANTATION SITE. Ai, Aii. Tissue slices were stained for collagen III after 3, 7 and 15 days of implantation. The double arrows depict the kidney capsule in the contralateral kidneys; the fibrous kidney capsule, i.e. Kc + Fc, in the control kidneys; the fibrous capsule for the experimental kidneys with gels 1 and 2; and the fibrous kidney capsule with possible gel remnants, Kc + Fc (+H), for the experimental kidneys with gel 3. All scale bars represent 100 µm. Contral. = contralateral kidney, ctrl. = control kidneys, C = cortex, Fc = fibrous capsule, H = (hydro)gel, Kc = kidney capsule. The amount of collagen III was quantified by morphometry in B. the cortex and C. the capsule.
The low αSMA-positivity and low Mφ infiltration after 15 days, together with the lack of differences with respect to the amount of collagen III between the kidneys with hydrogels 1 and 3 and contralateral kidneys, might be explained by the fact that enhanced ECM deposition, and thus deposition of collagen III, is only upregulated after more extensive damage or sustained inflammation. This indicates that subcapsular implantation of hydrogels 1 and 3 does hardly damage the renal cortex. The tissue response for hydrogel 2 was detected to be slightly less mild.

SUBCAPSULAR FOREIGN BODY REACTION

The introduction of a biomaterial in the body typically elicits a foreign body reaction (FBR), which aims at encapsulation of the biomaterial for the protection of the surrounding tissue and at its removal by macrophage-mediated phagocytosis. In our present subcapsular implantation model, saline-treated control kidneys were used to distinguish between capsular alterations caused by the pocket surgery and FBR-related phenomena. The generation of a subcapsular pocket and subsequent administration of saline in the pocket induced significant, capsule thickening after 3 and 7 days, although the process was reversed to a large extent by day 15 (Fig. 8). Upon implantation of hydrogels 1 and 2, thickening of the kidney capsule was observed as well, while a fibrous, FBR-related encapsulation was observed at the cortical side of the implants. This latter phenomenon was not observed after implantation of hydrogel 3, possibly due to its rapid degradation. Quantification of kidney capsule thickness revealed time and material-dependent differences. After implantation of hydrogel 1, the capsule thickness resembled that of the saline receiving control kidneys, but increased gradually until day 15. Implantation of hydrogel 2 led to significantly more capsule thickening than saline control injection. However, capsule thickness remained relatively stable over time. After implantation of hydrogel 3, the thickness of the capsule equalled that of saline controls and increased transiently by day 7 (Fig. 8).

Collagen IV is both present in the basement membranes of blood vessels, tubuli and glomeruli, consequently it is difficult to distinguish between these structures. Therefore, collagen IV staining was used to particularly investigate vascularisation in the capsule (Fig. 9). Whereas the distinctions between the groups were less clear after 3 days, the differences became more apparent after 7 and 15 days. The amount of vascularisation became higher for kidneys containing hydrogels 1 or 2, and progressed until day 15. This was already detected macroscopically during explantation of kidneys with hydrogels 1 and 2 after 15 days. In the case

FIGURE 8. Capsule Thickness. The thickness of the kidney capsule, foreign body reaction-induced fibrous capsule and combined capsules were determined by morphometry. Statistical differences were determined following two methods: i. for each time point between the contralateral cl, the control c, and the experimental groups with gel 1, 2 or 3 (indicated with a black line), and ii. for each group between the time points d3, d7 and d15 (depicted with a grey line).
of the control kidneys and the kidneys with hydrogel 3, the amount of blood vessels seemed to be reduced after 15 days.

**FIGURE 9. COLLAGEN IV IN THE CAPSULE AND CORTEX UNDER THE IMPLANTATION SITE.** Collagen IV is a component of blood vessel and tubular basal membranes. Tissue slices were examined for collagen IV, 3, 7 and 15 days after implantation. The double arrows depict the kidney capsule in the contralateral kidneys, the combined kidney capsule and fibrous capsule, i.e. Kc + Fc, in the control kidneys, the fibrous capsule for the experimental kidneys with gels 1 and 2, and the combined kidney capsule and fibrous capsule with possible gel remnants, i.e. Kc + Fc (+H) for the experimental kidneys with gel 3. All scale bars represent 100 µm. C = cortex, contral. = contralateral kidney, ctrl. = control kidney, Fc = fibrous capsule, H = (hydro)gel, Kc = kidney capsule.

