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**PAPER**

# Aggregation behaviour of peptide–polymer conjugates containing linear peptide backbones and multiple polymer side chains prepared by nitroxide-mediated radical polymerization†‡

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A series of peptides with an alternating sequence of alkoxyamine conjugated lysine and glycine residues were synthesized by classical solution phase peptide coupling. The resulting peptides containing up to eight alkoxyamine moieties were used as initiators in nitroxide-mediated polymerization (NMP) to obtain peptide–polymer conjugates with well defined linear peptide backbones and a defined number of polymeric side chains. Polymerization of styrene and *N*-isopropylacrylamide (NIPAM) occurred in a highly controlled fashion. Molecular weight and polydispersity index (PDI) were determined by gel permeation chromatography (GPC). Aggregation behaviour of these hybrid materials was investigated by dynamic light scattering (DLS) and atomic force microscopy (AFM). Depending on composition, number and length of the polymer side chains, the conjugates aggregate to different topologies. Whereas peptide–polystyrene conjugates may aggregate to so called honeycomb structures, peptide–poly-*N*-isopropylacrylamide conjugates show differentiated aggregation behaviour.

## Introduction

Peptide–polymer conjugates belong to a very interesting class of materials because they often show self-organizing properties and biological activities.[1–5] These hybrid materials offer the possibility to build up defined nano- and microstructures due to the general tendency of peptides to self-assemble.[6–13] This biomimetic behaviour facilitates the design of complex molecular architectures. Along with these properties, peptide–polymer conjugates can also show enzymatic activity, alter the conductivity of a material and spread or bound to surfaces they lead to bioactive surfaces.[7,10,13,14] In addition, they are also able to control crystallization processes, transport substances like dyes or drugs and are also used for molecular targeting.[15–17]

On the one hand, the synthesis of peptide–polymer conjugates can be accomplished by the "grafting to" or coupling strategy. In that approach, one or several polymers are covalently bound to a preformed peptide.[3,7–11,18–19] In the past years "click" chemistry has been successfully used along this line.[20] Especially the 1,3-dipolar cycloaddition of azides to alkynes has been widely applied for this purpose.[21–22] Disadvantages of using the "grafting to" strategy are selectivity problems if the peptide contains more than one reactive functionality. Moreover, full conversion in particular if more than one polymer should be attached to the peptide, is difficult to achieve. This method is only practical for conjugation of synthetic polymers with small molecular weights (up to $M_w \sim 5000$).[2]

The second approach to the synthesis of peptide–polymer conjugates is called the "grafting from" or polymerization approach.[12,16,23–24] A polymerization initiator is covalently attached to a peptide. This peptide–initiator is then used for polymerization to give the desired conjugate. This method allows the conjugation of polymer moieties with much higher molecular weights. An important issue for the applied polymerization technique is its tolerance towards many functionalities as they occur in peptides. Due to the great functional group tolerance of radical chemistry, controlled radical polymerization techniques such as reversible addition-fragmentation chain transfer (RAFT) and atom-transfer radical polymerization (ATRP) have widely been used for the synthesis of peptide–polymer conjugates.[12,16,24–26] Nitroxide-mediated polymerization (NMP) is also well established for the synthesis of these complex materials.[23,27–28] These methods lead to polymers with a controlled chain length and narrow molecular weight distribution.

Whereas most approaches aimed for the conjugation of a single polymer chain at one terminus of a peptide, examples of peptides bearing polymeric side chains attached at the middle of the

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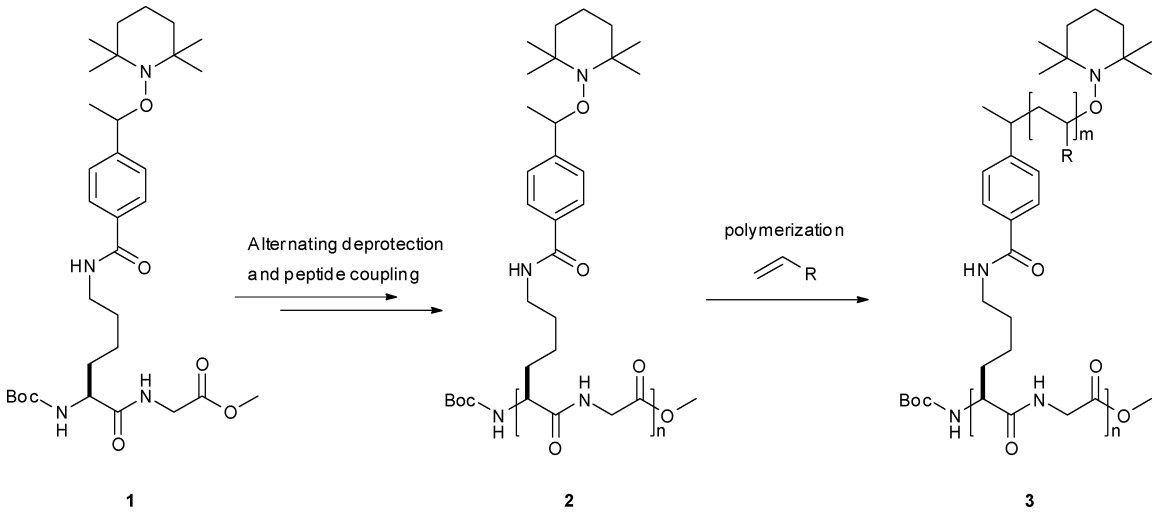



**Scheme 1** Synthesis strategy for peptide–polymer conjugates **3**.

peptide are rare. Even less investigated are peptides containing more than one polymer side chain.[12,29] Peptide–polymer conjugates consisting of a well defined linear peptide backbone and a multiple number of polymeric side chains have not been reported to date.

