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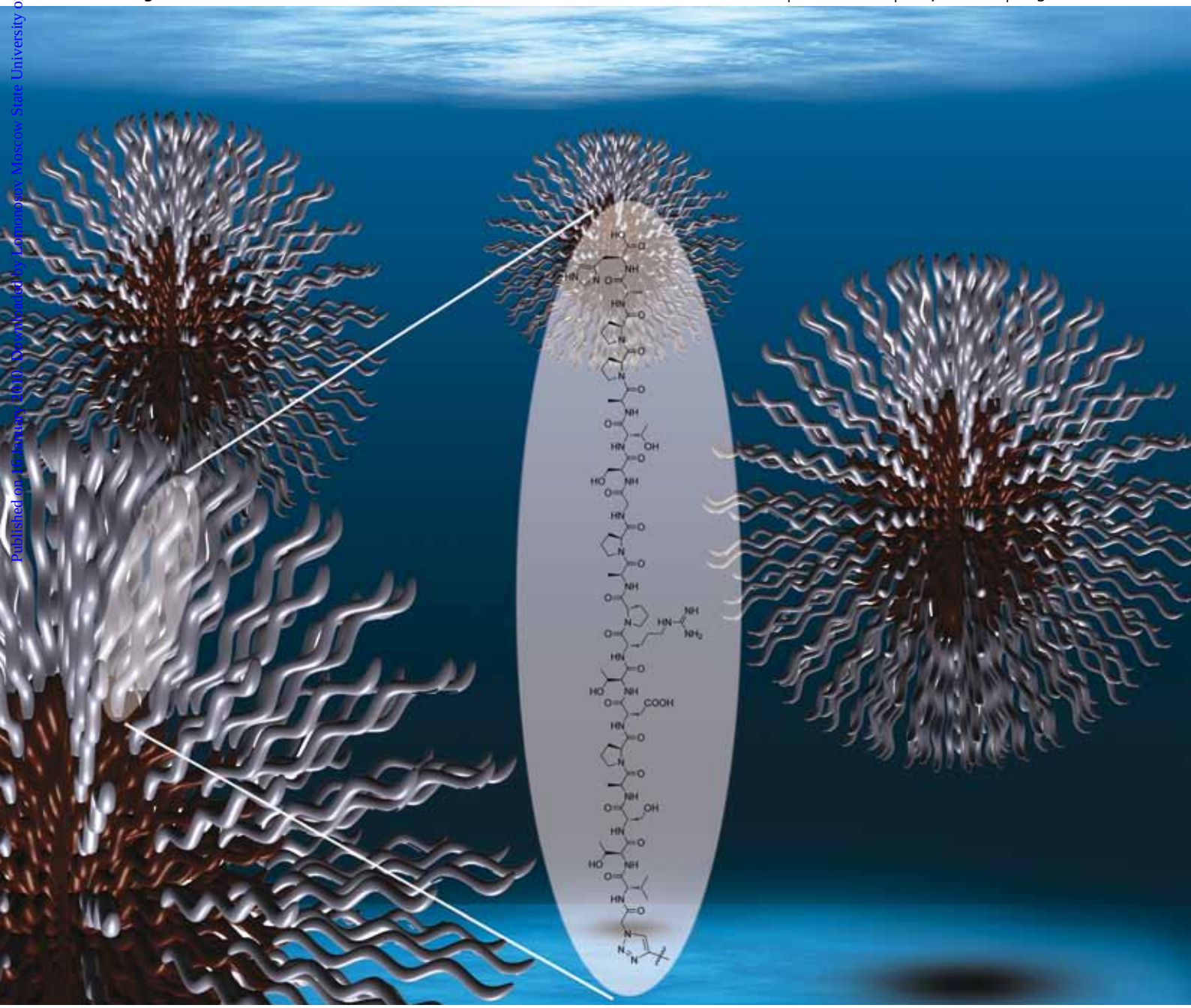

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Polymer–peptide chimeras for the multivalent display of immunogenic peptides

Polymer–peptide chimeras for the multivalent display of immunogenic peptides†

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Chimeras of poly(*n*-isopropyl acrylamide) and immunogenic peptides from the cancer-associated glycoprotein MUC1 were synthesised using a combination of solid-phase peptide synthesis, RAFT polymerisation and copper-catalysed alkyne–azide cycloaddition reactions.