As mentioned previously, the infiltration of Mφ is a hallmark of the FBR. In saline-treated kidneys, Mφ were present in the thickened kidney capsule and at the interface between the loosened kidney capsule and the cortex, indicating that the surgical procedure elicited a mild tissue response. Upon implantation of hydrogels 1-3, Mφ infiltration was detected in the thickened kidney capsules at all time points, suggesting that the subcapsular vascular network might be the entry route of these cells at the implantation site. For each hydrogel, the amount of FBR-related Mφ remained relatively stable in time (Fig. 5C). Occasionally, giant cells and foam cells were detected in the FBR associated with hydrogels 1 and 2 (Fig. 10).

Surgical and FBR-related renal capsule thickening was also investigated by detection and quantification of αSMA expressing myofibroblasts (Fig. 6C). In contralateral kidneys, myofibroblasts were detected as constituents of the renal capsule. In saline-treated kidneys, the amount of αSMA staining was higher at all time points studied, indicating that capsular thickening after generation of the subcapsular pocket was mediated by myofibroblasts. In general, αSMA-positivity in the kidney
capsule was comparable to that in control kidneys after implantation of all three hydrogels at the beginning (day 3) and at the end (day 15) of the observation period (Fig. 6C). Material-related differences in capsular αSMA-positivity were observed at day 7, where gel 2 elicited the highest myofibroblasts formation and implantation of gel 3 resulted in similar αSMA-positivity as saline injection.

**FIGURE 10. GIANT CELLS AND FOAM CELLS.** **Left.** Schematic representation of the kidney indicating the places where, **Right.** At the interface between renal tissue and hydrogel # giant cells (G-c) were detected in gel 2 at day 7, and where above the hydrogel * foam cells (F-c) were seen in gel 1 at day 15. These tissue sections were stained with ED1. All scale bars represent 100 µm.

### 6.3 DISCUSSION

Irrespective of the nature of the damage, kidney injury is invariably associated with inflammation. Improper resolution or chronic persistence of inflammation sets the stage for fibrosis, which has been identified as the common pathway to kidney failure. Inflammation and fibrosis of the tubulointerstitial space cannot be effectively addressed by systemic drug administration. Moreover, systemic drug administration is associated with the loss of the therapeutic compounds to irrelevant sites in the body and with undesired side effects, as is exemplified by immune-suppressive treatment after kidney transplantation. Therefore, various areas of kidney therapy would profit from a therapeutic device that allows local drug delivery into the renal interstitium in order to modulate tubulointerstitial inflammation and fibrosis, to promote renal repair, and in this way to prevent progression towards chronic renal damage and failure.

In this chapter we present the synthesis and *in-vivo* kidney compatibility of supramolecular hydrogels designed for intrarenal drug delivery. Intrarenal drug delivery from a hydrogel carrier implanted under the kidney capsule might be an innovative way to induce kidney tissue regeneration and/or prevent kidney inflammation or fibrosis. In order to investigate the feasibility of this approach it is important to first study the tissue response to these carriers when implanted under the kidney capsule. Subcutaneous implantation studies will not be adequate because it has been shown previously that the tissue response differ between different implantation sites.20

We designed and synthesised three different supramolecular UPy-modified poly(ethylene glycol) hydrogels for intrarenal implantation. We have chosen to use supramolecular hydrogelators because the directed non-covalent interactions taking care of network formation give the materials dynamic properties complying with intrarenal implantation, i.e. the requirement for distribution and spreading of the material under the kidney capsule. Two chain-extended UPy-polymers, with the UPy-units part of the main chain,
and one bifunctional UPy-polymer, in which the two chain ends were modified with UPy- moieties, were synthesized. The differences in properties were immediately clear from the method of formulation of these hydrogelators into hydrogels. Whereas the end-functionalized material could be readily dissolved in water at elevated temperatures, the chain-extended materials required the addition of organic solvent to be dissolved. Gelation took place after cooling down to room temperature (for the bifunctional hydrogelator), or after removal of the organic solvent under reduced pressure (for the chain-extended hydrogelators).

After hydrogel formation the bifunctional hydrogel erodes fast upon addition of water, while the chain-extended hydrogels remained stable in time. Rheology showed that the chain-extended materials form strong, flexible hydrogels, and the bifunctional material forms a weaker, soft hydrogel. The differences between these two classes of hydrogels with respect to their physico-chemical properties were also reflected in the results obtained after subcapsular implantation in kidneys.