Surface morphology of spin-coated polystyrene has been intensively investigated. If unsubstituted linear polystyrene is spread in solution on silicon substrates, it usually builds up droplet or net-like structures after evaporation of the solvent.[30–31] François *et al.* first described the formation of so called honeycomb structures by using the same approach with polystyrene-polyparaphenylene copolymers in $CS_2$.[32] After publication of this initial paper, numerous reports on honeycomb structures have appeared. Self-assembly properties have been achieved by the use of specially designed polymer architectures or by addition of polar additives. In practice a special evaporation technique, the so called breath figure method has mostly been used.[33] Furthermore, carbohydrate-polystyrene conjugates are also able to assemble in honeycomb structures.[34]

Poly-*N*-isopropylacrylamide (PNIPAM) has attracted great interests in the last years, because of its reversible thermoresponsive solution behaviour in water. It is soluble in water below the lower critical solution temperature (LCST) of 32 °C and it precipitates at higher temperature. Hence, it is highly attractive to be used as a component in biohybrid materials.[12,35–36] Several studies on PNIPAM and its phase transition have been performed on covalently bound PNIPAM-brushes and on spin-coated films. Homogeneous films and beads of PNIPAM on silicon, gold and mica substrates have been studied.[37–38] Spin-coated copolymers of PNIPAM and polyacrylic acid showed homogeneous structures with stick type morphology whereas copolymers with polymethylmethacrylate (PMMA) delivered honeycomb like structures.[39–40]

Herein we present the synthesis of peptide–polymer conjugates containing well defined linear peptide backbones with a defined number of polymeric side chains using NMP (Scheme 1). Our report includes the synthesis of polymerization initiators, polymerization studies using styrene and NIPAM as monomers, DLS-investigations on PNIPAM-conjugates and imaging of morphologies of the conjugates on silicon surfaces by AFM.

## Results and discussion

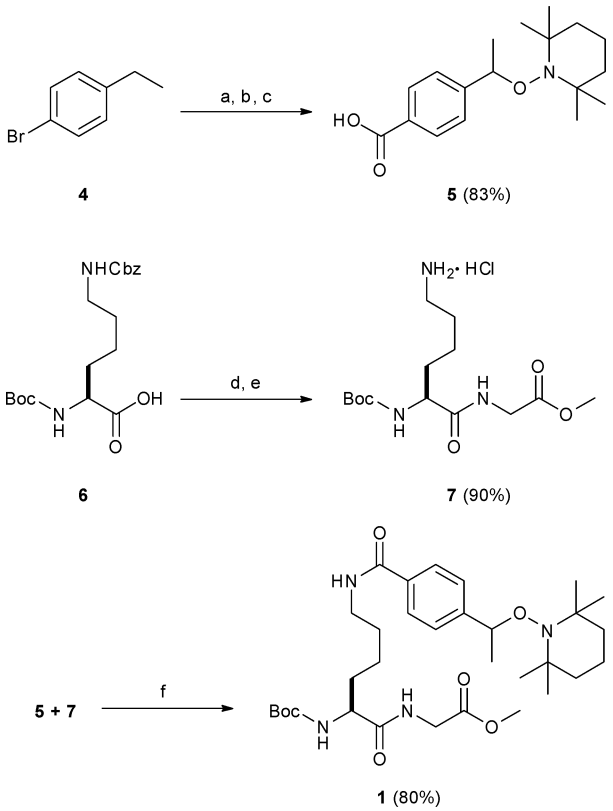
The synthesis of all polymer conjugated peptides discussed herein started with polymerization initiators like **1** which consists of an alkoxyamine, covalently bound to a lysine–glycine dipeptide. This modified dipeptide was homocoupled *via* conventional solution phase peptide coupling procedures to give oligopeptides **2**. Because the number of initiator moieties doubles after each coupling reaction, well defined peptides bearing multiple alkoxyamine functionalities can be obtained in a few steps by this fragment coupling strategy. These alkoxyamine derived peptides were then used as initiators for NMP. As TEMPO-derived alkoxyamines are only able to control polymerization of styrene or styrene derivatives, synthesis of analogous alkoxyamine initiators bearing a more sterically hindered nitroxide was necessary. The same fragment coupling route was followed for immobilization of a more sophisticated tetraethyl-substituted nitroxide into the peptide.

### Initiator synthesis

The synthesis of the alkoxyamine moiety started with 1-bromo-4-ethylbenzene (**4**) (Scheme 2). Radical bromination, alkoxyamine formation under atom transfer conditions with 2,2,6,6-tetramethylpiperidin-*N*-oxyl radical (TEMPO) and introduction of the carboxyl group by bromine-lithium-exchange and subsequent addition of $CO_2$ delivered **5** in a good overall yield. Reaction of double protected lysine **6** with glycinemethylester hydrochloride using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBt) and *N*-methylmorpholine (NMM) followed by deprotection of the side chain amino group led to dipeptide **7**. Hydrogenation of the benzylcarbamate group was conducted out under slightly acidic conditions. The alkoxyamine functionality was introduced by acylation of the lysine side chain of **7** with acid **5** using EDCI. Building block dipeptide **1** was obtained in 80% yield.

Deprotection of the Boc-group was readily accomplished by treatment of **1** with HCl in 1,4-dioxane (Scheme 3). Amine