Chimeras are macromolecular structures that combine biomolecules with synthetic polymers.[1,2] These hybrid molecules have recently triggered widespread enthusiasm among physicists, chemists and biologists due to their ability to amalgamate the properties of synthetic polymers with the activity of biomolecules. This has seen their application in a diverse spectrum of areas such as nanotechnology, photonics, biotechnology and medicine.[3] The major approaches to the synthesis of these molecular structures are either *via* direct polymerisation of functional biomolecule-based monomers,[4] or *via* the coupling of biomolecules to preformed polymeric scaffolds. The direct polymerisation approach has received much attention since the early 1990s, with the development of living radical polymerisation (LRP) techniques allowing rapid access to well-defined functional polymers of varying molecular architecture.[5] Among the LRP methods, reversible addition–fragmentation chain transfer (RAFT[6,7]) polymerisation represents an extremely versatile means for the generation of well defined chimeras.[2] However, from a synthetic standpoint, the direct polymerisation approach presents some challenges. For example, the construction of low molecular weight polymers demands large quantities of biomolecule initiators for the polymerisation to be carried out on a reasonable laboratory scale. Additionally, biomolecules usually bear a range of functional groups which may disrupt the polymerisation process *e.g.* the thiocarbonyl thio chain transfer reagents used in RAFT polymerisation can be degraded in the presence of amine groups, thus precluding their use in peptide and protein applications.[2,8] Another potential drawback of this strategy is the potential loss of activity of a given biomolecule due to unwanted side reactions.

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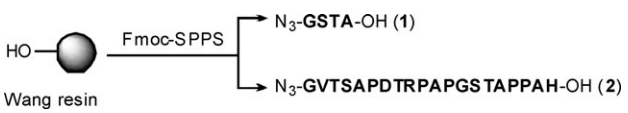
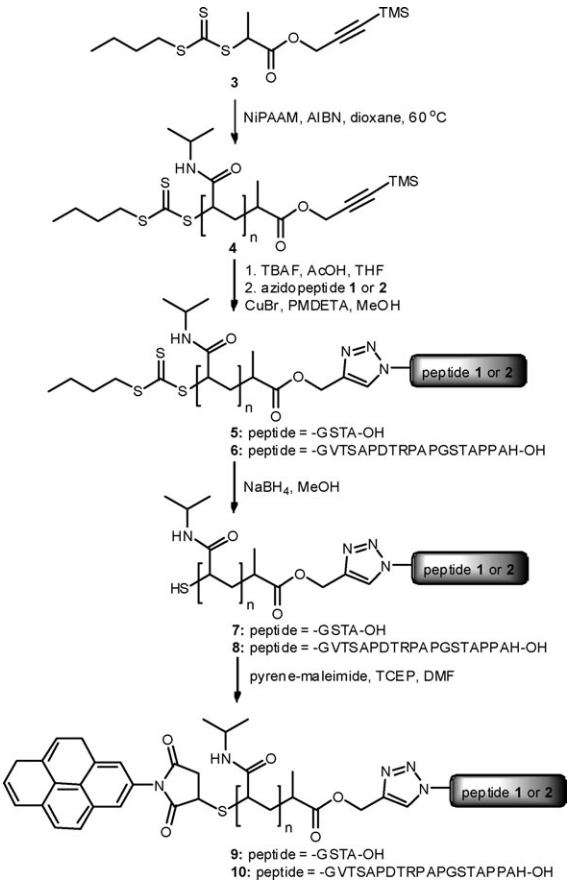
In this study, we were interested in developing a synthetic methodology for the generation of self assembling polymer–peptide chimeras. Specifically, we proposed to link immunogenic peptides from the extracellular variable number tandem repeat (VNTR) region of the cancer associated glycoprotein MUC1[9] to a preformed functional polymer in a convergent manner. A hydrophobic polymer of tailored length was designed to complement the hydrophilic peptide segment to afford amphiphilic chimeras. It was anticipated that these features would afford self-assembled nanostructures in aqueous media with the hydrophilic peptide presented at their surface. This approach provides a novel avenue for the multivalent display of immunogenic peptide epitopes, a desirable feature for a strong and sustained response in immunological studies.[10,11] The resulting nanostructures can then be utilised to investigate the generation of immunostimulating antigens with a view to developing vaccines for a variety of epithelial cancers, an area which is currently under investigation with MUC1 peptides and glycopeptides.[12–18] Our synthetic strategy was to utilise the Cu(I)-catalysed variant of the Huisgen 1,3-dipolar cycloaddition reaction,[19] namely the Cu(I)-catalysed azide–alkyne cycloaddition (CuAAC),[20,21] to generate these constructs in an efficient and concise manner. CuAAC was chosen by virtue of its tolerance to a wide variety of solvents and functionalities and because the reactions are chemoselective and high yielding. The reaction has also been successfully employed in the preparation of a number of other polymer–peptide conjugates.[3,22–25]