The subcapsular application of a drug delivery device should ideally not affect the structure and function of the kidney or evoke a tissue response such as inflammation or fibrosis. This is crucial for implantation in the kidneys since these organs are sensitive to stress. We showed that partial surgical detachment of the kidney capsule in order to generate a pocket for hydrogel implantation leads to capsular thickening, which did not appear to be deleterious to kidney structure or function. Importantly, both the subcapsular FBR and the cortical presence of macrophages and myofibroblasts were highly restricted to the site of implantation, irrespective of hydrogel type, although variations between hydrogels were observed. Differences in tissue response to the two chain-extended hydrogels could be detected; the more elastic hydrogel induced a tissue response which was located at the interface between hydrogel and kidney tissue, while the tougher, more shape-persistent hydrogel additionally induced slight damage to the cortex. It is proposed that the tougher hydrogel cannot easily adjust itself to the pocket created between the renal capsule and the cortex; therefore we reason that this hydrogel exerts more pressure on the cortex thereby inducing more damage. Fifteen days after implantation, the bifunctional hydrogel ultimately showed the lowest tissue response of all three hydrogels tested. After injection it was immediately observed that this hydrogel could fill up the complete pocket by spreading. In this way, the hydrogel was homogeneously distributed and did not exert force on the kidney cortex. The tissue response was similar to the control kidneys in which only the kidney capsule was loosened. The damage to the cortex was minimal, and a fibrous capsule was hardly formed. Additionally, the erosion behaviour of the two classes of hydrogels in vivo was different. The chain-extended UPy-hydrogels did not show a sign of degradation after two weeks. The end-functionalized was resorbed within days, which we address to supramolecular erosion, i.e. complete dissolution of the supramolecular polymer aggregates without chemical cleavage of bonds by hydrolysis. This also explains the minimal tissue response induced by this hydrogel. Furthermore, from our rheological data in combination with the in-vivo data we derive that kidneys are able to deal with soft hydrogels, or more particularly, (visco)-elastic hydrogels that have $G'$-values that are preferably lower than $10^3$ Pa.

With respect to future clinical applications as kidney drug delivery vehicles, the minimal tissue response to the bifunctional hydrogel and the fast degradation behaviour makes it suitable for short-time boost release of drugs. Moreover, the stability of this hydrogel can be easily adjusted by introduction of additional carbon atoms in the alkyl spacer and/or shortening the length of the PEG chain. The green, organic-solvent free preparation method of the bifunctional hydrogel makes it eminently suitable as (growth factor) protein drug delivery
vehicle. Very recently we provided the proof-of-concept that the formulation of the anti-fibrotic growth factor BMP7 is feasible in this hydrogel with preservation of its biological activity.\textsuperscript{18} Moreover, local intrarenal delivery of BMP7 clearly inhibited formation of myofibroblasts in the renal cortex after subcapsular administration in the bifunctional hydrogel.\textsuperscript{18}

We propose that the chain-extended hydrogels can be used for long-term release studies, although incorporation of protein drugs into these chain-extended hydrogels might be a challenge because of the need for organic solvent in the formulation process. On the contrary, the release of organic drugs from the chain-extended hydrogels is very much possible as such drugs can in general withstand harsher formulation conditions. Moreover, the mechanical and solution vs. hydrogel performance of these chain-extended hydrogels can also be easily adjusted by tuning the chain length of the PEG-block and the nature of the alkyl linker. The minimal cortical damage and the mild tissue response to all three hydrogel systems show that these supramolecular hydrogels can be applied as subcapsular vehicles for intrarenal drug delivery. Currently, we are investigating the administration of BMP7 from subcapsularly implanted supramolecular hydrogels after kidney ischemia-reperfusion injury.

6.4 CONCLUSIONS

We have successfully synthesized three different supramolecular hydrogelators based on PEG chains and UPy-moieties. Two chain-extended hydrogelators possessing UPy-units in the main chain, and one bifunctional hydrogelator with UPy-groups at the chain end, were produced. A mixture of water and organic solvent was needed to dissolve the chain-extended hydrogelators. Gelation took place after removal of the organic solvent. In contrast, the bifunctional hydrogelator could be dissolved in only water at elevated temperature. Subsequently, the hydrogel was formed after cooling to ambient temperature. The chain-extended polymers both formed strong, elastic hydrogels; one was more flexible, while the other was tougher. The end-functionalized UPy-hydrogelator formed a weaker and softer hydrogel. Implantation of these three different hydrogels under the renal capsule resulted in mild tissue responses. The reaction to the more flexible chain-extended hydrogel was located at the interface between renal tissue and hydrogel, whereas the tougher chain-extended hydrogel also induced slight damage to the renal cortex. The bifunctional hydrogel showed a minimal tissue response which was comparable to the control kidneys in which only the renal capsule was loosened. The chain-extended UPy-hydrogels did not degrade after two weeks, while the bifunctional hydrogel was resorbed within days, which is proposed to occur by dissolution of entire polymer aggregates. The properties and \textit{in-vivo} behaviour of the bifunctional hydrogel makes it an attractive intrarenal drug carrier for short term protein delivery, while the properties of one of the chain-extended hydrogels makes this hydrogel possibly suitable for long term intrarenal delivery of organic drugs.
6.5 EXPERIMENTAL SECTION