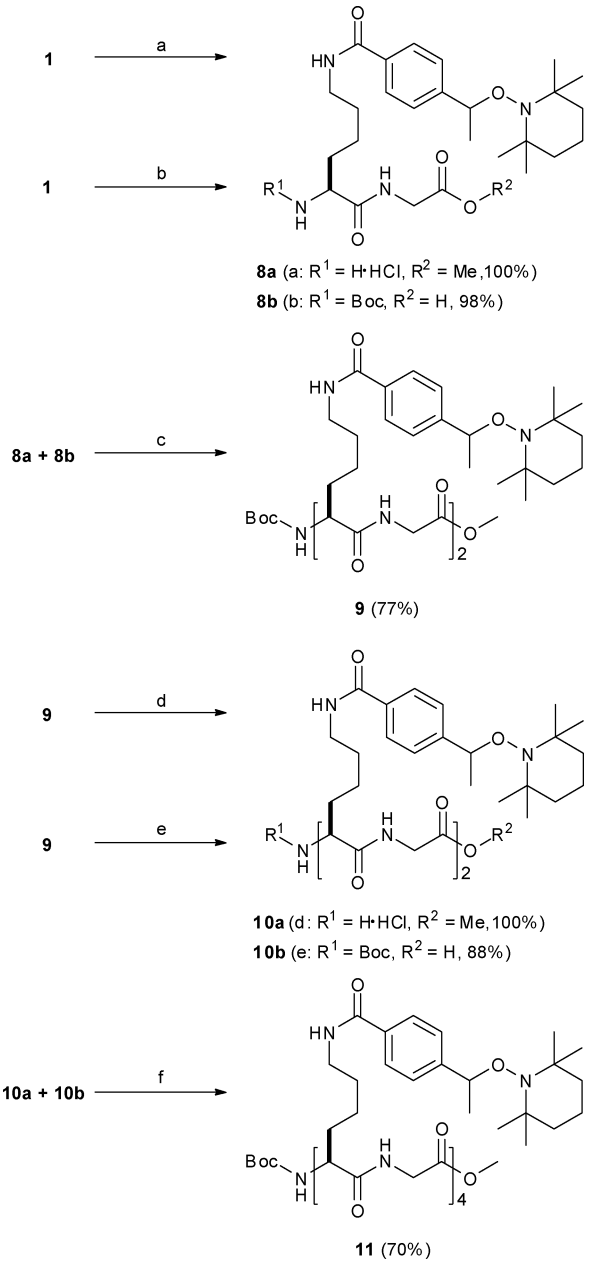



**Scheme 2** Reagents and conditions: (i) $Br_2$, $CCl_4$, 50 °C, hν, 1 h; (ii) TEMPO, Cu (powder), $Cu(OTf)_2$, 4,4′-di-*tert*-butyl-2,2′-dipyridyl, benzene, 75 °C, 16 h; (iii) *t*-Buli (2 eq.), $CO_2$, THF, −78 °C → RT, 1 h; (iv) HCl·H-Gly-OMe, EDCI, HOBt, NMM, $CH_2Cl_2$, RT, 18 h; (v) $H_2$, (1 bar), Pd/C, MeOH, HCl in MeOH (0.2 eq.), RT, 18 h, then HCl in MeOH (0.8 eq.); (vi) EDCI, HOBt, NMM, $CH_2Cl_2$, RT, 16 h.

hydrochloride **8a** was isolated in a quantitative yield. Ester hydrolysis under basic conditions in a mixture of water and methanol afforded the free acid **8b** in excellent yield. Coupling of the dipeptide **8a** with **8b** was achieved with EDCI to give tetrapeptide **9**, containing two alkoxyamine moieties.

Removal of the Boc-group in **9** was performed with HCl in methanol. Deprotection of the *C*-terminus was achieved applying the same conditions used for ester hydrolysis of **1**. The coupling reaction between tetrapeptides **10a** and **10b** was again carried out with EDCI, HOBt and NMM. Due to solubility problems, this coupling reaction was conducted in DMF. The resulting octapeptide **11** was isolated by reversed-phase flash chromatography in 70% yield.

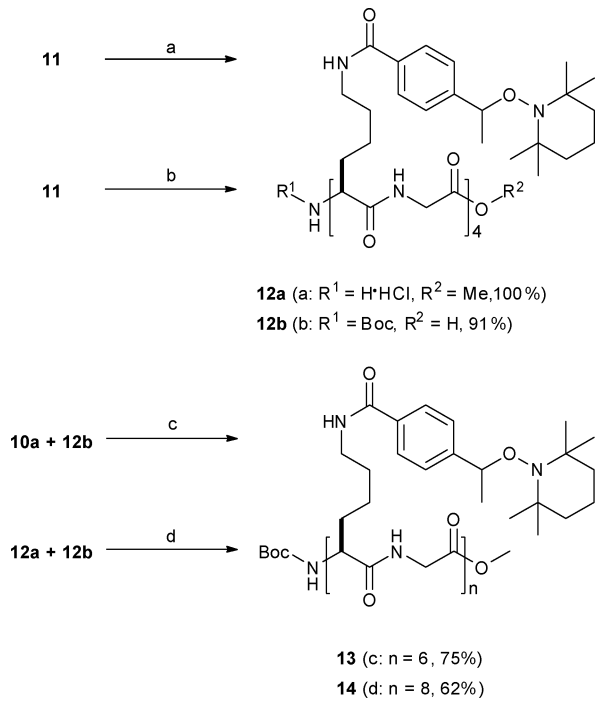
Selective deprotection of octapeptide **11** turned out to be very challenging. After careful optimization we found that Boc-cleavage was best conducted at 0.05 mmol scale with 12.5 equivalents of HCl in 5.5 mL methanol for 48 h at room temperature (Scheme 4). Reaction conditions had to be strictly followed in order to achieve full conversion and to avoid methanolysis of the peptide backbone. For the deprotection of the *C*-terminus, peptide **11** was dissolved in methanol (0.167 mM) and 20 mL of NaOH (1 M *aq*.) was added. High dilution was necessary due to low solubility of the octapeptide **11**. Full conversion for that ester hydrolysis was achieved after 40 h.




**Scheme 3** *Reagents and conditions*: (a) HCl (4 M in 1,4-dioxane), 1 h; (b) NaOH (0.25 M, *aq*.), MeOH, 15 h; (c) EDCI, HOBt, NMM, $CH_2Cl_2$; (d) HCl (1.25 M in MeOH), 16 h; (e) NaOH (0.25 M *aq*.), MeOH; (f) EDCI, HOBt, NMM, DMF.