In order to generate the desired chimeras, we first embarked on the synthesis of the requisite building blocks. To this end, tetrapeptide N_3-GSTA-OH **1** and eicosapeptide N_3-GVTSAPDTRPAPGSTAPPAH-OH **2** (representing the complete repeat unit of the VNTR sequence) were first synthesised *via* Fmoc-strategy SPPS (Scheme 1, ESI†). Notably, azidoglycine was incorporated as the *N*-terminal amino acid to facilitate conjugation to an alkyne-bearing polymer construct *via* CuAAC chemistry (ESI†).[26]

Poly(*n*-isopropyl acrylamide) (PNiPAAM), a non-toxic and biocompatible polymer, was chosen as the polymeric unit for the generation of chimeras, and was generated *via* RAFT



Scheme 1 SPPS of MUC1 peptides **1** and **2** bearing *N*-terminal azides.



Scheme 2 Synthesis of PNiPAAM–MUC1 peptide chimeras **5–10**.

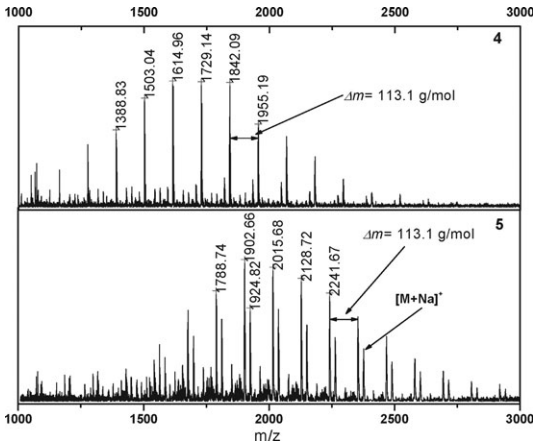


Fig. 1 MALDI-TOF spectra of protected PNiPAAM **4** (top) and polymer–peptide chimera **5** (bottom) after PNiPAAM deprotection and CuAAC reaction.

polymerisation.[27] PNiPAAM typically exhibits a temperature-response in water by changing its character from hydrophilic below the lower critical solution temperature (LCST), to hydrophobic above this temperature.[28] This LCST is strongly dependent on the molecular weight of the polymer, and short chain PNiPAAMs have been shown to exhibit hydrophobic properties at room temperature.[29] Polymerisation of NiPAAM, mediated by 3-(trimethylsilyl)prop-2-ynyl-2-(butylthiocarbonothioylthio) propanoate **3**, provided a low molecular weight polymer **4** (M_n = 2000 g mol^{-1}) with polydispersity below 1.2 (Scheme 2). Investigation of the LCST by DSC[28] revealed a transition temperature of 8 °C, thus confirming the hydrophobic character of the PNiPAAM chains at the temperatures of interest (ESI†). We chose to incorporate a TMS-protected alkyne in **4** to avoid potential side reactions during radical polymerisation. The silyl ether was removed in a subsequent step by treating with TBAF, providing the desired alkyne-terminated PNiPAAM with quantitative conversion as determined by ^{1}H NMR spectroscopy (ESI†).[30]

With the desired building blocks in hand, we next investigated the conjugation of alkynyl-PNiPAAM to tetrapeptide **1** using the CuAAC reaction. The reaction was conducted using CuBr and PMDETA in methanolic solvent and proceeded to completion after 60 h as confirmed by FT-IR, MALDI-TOF and size exclusion chromatography (SEC) analyses (Scheme 2). Analysis by MALDI-TOF clearly showed the successful formation of conjugate **5** (Fig. 1). SEC analysis confirmed an increase in molecular weight of the conjugate (ESI†). In addition, the ^{1}H NMR spectrum was consistent with the formation of the chimera with resonances present for polymer **4** and peptide **1** and an additional resonance at 8 ppm corresponding to the triazole ring proton in **5** (ESI†).

Identical CuAAC conditions were subsequently employed for the conjugation of the azidoeicosapeptide **2** to alkyne-functionalised PNiPAAM **4**. Formation of conjugate **6** was confirmed by ^{1}H NMR spectroscopy and HPLC analysis (ESI†). Unfortunately, the low solubility of **6** in SEC eluents prevented the use of this technique for characterisation.