GENERAL MATERIALS
The hydroxy-terminated PEGs and PCL were purchased from Acros. 1,1-carbonyldiimidazole was obtained from Sigma, and 1,6-hexanediame and 1,12-dodecyldiamine were purchased from Acros. Commercial products were used without further purification. All solvents purchased from Acros, Sigma-Aldrich or Biosolve were of p.a. quality. Deuterated chloroform was obtained from Cambridge Isotope Laboratories.

GENERAL METHODS
$^1$H-NMR spectra were recorded on a Varian Gemini (300 MHz) or Varian Mercury (400 MHz) spectrometer at 298 K. Chemical shifts ($\delta$) are given in ppm values relative to tetramethylsilane (TMS). MALDI-TOF mass spectrometry was performed on a PerSeptive Biosystems Voyager-DE PRO spectrometer using an α-cyano-4-hydroxycinnamic acid matrix. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer with a Universal ATR Sampling Accessory for solids. Micrographs of tissue slices were taken on a Leica DMLB microscope with Leica DC300 camera and Leica QWin 2.8 software.

SYNTHESISES OF UPy-MODIFIED HYDROGELATORS

CDI-ACTIVATION OF PEG PREPOLYMERS 4A, 4B, 4D. All telechelic hydroxy-terminated poly(ethylene glycols) ($M_n=3000$ g/mol – PEG3000 a, $M_n=6000$ g/mol – PEG6000 b, $M_n=20,000$ g/mol – PEG20000 d; 22 g, 7.3 mmol a; 22 g, 3.7 mmol b; 12 g, 0.6 mmol d) were dried by heating in vacuo at 60 °C for 2 h for a, b, and at 120 °C for 3 h for d. The CDI-activation was performed in a similar way for all polymers a, b, d as described: the solid polymers were added in portions to a solution of 1,1-carbonyldiimidazole (CDI; 9.8 g, 60 mmol; 4.9 g, 30 mmol b; 0.7 g, 4.3 mmol d) in chloroform. The mixtures were stirred at 21 °C under an inert argon atmosphere for 18 h for a, b, and for 8 h in the case of d. Then they were precipitated in an excess of diethyl ether. The white solids were isolated by filtration. The products were instantly used for the next synthesis steps. $^1$H-NMR (300 MHz, CDCl3): $\delta$ = 8.2 (2H, CDI), 7.4 (2H, CDI), 7.0 (2H, CDI), 4.5 (4H, EG next to CDI), 3.8-3.4 (4nH, PEG) ppm.

CDI-ACTIVATION OF PCL PREPOLYMERS 4C. Telechelic hydroxy-terminated polycaprolactone ($M_n=1250$ g/mol – PCL1250 c; 3.6 g, 2.9 mmol) was dried by heating in vacuo at 60 °C for 2 h. The solid polymer was added in portions to a solution of CDI (3.7 g, 23 mmol) in chloroform. The reaction mixture was stirred overnight at 21 °C and kept under an inert argon atmosphere. The solution was washed twice with water, and four times with a saturated NaCl solution. Then the organic phase was dried over Na$_2$SO$_4$. The chloroform was evaporated under reduced pressure and the resulting polymer material was dried in vacuo to yield a white waxy solid. The isolated product was instantly used for the next synthesis step. $^1$H-NMR (300 MHz, CDCl3): $\delta$ = 8.1 (2H, CDI), 7.4 (2H, CDI), 7.0 (2H, CDI), 4.5 and 4.4 (4H, next to CDI), 4.2 (4H, initiator), 4.0 (2nH, PCL), 3.8 and 3.7 (4H, initiator), 2.3 (2nH, PCL), 1.8-1.2 (4nH + 2nH, PCL and 16H, next to CDI) ppm.