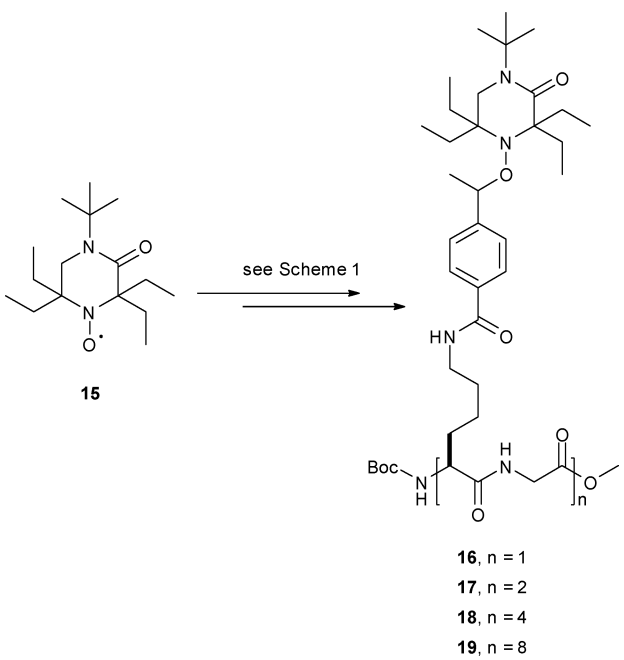
For the coupling reaction of peptide **10a** with **12b**, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HATU) was chosen as coupling reagent. The reaction was performed in DMF as solvent and the targeted peptide **13** was precipitated directly from the reaction mixture by addition of methanol and acetone. The same procedure was used also for coupling of octapeptides **12a** and **12b** using *N*-methylpyrrolidone as solvent.

As mentioned in the introduction, TEMPO-derived alkoxyamines are only suited for the controlled polymerization of styrene.[41] To enlarge the range of possible monomers which can be polymerized in a controlled way, we also synthesized




**Scheme 4** *Reagents and conditions*: (a) HCl (1.25 M in MeOH, 12.5 eq.), MeOH (0.01 M solution of **11**), 48 h; (b) NaOH (1 M, *aq*., 400 eq.), MeOH (0.167 mM solution of **11**), 40 h; (c) HATU, HOAt, DIPEA, DMF; (d) HATU, HOAt, DIPEA, *N*-methylpyrrolidone.

the corresponding peptides bearing tetraethyl-substituted alkoxyamines. To this end, TEMPO was replaced by nitroxide **15** (Scheme 5).[42] The synthesis of **15**-containing peptides were performed in analogy to those used for the preparation of the TEMPO-conjugated peptides. In most cases the yields were slightly lower (see the ESI‡).




**Scheme 5** Polymerization initiators derived from nitroxide **15**.

### Polymerization studies with styrene as monomer

Polymerization of styrene was conducted at initiator loadings from 0.125 to 1.0 mol% at 125 °C in neat solution under argon atmosphere (Table 1). Excess of unreacted monomer was removed in a vacuum drying cabinet at 60 °C for at least 18 h and conversion was determined gravimetrically. Experimental molecular weights ($M_{n,exp}$) and polydispersity indices (PDI) were measured by gel-permeation chromatography (GPC). Styrene polymerization proceeded highly controlled with alkoxyamines **1**, **9**, **11**, **13**. Best results for polymerizations conducted with **1** were obtained with initiator concentrations of 0.5 or 1.0 mol% (Table 1, entries 1–8). PDIs were in most cases below 1.20 and tended to decrease at longer polymerization time. Molecular weight distributions were narrow if polymerization was allowed to proceed for 3 h up to 24 h. Lower initiator loadings led to larger polymers and to larger PDIs.

Under analogous conditions peptide initiator **9** delivered polymers with roughly doubled molecular weights as compared to those synthesized with initiator **1** (entries 6–8, 9–12). This indicates that both alkoxyamine moieties in **9** regulate NMP independently from each other. Aberration of this relationship may occur up to 15%, due to experimental and analytical variation. Pleasingly, polymerization also took place in a highly controlled fashion with alkoxyamine functionalized peptides **9** and **11** bearing up to four initiator residues. In these cases, polymerizations were conducted for up to 24 h. Because of statistical effects, PDIs decreased with increasing number of polymer chains. When alkoxyamine hexamer **13** was used as a regulator at loadings of 0.083 or 0.167 mol%, polymerization had to be stopped after 12 h (entries 18–20). If reactions were allowed to run for longer times, bimodal molecular weight distributions were observed. We assume this is a result of dimerization of polymeric radicals, because the size of polymers with higher molecular weight distribution is roughly about twice the size of the smaller targeted ones. Due to the higher number of potential radicals per molecule in polymers bearing multiple alkoxyamines, dimerization is more likely.

This problem was even more pronounced when hexadecapeptide **14**, containing eight alkoxyamine functionalities, was used as a polymerization initiator. Bimodal molecular weight distributions resulted at initiator loadings of 0.0625 and 0.125 mol% even for short polymerization times. However, this problem was solved by further decreasing of initiator loading. Monomodal size distributions of polymers were obtained at concentrations from 0.008 to 0.03 mol% (entries 21–25). However, the resulting polymers showed larger PDIs. Nevertheless, we were able to synthesize peptide–polystyrene conjugates with up to eight polymer side chains and molecular weights with up to 300.000 g mol$^{-1}$ with narrow size distributions (see ESI‡ for additional polymerization experiments).

### Polymerization studies with NIPAM as monomer

Polymerization studies with NIPAM were conducted using peptide initiators bearing the more sophisticated nitroxide **15**. Experiments were conducted in 1.78 M solution of NIPAM in benzene at 125 °C in sealed tubes under argon atmosphere. The resulting PNIPAM was separated from non reacted monomer by precipitation from an acetone solution upon addition of diethyl ether.