The amphiphilic properties of chimeras **5** and **6** were exploited to generate self-assembled particles in water. The presence of aggregates in water was initially ascertained for **5** *via* dynamic light scattering (DLS). This revealed the presence of structures with an average diameter of 136 nm and high polydispersity. This suggested that the structures were non-uniform, a result confirmed by transition electron microscopy (TEM) imaging, which illustrated the presence of large and poorly defined aggregates (Fig. 2). The uncontrolled self-assembly of chimera **5** suggested that this construct would not be useful for the multivalent presentation of the immunogenic MUC1 tetrapeptide. In contrast chimera **6**, containing the full eicosapeptide tandem repeat sequence of MUC1, formed nearly uniform micelles with a size of 60 ± 3 nm, according to DLS measurements, and 20 nm according to TEM imaging. The difference observed between DLS and TEM measurements arises from the fact that DLS measures the hydrodynamic volume of fully hydrated micelles whilst TEM measures the diameter of non-hydrated particles, obtained after deposition on a slide and drying.[31] The TEM images of **6** in water clearly show well-ordered self-assembled particles and, given the hydrophobic nature of PNiPAAM, multiple copies of the MUC1 VNTR peptide are expected to be presented on the surface of the micelle. Given that **5**, bearing a tetrapeptide moiety, did not form well-defined micelles, it appears that a critical length of the hydrophilic peptide segment must be reached in order to synergise with

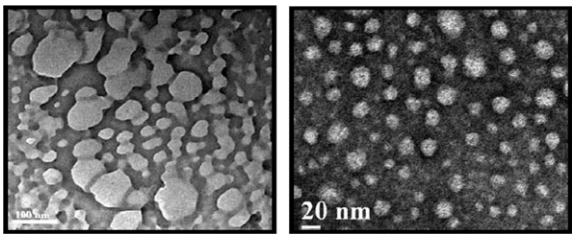


Fig. 2 TEM images of **5** (left) and **6** (right) after self-assembly in water. The scale bar on the images is 100 nm (left) and 20 nm (right).

the hydrophobic polymer segment to generate a self-assembled chimera.

For certain applications, the micelles obtained from the self-assembly of **6** may require further stabilisation to avoid loss of structure due to variations in conditions *e.g.* temperature, pH, *etc*. One of the key benefits of the RAFT process is that it offers polymeric chains end-capped with a thiocarbonyl thio group, which can be reduced to a thiol and utilised as a handle for further functionalisation of the resulting polymer chain end.[32] Since the thiol end group is located in the hydrophobic core of the micelles, we envisioned its utility in the covalent crosslink of aggregates in order to lock their structure. To investigate whether further functionalisation of the chimera end groups was viable, the thiocarbonyl thio groups of **5** and **6** were reduced to terminal thiol moieties using sodium borohydride. The resulting thiol-functionalised polymer–peptide chimeras (**7** and **8**) were subsequently reacted with pyrene maleimide (Scheme 2, ESI†).[32] Pyrene was chosen as an end group as it is non-fluorescent in aqueous solution until it is conjugated to thiols, thus offering a simple means to assess the success of the functionalisation.[33] The resulting tagged polymer–peptide chimeras **9** and **10** were analysed by fluorescence spectroscopy. The fluorescence emission spectra of **9** and **10** exhibited typical emission spectra for pyrene with λ_{max} emissions at 375 nm and 400 nm, thus indicating the thiol–maleimide conjugation reaction was successful (ESI†, Fig. S11).[33] A reaction yield of 33% was inferred by measuring the absorbance at 338 nm in methanol (ε = 40 000 cm^{-1} M^{-1} in MeOH, ESI†). These studies suggest that, while the chimera end groups can be functionalised, further optimisation is required before crosslinking is employed to stabilise such aggregates. This is currently being investigated in our laboratories.

In summary, well defined, low molecular weight alkyne-functionalised PNiPAAM polymers were conjugated to immunogenic peptides found within the VNTR sequence of the cancer associated protein MUC1 *via* CuAAC thus generating polymer–peptide chimeras. Chimera **6**, bearing the full length eicosapeptide repeat unit, formed well defined nanoparticles thus providing a multivalent display of the peptide epitope at its periphery. The versatility of the RAFT polymerisation–click approach was further demonstrated by modifying the RAFT end groups on the polymer–peptide conjugates to thiols, which were subsequently conjugated to pyrene maleimide, yielding fluorescently labelled biohybrids. The methodology developed here is currently being employed in our laboratories for the generation of stable, crosslinked glycopeptide–polymer chimeras for the generation of novel vaccine candidates.

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