DIAMINE-TERMINATION OF PEG PREPOLYMERS 5A, 5B, 5D. All diamine-terminated PEG-prepolymers were prepared as described: CDI-activated 4a, 4b, 4d (17 g, 5.5 mmol 4a; 17 g, 2.8 mmol 4b; 11.5 g, 0.57 mmol 4d) were dissolved in dichloromethane. The resulting solutions were slowly added to a solution of 1,6-hexanediame (5.2 g, 45 mmol for 5a; 2.7 g, 23 mmol for 5b) in dichloromethane, or to obtain 5d to a melt of 1,12-dodecyldiamine (0.92 g, 4.6 mmol) which was molten at 80 °C under an argon atmosphere. After the additions were complete, the solutions were stirred under argon at 21 °C for 1.5 h in the case of 5a and 5b, and at 60 °C for 4 h for 5d. Verification by $^1$H-NMR that conversion was complete was followed by precipitation of the products in an excess of diethylether. Redissolution in dichloromethane for 5a and 5b, or in chloroform for 5d, filtration of the mixtures over celite, and second precipitation of the filtrates in an excess of diethyl ether gave white solid products that were isolated by filtration. The products were instantly used for the next synthesis steps, although they were quite stable. In MALDI-TOF MS analysis sets of masses around the expected molecular weight of 3-4 kDa, 6-7 kDa, 20-21 kDa were observed. No other arrays were recorded, also not at doubled molecular weights. This proves that the prepared prepolymers have a well-defined telechelic structure. $^1$H-NMR (400 MHz, CDCl3): $\delta$ = 4.9 (2H, urethane), 4.2 (4H, next to urethane), 3.9-3.2 (4nH, PEG), 3.1 (4H, next to urethane), 2.7 (4H, next to amine), 2.0 (4H, broad, next to urethane), 1.5-1.1 (16H, hexyl spacer or 40H, dodecyl spacer) ppm.

DIAMINE-TERMINATION OF PCL PREPOLYMER 5C. CDI-activated 4c (2.8 g, 2.1 mmol) was dissolved in dichloromethane and was slowly added to a solution of 1,6-hexanediame (2.1 g, 18 mmol) in dichloromethane. After addition of 4c, the solution was stirred at 21 °C for 1.5 h while keeping the reaction mixture under argon. The conversion was checked with $^1$H-NMR. The product was precipitated in pentane, and redissolved in dichloromethane. The solution was washed with a saturated NaCl solution, and was dried over Na$_2$SO$_4$. Evaporation of the solvent under reduced pressure at 21 °C yielded a waxy white product. The isolated product could not be stored conveniently at 21 °C and was instantly used for the next synthesis step. $^1$H-NMR (300 MHz, CDCl3): $\delta$ = 4.9-4.6 (2H, urethane), 4.2 (4H, initiator), 4.1 (2nH, PCL), 3.7 (4H, initiator), 3.2 (4H, next to urethane), 2.7 (4H, next to amine), 2.3 (2nH, PCL), 1.7-1.2 (4nH + 2nH, PCL and 16H, hexyl spacer) ppm. The integral values of the end groups indicated a well-defined telechelic structure.

UREIDO-PYRIMIDINONE SYNTHONS. The UPy-synthons were synthesized as described before. $^{11,14}$ IPDI-UPy-IPDI was synthesized from 5-(2-hydroxyethyl)-6-methyl-isocytosine and isophorone diisocyanate (IPDI), and UPy-hexyl-
isocyanate from 2-amino-4-hydroxy-6-methylpyrimidine and 1,6-hexylisocyanate. For convenience, only one isomer of the IPDI-UPy-IPDI product is shown. IPDI exists in different regio- and stereoisomers, and the coupling is not selective for one of the isocyanates in IPDI.

**CHAIN-EXTENDED UPY-HYDROGELATOR 1.** Diamine-terminated PEG 5a (2.6 g, 0.75 mmol) and diamine-terminated PEG 5b (2.6 g, 0.40 mmol) were dried in vacuo at 80 °C for 2 h. They were subsequently dissolved in chloroform to ultimately obtain a co-polymer with a 1:1 w/w% PEG3000:PEG6000 ratio. The IDPI-UPy-IDPI synthon (0.72 g, 1.2 mmol, 1 equivalent) was added. The reaction mixture was heated to 40 °C and stirred under argon for 18 h. The completion of the reaction was checked by the disappearance of the isocyanate bands in FT-IR. The reaction mixture was diluted with ethanol, and extensive evaporation of solvents under reduced pressure, resulted in a white, semi-crystalline, elastic and tough material. ^1H-NMR (400 MHz, CDCl3): δ = 13.0 (UPy), 11.9 (UPy), 10.1 (UPy), 5.2-4.8 (urea, urethane), 4.2 (next to urea or urethane), 3.7-3.5 (PEG), 3.2 (next to urea or urethane), 2.3 (methyl at UPy), 2.0-1.6 (next to urea or urethane), 1.5 (hexyl spacer, isophorone), 1.3 (hexyl spacer), 1.1 (isophorone), 0.9 (isophorone) ppm. Relative integral values of PEG-units and those of the apolar isophorone and hexyl spacers are in good agreement.