**Table 1** Polymerization of styrene at 125 °C, neat, sealed tube

| Entry | Initiator | Conc. (%mol) | Time (h) | Conversion (%) | $M_{n,th.}$ (g mol$^{-1}$) | $M_{n,exp.}$ (g mol$^{-1}$) | PDI |
|---|---|---|---|---|---|---|---|
| 1 | **1** | 0.125 | 1 | 10 | 9300 | 10100 | 1.20 |
| 2 | **1** | 0.25 | 1 | 6 | 2900 | 2600 | 1.20 |
| 3 | **1** | 1.0 | 3 | 15 | 2200 | 2400 | 1.11 |
| 4 | **1** | 0.5 | 6 | 16 | 4000 | 5700 | 1.19 |
| 5 | **1** | 1.0 | 6 | 24 | 3000 | 3200 | 1.15 |
| 6 | **1** | 1.0 | 12 | 40 | 4800 | 5000 | 1.09 |
| 7 | **1** | 0.5 | 24 | 64 | 13800 | 11400 | 1.10 |
| 8 | **1** | 1.0 | 24 | 56 | 6400 | 5000 | 1.11 |
| 9 | **9** | 0.5 | 3 | 19 | 4900 | 5000 | 1.13 |
| 10 | **9** | 0.5 | 12 | 42 | 9800 | 8800 | 1.18 |
| 11 | **9** | 0.25 | 24 | 72 | 31000 | 25000 | 1.12 |
| 12 | **9** | 0.5 | 24 | 62 | 13900 | 11200 | 1.16 |
| 13 | **11** | 0.25 | 3 | 16 | 8700 | 8100 | 1.08 |
| 14 | **11** | 0.125 | 9 | 42 | 36900 | 32000 | 1.08 |
| 15 | **11** | 0.25 | 12 | 59 | 26500 | 24900 | 1.08 |
| 16 | **11** | 0.125 | 24 | 64 | 55200 | 31200 | 1.07 |
| 17 | **11** | 0.25 | 24 | 62 | 22600 | 22900 | 1.09 |
| 18 | **13** | 0.167 | 3 | 27 | 20000 | 17000 | 1.07 |
| 19 | **13** | 0.083 | 12 | 51 | 65600 | 95200 | 1.06 |
| 20 | **13** | 0.167 | 12 | 43 | 29800 | 33100 | 1.06 |
| 21 | **14** | 0.008 | 2 | 22 | 294200 | 297500 | 1.30 |
| 22 | **14** | 0.016 | 0.5 | 9 | 62000 | 64300 | 1.18 |
| 23 | **14** | 0.016 | 1 | 15 | 103700 | 87100 | 1.14 |
| 24 | **14** | 0.016 | 3 | 22 | 150600 | 70700 | 1.15 |
| 25 | **14** | 0.031 | 0.5 | 7 | 28800 | 30300 | 1.19 |

Conversion was determined gravimetrically, $M_{n,exp}$ and PDIs were determined by GPC.

Polymerization of NIPAM regulated by **16** occurred well controlled with initiator loadings of 0.5 and 1.0 mol% (Table 2, entries 1–5). Low PDIs (<1.20) were obtained for these polymerizations. Similar results were observed using tetrapeptide **17**. As for the styrene polymerizations, a doubling of the molecular weight was achieved by switching from **16** to **17** containing two active initiator moieties under analogous conditions (entries 6–10). The polydispersities measured indicated a controlled polymerization. Tetraalkoxyamine-substituted peptide initiator **18** also allowed controlled NIPAM polymerization (entries 11–15).

Polymerization of NIPAM initiated with octaalkoxyamine **19** was difficult to control. Polymerizations occurred fast and side reactions were observed. Acceptable results were obtained by stopping the reactions at low conversions (reaction time < 6 h). In contrast to the styrene polymerizations, we did not observe bimodal molecular weight distributions for the polymerization of NIPAM conducted by octaalkoxyamine substituted peptide **19** (see ESI‡ for additional polymerization results).

**Table 2** Polymerization of NIPAM at 125 °C, 1.78 M in benzene, sealed tube

| Entry | Initiator | Conc. (mol%) | Time (h) | Conversion (%) | $M_{n,th.}$ (g mol$^{-1}$) | $M_{n,exp.}$ (g mol$^{-1}$) | PDI |
|---|---|---|---|---|---|---|---|
| 1 | **16** | 0.5 | 6 | 24 | 6100 | 10000 | 1.21 |
| 2 | **16** | 1.0 | 6 | 15 | 2500 | 6900 | 1.17 |
| 3 | **16** | 1.0 | 12 | 38 | 5000 | 7400 | 1.18 |
| 4 | **16** | 0.5 | 24 | 56 | 13500 | 20200 | 1.12 |
| 5 | **16** | 1.0 | 24 | 76 | 9300 | 12600 | 1.20 |
| 6 | **17** | 0.5 | 3 | 16 | 5000 | 5900 | 1.20 |
| 7 | **17** | 0.5 | 6 | 21 | 6200 | 8200 | 1.18 |
| 8 | **17** | 0.5 | 12 | 37 | 9700 | 10300 | 1.17 |
| 9 | **17** | 0.25 | 24 | 64 | 30200 | 26500 | 1.14 |
| 10 | **17** | 0.5 | 24 | 71 | 17400 | 19500 | 1.14 |
| 11 | **18** | 0.25 | 3 | 25 | 13600 | 12100 | 1.17 |
| 12 | **18** | 0.25 | 6 | 29 | 15700 | 21200 | 1.10 |
| 13 | **18** | 0.25 | 12 | 46 | 23100 | 23800 | 1.11 |
| 14 | **18** | 0.125 | 24 | 57 | 54300 | 45500 | 1.09 |
| 15 | **18** | 0.25 | 24 | 74 | 35700 | 33600 | 1.11 |
| 16 | **19** | 0.125 | 1 | 33 | 35500 | 36600 | 1.24 |
| 17 | **19** | 0.125 | 2 | 44 | 40000 | 39000 | 1.18 |
| 18 | **19** | 0.125 | 6 | 71 | 70000 | 43400 | 1.20 |
| 19 | **19** | 0.125 | 12 | 78 | 76400 | 43400 | 1.20 |
| 20 | **19** | 0.063 | 24 | 85 | 160100 | 149300 | 2.40 |
| 21 | **19** | 0.125 | 24 | 87 | 84500 | 85900 | 1.78 |