**CHAIN-EXTENDED UPY-HYDROGELATOR 2.** Diamine-terminated PEG 5b (4.4 g, 0.68 mmol) was dried in vacuo at 80 °C for 1 h, and was dissolved in chloroform. Next, solutions of the IDPI-UPy-IDPI synthon (0.63 g, 1.0 mmol, 1 equivalent) in chloroform, and the diamine-terminated PCL 5c (0.48 g, 0.31 mmol) were added, to obtain a co-polymer with a 9:1 w/w% PEG6000:PCL1250 ratio. The reaction mixture was heated to 40 °C and stirred for 18 h under an argon atmosphere. The solution was cooled down and ethanol was added to reduce the viscosity of the solution. Solvents were evaporated resulting in a white, semi-crystalline, elastic and tough material, which was extensively dried in vacuo. ^1H-NMR (400 MHz, CDCl3): δ = 13.0 (UPy), 11.9 (UPy), 10.1 (UPy), 5.2-4.8 (urea, urethane), 4.0 (PCL), 3.7-3.5 (PEG), 3.2 (next to urea or urethane), 2.3 (PCL, methyl at UPy), 2.0-1.6 (next to urethane or urea), 1.5 (hexyl spacer, isophorone), 1.3 (hexyl spacer), 1.1 (isophorone), 0.9 (isophorone) ppm. Relative integral values of PEG-units, PCL-units and those of the apolar isophorone and hexyl spacers are in good agreement.

**BIFUNCTIONAL UPY-HYDROGELATOR 3.** The UPy-hexyl-isocyanate synthon (0.24 g, 0.83 mmol, 2.0 equivalents) was added as a powder to a solution of pre-dried diamine-terminated PEG 5d (8.3 g, 0.41 mmol) in chloroform. The reaction mixture was allowed to stir at 70 °C for 3 h under an argon atmosphere. After cooling down to 21 °C, ethanol was then added, and the hazy mixture was filtered over celite. The clear filtrate was concentrated under reduced pressure. Then, the filtrate was stirred and diluted with diethyl ether. A white precipitate was formed, and the mixture was stirred for 20 minutes. Then the precipitate was allowed to settle prior to filtration. The product was washed with an excess of diethyl ether, and was extensively dried in vacuo to obtain a white powder. ^1H-NMR (400 MHz, CDCl3): δ = 13.1 (2H, UPy), 11.8 (2H, UPy), 10.1 (2H, UPy), 5.8 (2H, UPy alkyldiene), 4.9 (2H, urethane), 4.7 (2H, urea), 4.5 (2H, urea), 4.2 (4H, next to UPy), 3.8-3.3 (4nH, PEG), 3.3 (4H, next to urethane), 3.1 (12H, next to urethane and urea), 2.2 (6H, methyl at UPy), 1.6-1.1 (56H, hexyl and dodecyl spacer) ppm.

**PREPARATION OF HYDROGELS**

UPy-polymer 1 was dissolved in THF (Biosolve) and a 0.9% NaCl (B. Braun Melsungen) aqueous solution was added in a ratio 1:1 to obtain a 5 w/w% hydrogel. The clear solution was subsequently put in vacuo to slowly remove the THF, resulting in a very elastic hydrogel. The same procedure was applied to UPy-polymer 2, resulting in an elastic, rigid and shape-persistent 5 w/w% hydrogel. After preparation, gel 1 and 2 were incubated in a 0.9% NaCl solution for 16 h at 4 °C to ensure that all organic solvents were washed out. A 10 w/w% hydrogel of 3 was prepared by mixing the hydrogelator powder and a 0.9% NaCl aqueous solution in the appropriate loading ratio. The mixture was vigorously mixed by stirring at 55-65 °C for 1 h. The polymer solution was removed from the water bath and placed on the bench at 21 °C to set over a 16-24 h period, which resulted in a soft hydrogel. Subsequently, the gel could immediately be used. All gels were sterilized with UV for 1 h prior to use.