**Table 3** Hydrodynamic diameters of peptide–polystyrene conjugates in THF, $c = 1$ mg mL$^{-1}$, $T = 25$ °C

| Entry | Entry in Table 1 | Initiator | $M_{n,exp}$ (g mol$^{-1}$) | Diameter (nm) |
|---|---|---|---|---|
| 1 | 6 | **1** | 5000 | 1.74 |
| 2 | 11 | **9** | 25000 | 7.24 |
| 3 | 16 | **11** | 31200 | 6.88 |
| 4 | 18 | **13** | 17000 | 6.30 |
| 5 | 19 | **13** | 95200 | 10.55 |
| 6 | 25 | **14** | 30300 | 8.43 |
| 7 | 21 | **14** | 297500 | 27.27 |

## DLS-analysis of conjugates

Investigations of peptide–polystyrene conjugates *via* DLS-measurements revealed that no aggregation of these conjugates occurred in THF or dichloromethane under the tested conditions. Variation of concentration and temperature, as well as equilibration of the samples for several hours did not lead to any aggregation. We were able to determine hydrodynamic diameters in THF. Measurements were performed at room temperature in THF ($c = 1$ mg mL$^{-1}$). Diameters correlate roughly with molecular weights determined by GPC (Table 3, entries 1, 2, 5, 7).

DLS-measurements turned out to be very useful to investigate phase transition of peptide–PNIPAM conjugates in water. We studied aggregation behaviour as a function of temperature with peptide–PNIPAM conjugates bearing one, two, four and eight PNIPAM side chains. These four samples derived from polymerizations conducted under analogous conditions (1.0 mol% with respect to alkoxyamine moieties, concentration of PNIPAM = 1.78 M in benzene, 125 °C, 6 h, Table 2, entries 2, 7, 12, 18). Particle size distributions were analyzed either by their volume maxima ($v_{max}$) or their number ($n_{max}$) maxima (Fig. 1, Fig. 2). At 10 °C all samples were well dissolved and particle sizes represent monomeric conjugates and correlate with their molecular weights. By increasing the temperature aggregation of the conjugates was observed. In comparison to unsubstituted PNIPAM, which precipitates at a temperature of 32 °C, our conjugates generally showed a lower phase transition temperature. The monopolymer substituted conjugate started to precipitate at 23 °C. Phase transition occurred in a narrow temperature range and particles formed grew with increasing temperature to large aggregates ($v_{max} = 708$ nm, $T = 34$ °C; $n_{max} = 387$ nm, $T = 30$ °C).

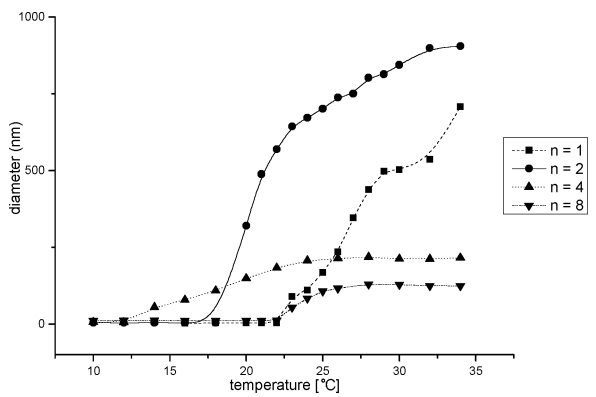



**Fig. 1** Size distribution maxima by volume of peptide–PNIPAM conjugate particles ($v_{max}$), determined by DLS-measurement in water ($c = 1$ mg mL$^{-1}$). *n* indicates the number of attached polymers.

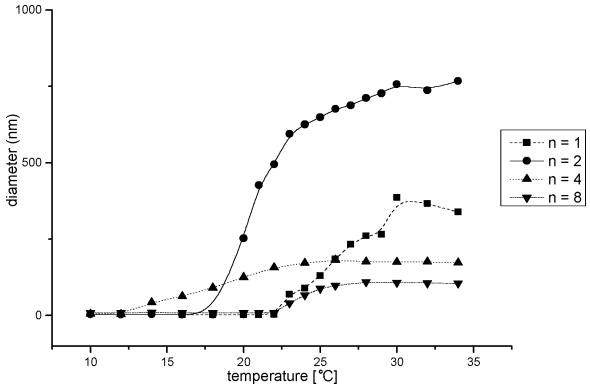



**Fig. 2** Size distribution maxima by number of peptide–PNIPAM conjugate particles ($n_{max}$), determined by DLS-measurement in water ($c = 1$ mg mL$^{-1}$). *n* indicates the number of attached polymers.

The conjugate containing two PNIPAM-chains, which can be regarded as a linear PNIPAM polymer containing a peptide spacer, showed a similar aggregation behaviour. Phase transition started at 20 °C ($v_{max} = 905$ nm, $T = 34$ °C; $n_{max} = 768$ nm, $T = 34$ °C). For linear peptide–polymer conjugates a steady increase of the particle size was observed upon increasing the temperature.

Comb type peptide–PNIPAM conjugates showed a different aggregation behaviour. A smooth phase transition over a large temperature range was observed. In addition, aggregation to large particles did not occur at higher temperature. The conjugate with four PNIPAM-chains started to aggregate at the lowest temperature ($T \sim 14$ °C). Particle size maxima did not exceed 220 nm ($v_{max} = 219$ nm, $T = 28$ °C; $n_{max} = 182$ nm, $T = 26$ °C). The conjugate containing eight polymer moieties showed a similar aggregation behaviour, but interestingly, phase transition started at a higher temperature ($T \sim 23$ °C) and the maximal particle diameter was far smaller ($v_{max} = 130$ nm, $T = 28$ °C; $n_{max} = 110$ nm, $T = 28$ °C).