**RHEOLOGY**

For rheology measurements, 5 w/w% gel 1 and 10 w/w% gel 3 were heated and melted on the rheometer in order to be moulded into the proper shape for measurement. The samples were left to set for several hours before the measurements were performed. The 5 w/w% gel 2 could not be treated similarly, as it was too rigid. Therefore, the 5 w/w% gel 2 was dissolved in a small amount of acetone, and was poured in a teflon mould that was placed in a petridish containing water. The whole set-up was placed under a beaker to allow slow evaporation of the acetone for 3 days, while leaving the hydrogel saturated with water. A sample (25 mm in diameter) could be cut from the resulting hydrogel film. NMR analysis of the film showed no residual acetone. Drying and weighing of the film showed that it had developed into a 7 w/w% hydrogel, indicating that water had evaporated from the hydrogel. Rheology measurements were performed on Rheometrics ARES equipment. The linear viscoelastic region was determined for these samples at 20 °C, at frequencies of ω = 1 and 10 rad/s using strains of 1% to 100%. Gel 2 gave a constant response up to strains of 30%, while gel 1 and 3 gave constant moduli up to 100% strain. Frequency sweep measurements were performed using a strain of 2% (= 0.02 rad) and applying a sweep from 0.1 rad/s to 100 rad/s. Mastercurves at 20 °C were constructed from the measurements performed at various temperatures between 20 °C and 40 °C by using the time-temperature superposition principle.
Fischer rats (F344, Harlan) with a weight of 230-290 g were used for this study (in total 45 rats). The rats were housed in Groningen, and performed according to governmental guidelines on animal experimentation. Ten weeks old male rats were fed under conventional conditions, in a temperature-controlled and humidity-controlled room with 12 hours light/dark cycles. They had access to water and standard rat food ad libitum. A consent form according to Dutch legal requirements. Isolation, culture and characterization were performed as described in the Chapter 3. PTEC (passage 2-3) from 3 different donors were seeded in 24-wells plates at a density of 8.5·10⁴ cells/cm² in 0.6 mL medium. The cells were cultured for 3 days (in the case of the ‘dry hydrogelator’ test) or 4 days (in the case of the ‘hydrogel’ test) at 37 °C and 5% CO₂ with intermittent medium changes every 2 days. After 3 days, the cells were washed with PBS and the solid hydrogelator material, sterilized with UV for 1 h, was added in 0.6 mL medium: 50-70 mg/well of hydrogelator 1 or 2 (end concentration of approx. 10 w/w%), 100-150 mg/well of hydrogelator 3 (end concentration of approx. 20 w/w%). Alternatively, after 4 days, the cells were washed with PBS and the hydrogels, prepared as described above, were added: 160 mg gel/well in 0.6 mL medium, i.e. end concentration of 1 w/w% 1 or 2, and 2 w/w% 3. The incubations were performed at least in triplicate for every donor-hydrogelator/hydrogel combination. As a reference, cells were cultured in the absence of hydrogelator/hydrogel. The cells were cultured for another 16 h at 37 °C and 5% CO₂. Optical microscopy pictures of the cells were taken on a Leica DM1L microscope with Leica DC300F camera. The cells were incubated for 2 h at 37 °C in fresh culture medium containing 44 μM resazurin (Sigma Aldrich). Viable cells convert non-fluorescent resazurin into fluorescent resorufin (λex = 540 nm, λem = 590 nm) which was measured with a Varioskan plate reader. Bacterial endotoxin contamination was determined by the chromogenic Limulus amebocyte lysate (LAL) test, as described. Endotoxin limits in EU (endotoxin unit) as set by the FDA are 0.5 EU/mL or 20 EU/medical device, with 10 EU corresponding to 1 ng of endotoxin.

**ENDOTOXIN DETERMINATION**

The hydrogels were sterilized with UV for 1 h, and subsequently extracted by shaking in water at 37 °C for 2 h (5 w/w% 1, 5 w/w% 2, 10 w/w% 3 in water). Bacterial endotoxin contamination was determined by the chromogenic Limulus amebocyte lysate (LAL) test, as described. Endotoxin limits in EU (endotoxin unit) as set by the FDA are 0.5 EU/mL or 20 EU/medical device, with 10 EU corresponding to 1 ng of endotoxin.

**ANIMALS**

All animal procedures were approved by the committee for care and use of laboratory animals of the University of Groningen, and performed according to governmental guidelines on animal experimentation. Ten weeks old male Fischer rats (F344, Harlan) with a weight of 230-290 g were used for this study (in total 45 rats). The rats were housed under conventional conditions, in a temperature-controlled and humidity-controlled room with 12 hours light/dark cycles. They had access to water and standard rat food ad libitum.

**SURGICAL PROCEDURES**

Rats were anesthetized with a mixture of isoflurane (Abbott) and O₂. A retroperitoneal incision was made, and the left kidney was gently lifted through this incision. A subcapsular pocket was generated distal to the urether, using a blunt needle. The hydrogels 1, 2 and 3, 30 μL per kidney, were implanted into the subcapsular pocket. The control kidneys received 30 μL physiological salt solution (0.9% NaCl solution; B. Braun Melsungen) in the subcapsular pocket. The contralateral kidneys were left untouched. At 3, 7 or 15 days after subcapsular implantation, rats were anesthetized and blood was drawn. The kidneys were perfused in situ with PBS, after which they were excised. The kidneys were cut into halves along the sagittal plane. One part was fixed in zinc fixative for 18 h (0.1 M Tris-buffer, 3.2 mM calcium acetate, 23 mM zinc acetate, 37 mM zinc chloride, pH 6.5-7; Merck) and embedded in paraffin. The other part was snap-frozen in liquid N₂ and stored at −80 ºC.