## Aggregation behaviour on surfaces

We first studied aggregation behaviour at surfaces using the non polymerized peptide initiators. To this end, a drop of a solution of the peptide initiator in DMF ($c = 1$ mg mL$^{-1}$) was spin-coated on a silicon wafer. After evaporation of the solvent AFM-images of the resulting surfaces were recorded.

TEMPO-substituted peptides **1**, **9** and **11** did not show any regular structures. After one week equilibration of the peptide solution, peptide **13** bearing six alkoxyamine moieties formed sharp fibres with heights of about 2–4 nm, widths of 20–40 nm

and lengths of 100–1000 nm, which aggregated to fingerprint type like structures at the silicon surface (Fig. 3, left side).

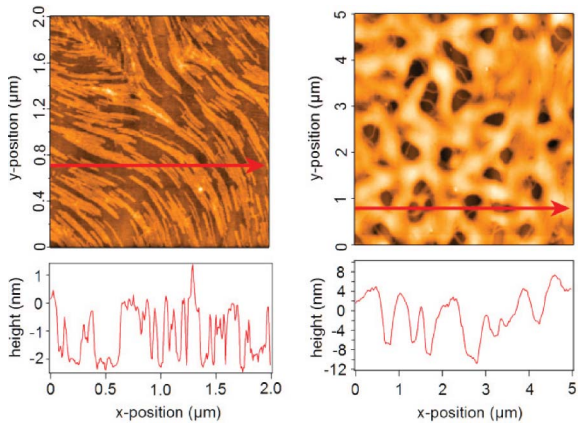



**Fig. 3** AFM-images of peptides **13** (left) and **19** (right) after spin-coating in DMF on silicon wafer.

Hexadecapeptide **19**, bearing eight alkoxyamine functionalities was dissolved in DMF and the solution was spread on a silicon substrate. AFM-imaging after evaporation of the solvent revealed a net-like structure consisting of peptide fibres. In comparison to **13**, these fibres exhibit larger diameters of about 100 nm (Fig. 3, right side).

These initial results encouraged us to study peptide–polystyrene conjugates in the same manner. Instead of DMF we used THF as solvent for spin-coating of the hybrids. AFM-measurement revealed formation of so called honeycomb structures. Conjugate **20** (Table 1, entry 18), obtained from polymerization with tetraalkoxyamine functionalized peptide **11**, showed first tendencies toward layered structures with holes (Fig. 4, left side), but the layer is cracked and the holes have irregular distributions and diameters. Conjugate **21** bearing six polystyrene chains (Table 1, entry 24) showed a more defined morphology (Fig. 4, right side). There were fewer but more regular holes in these layers. Layer thickness varied from about 5 to 10 nm in both cases.

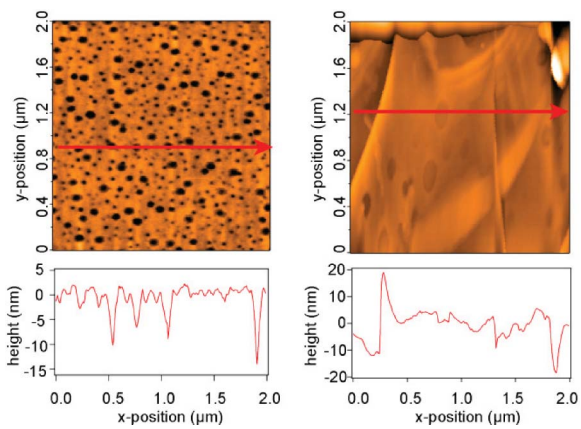



**Fig. 4** AFM-images of peptide–polystyrene conjugates **20** (left) and **21** (right) after spin-coating in THF on a silicon wafer.

Peptide conjugates with eight polystyrene chains showed different aggregation architectures. If the attached polymer chains are short, formation of honeycomb structures was still favoured. Conjugate **22** (Table 1, entry 29) was able to assemble to a honeycomb structure containing up to three layers with a regular hole distribution (Fig. 5, left side). Layer thickness was about 4 nm and diameters of the holes showed sizes from 20 up to 40 nm. Surprisingly, conjugate **23** assembled after spin-coating completely different than **22**. The structures have a coral like morphology (Fig. 5, right side). Its height is about 4 nm.

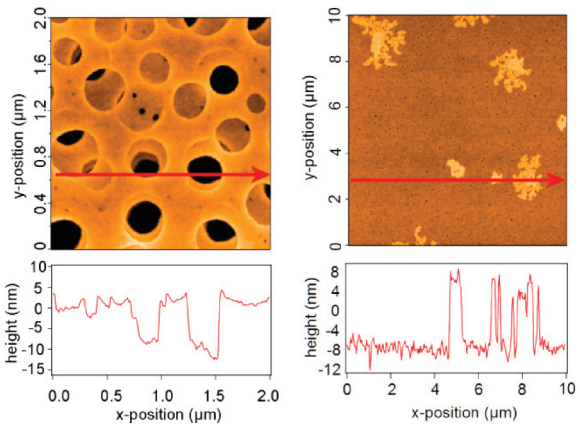



**Fig. 5** AFM-images of conjugates **22** (left) and **23** (right) after spin-coating in THF a silicon wafer.

A possible reason for this behaviour could be that the long non-polar polystyrene side chains hinder the polar peptide moieties aggregating over large areas. Then the conjugates may only form small aggregates which potentially show these structures. This explanation is plausible considering that literature reports polar additives or moieties in conjugates to be responsible for the formation of the holes in honeycomb structures under humid conditions.