**KIDNEY FUNCTION**

The concentration of plasma creatinine in the blood samples was determined using the enzymatic colorimetric CREA plus assay (Roche).

**HISTOLOGICAL EXAMINATION**

All histological stainings were performed on 5 μm zinc-fixed paraffin-embedded sections, which were deparaffinised before use, or on 5 μm cryo-sections fixed in acetone in the case of the R73-staining. All stainings were performed at room temperature. Standard washing steps were performed between incubations. After staining, the sections were mounted in permount (Fisher Scientific). Renal morphology was evaluated by periodic acid-Schiff (PAS) staining (15 min 1% periodic acid, 30 min Schiff’s reagent (Merck), 5 min hematoxylin 37 °C, 10 s 70% ethanol with 1% hydrochloric acid). For immunohistochemistry, antigen-retreival was performed on deparaffinised sections, either by incubation in 0.1 M Tris-buffer, pH 9.0 at 80 °C for 18 h (for the ED1 and αSMA stainings), or by incubation in 0.1% protease (Sigma) for 10 min (for the collagen IV and collagen III stainings). Aspecific staining was blocked with the appropriate 2% animal serum or with 2% bovine serum albumin for 30 min. Endogenous peroxidase was blocked with 0.5-1% hydrogen peroxide (Merck) for 30 min, or with 0.5% phenylhydrazine (Fluka) for 30 min in the case of...
the collagen IV staining. Endogenous biotin was blocked with a biotin blocking kit (Dako). The sections were incubated with the following primary antibodies for 1 h, and subsequently with the appropriate secondary antibodies for 30 min: mouse anti-rat CD68 (1:200; anti-ED1; Serotec) with secondary antibody rabbit-anti-mouse-peroxidase (1:100; Dako); goat anti-collagen IV (1:150; Southern Biotechnology Associates) with secondary antibody rabbit-anti-goat-peroxidase (1:100; Dako); mouse α-smooth muscle actin (1:200; Dako) with secondary antibody rabbit-anti-mouse-peroxidase (1:100; Dako) and additional swine-anti-rabbit-peroxidase (1:100; Dako) for 30 min; rabbit anti-rat collagen III (1:100; Serotec) with secondary antibody goat-anti-rabbit-peroxidase (1:100; Dako) and additional rabbit-anti-goat-peroxidase (1:100; Dako) for 30 min. Colour development was performed using 3,3-diaminobenzidine tetrachloride (brown; Sigma). If necessary, sections were counterstained with haematoxylin (blue; Merck).

**QUANTIFICATIONS**

The tissue area studied was in the order of the red box as shown in Fig. 3. The thickness of the fibrous capsule formed around the hydrogel implant during the tissue reaction was measured microscopically at the site of implantation in at least three areas at 200 times magnification using computerized morphometry with Leica QWin 2.8 software. The amount of macrophages in the cortex and the capsule at the site of implantation was determined on tissue sections stained for ED1. Scores in arbitrary units (A.U.) between 0 and 10 were given, where 0 means not present and 10 signifies abundantly available in the tissue section. Staining of αSMA and of collagen III was measured using computerized morphometry on a Leica DMLB microscope with Leica DC300 camera and Leica QWin 2.8 software. Both the amount of positive staining in the capsule and in the kidney cortex adjacent to the capsule, were measured. The percentage of positive staining of the total area was quantified in at least 5 areas at 200 times magnification. Vascular expression of αSMA, and vascular and glomerular expression of collagen III were excluded from the measurements.

**STATISTICAL ANALYSIS**

Differences in data were evaluated with the Kruskal-Wallis test followed by Dunns post-hoc multiple comparison analysis (for the in-vitro biocompatibility tests, and kidney function measurements), or using one-way ANOVA followed by post-hoc Newman-Keuls multiple comparison test for data with Gaussian distribution (for the morphometry data). In all cases a 95% confidence interval was used. All data is expressed as mean ± standard error of mean. Probabilities of P < 0.05 were considered to be statistically significant; P < 0.05 is depicted as *, P < 0.01 is shown as **, P < 0.001 is indicated as ***.

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