We next investigated the surface self-organizing behaviour of peptide–PNIPAM conjugates. First spin-coating attempts were performed with solutions of the conjugates in acetone ($c$ = 1 mg mL$^{-1}$). Conjugate **24** (Table 2, entry 24), bearing a hexadecapeptide chain with eight PNIPAM side chains attached, formed ring shaped structures (Fig. 6, left side). These larger aggregates also showed internal void areas. Outer rings of these morphologies were about 10 nm high whereas core rings could overtop the rings with around 20 nm height. Diameters of these rings vary in size up to 1 µm and thicknesses of the rings were approximately all around 100 nm. These structures were observed over large surface areas of about 250 µm$^2$ (Fig. 6, right side).

By increasing the length of the PNIPAM polymers, morphology of the aggregates changed. Conjugate **25** (Table 2, entry 26) aggregated to large objects (Fig. 7, left side).

These structures with diameters of 200–500 nm, did not show any inner void space. This morphology is also regular over large areas (Fig. 7, right side). Size and morphology of these structures are similar to those observed for PNIPAM-polyacrylic acid copolymers.[39] Obviously surface behaviour of peptide–PNIPAM conjugates switches to properties of unsubstituted PNIPAM if chain length increases. The influence of the peptide backbones regarding aggregation behaviour then seems to fade.

We were also interested in the aggregation behaviour of peptide–PNIPAM conjugates in water. This was studied at the interface between an aqueous solution and the silicon surface by coating. To

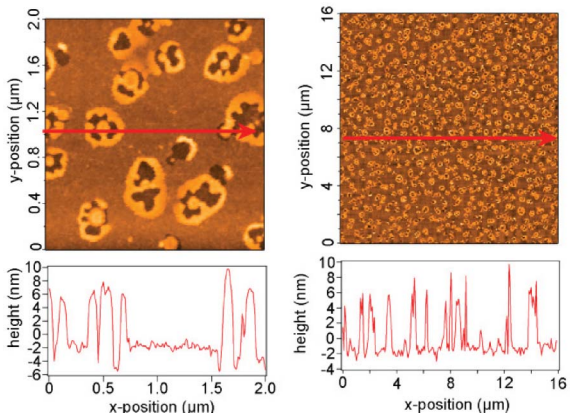



**Fig. 6** AFM-images of peptide–PNIPAM conjugate **24** after spin-coating in acetone on a silicon wafer.

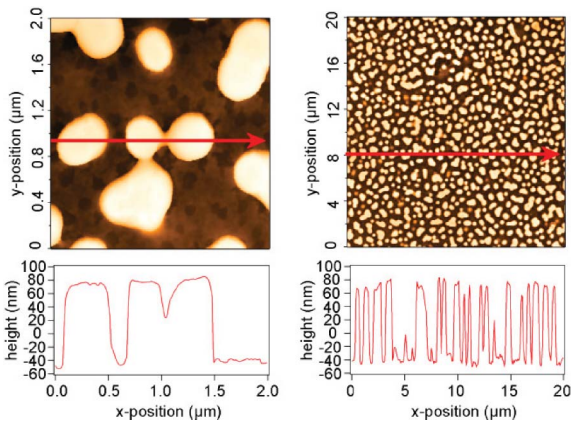



**Fig. 7** AFM-images of conjugate **25** after spin-coating in acetone on a silicon wafer.

this end, several conjugates were dissolved in water in an ice bath ($c = 1$ mg mL$^{-1}$). Evaporation of the solvent was conducted at 6 °C to avoid precipitation of PNIPAM conjugates from solution. We chose again conjugates **24** and **25** for these studies and we obtained for both samples homogeneous structured surfaces coated with spherical beads that accumulate to clusters (Fig. 8). These two conjugates showed in water in contrast to acetone very similar aggregation behaviour. Diameters of the observed beads varied between 100 and 150 nm. As it is known that PNIPAM can aggregate to beads[38] and because peptide chain length does not seem to have any influence on the morphology, our conclusion is that bead formation of these conjugates is fully driven by the PNIPAM residues and the peptide backbones do not exert a large effect on the self-assembly process.

## Conclusions

In this article we presented the synthesis of peptide–polymer conjugates composed of well defined linear peptide backbones and multiple defined numbers of polystyrene or PNIPAM side chains *via* NMP and studies on their aggregation behaviour. We showed that NMP is a reliable method to attach up to eight polymer chains onto a peptide backbone. Polymerizations occurred well controlled over a wide range of initiator loadings

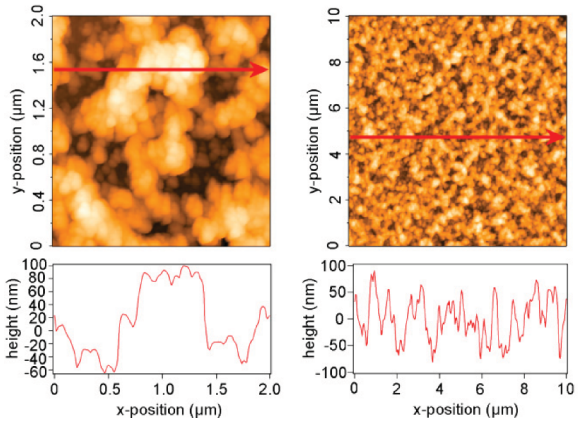



**Fig. 8** AFM-images of conjugate **25** after spin-coating in water on a silicon wafer.

and polymerization times. DLS-measurements revealed no aggregation of peptide–polystyrene conjugates in solution. Aggregation of peptide–PNIPAM conjugates in water at their critical solution temperature was observed. Linear conjugates formed much larger aggregates and showed sharp phase transitions whereas comb type conjugates precipitated to particles with limited sizes. AFM-imaging on silicon surfaces showed tendencies of peptide initiators to build up fibre like structures. Peptide–polystyrene conjugates bearing small polymer chains aggregated to so called honeycomb structures and showed coral like morphologies for systems bearing longer side chains. Spin-coated from acetone on a silicon wafer, peptide–PNIPAM conjugates aggregated to ring shaped topologies if PNIPAM chains were short. Conjugates containing longer PNIPAM chains showed typical PNIPAM-like aggregation behaviour. In water these conjugates accumulated to spherical beads.

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