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Stimulus responsive peptide based materials†

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In this *tutorial review* we give an introduction into the field of stimulus responsive peptide based materials illustrated by some recent and current developments. We have tried to categorize them according to the stimulus the materials are responsive to, being pH, temperature, metal ions, enzymes and light. Because we have focused on the structural changes that these stimuli effect we have further classified the topics according to the secondary structures that are involved. These changes in molecular structure in turn cause a change in the macroscopic properties of the material they constitute. It is believed that these materials, often referred to as smart materials, have a great potential being applicable in areas like drug delivery, tissue engineering and bio-sensors.

1. Introduction

Nature is an excellent source of inspiration in the design of stimulus responsive materials. Switching mechanisms found in proteins, where a response to environmental changes leads to conformational transitions, are particularly extensively studied. Proteins themselves are however not the most likely structures to prepare materials from due to their fragile nature and high cost of production. Therefore, peptides as a more synthetically accessible class of compounds have been evaluated as building blocks for stimuli responsive materials.^{1,2} Often a change in the peptide properties is caused by a conformational transition, leading to *e.g.* a spatial redistribution of hydrophobic and hydrophilic residues. Such an induced change can be employed

to control the self-assembly of peptides. These switchable peptides are considered as potentially useful in the field of tissue engineering, drug delivery, biosensing and so-called smart biomaterials, *i.e.* materials with well defined and controlled properties. The stimuli that can induce the desired changes are *e.g.* pH, temperature, metal ions, light, enzymes and ion concentrations. Due to the diversity in properties of their constituent amino acids, peptides are easily designed to behave as stimulus dependent materials. Moreover, by the incorporation of non-natural amino acids or other small organic moieties the scope can be extended even further.

The basic properties of peptides that ultimately also define their structure are determined by the primary amino acid sequence. The residues provide diversity *via* non-covalent and covalent interactions including hydrophobic interactions, aromatic stacking, hydrogen bonding, disulfide bridges and electrostatic interactions. The interactions of the individual amino acids are weak, but as an ensemble they can give rise to a stable secondary structure. The most common secondary

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structures are α -helices and β -sheets though other less abundant structures such as the 3_{10} helix and the π -helix also exist. Which secondary structure is formed depends on the primary structure as different amino acids have different secondary structure propensities. By folding into an α -helix, hydrogen bonds between backbone NH and C=O in residues i and $i + 4$ can be formed resulting in a stable secondary structure (Fig. 1A).

A tertiary structure that can arise from α -helices is the coiled-coil, often encountered in peptide-based materials, consisting of two or more α -helices that intertwine to form a stable structure. Peptides that adopt a coiled coil structure usually consist of repeats of seven residues. The residues in this heptad repeat labelled $a-g$ are usually hydrophobic (typically Leu, Ile or Val) at positions a and d (Fig. 2). Together these amino acids form a hydrophobic interface between the peptides providing most of the stability to the assembly. Incorporation of charged residues at positions e and g further stabilizes the

Fig. 1 Schematic representation of the most common secondary structures. (A) The α -helix and (B) the β -sheets, parallel and antiparallel. Figure adapted from Encyclopedia of Life Sciences 2002.

structure and provides directionality (*i.e.* parallel or anti-parallel association) and specificity to the interaction (hetero- or homo-oligomerization).³

In β -sheets a stable secondary structure is formed *via* backbone hydrogen bonds between two neighbouring peptide strands. The assembly of the β -strands which can be within or between peptides can be anti-parallel or parallel given by the directionality of the peptide backbone that interacts (Fig. 1B). Extended β -sheet formation may even result in amyloid formation, insoluble aggregates that are the basis for materials like spider silk but also diseases such as Alzheimer's. In the β -sheet conformation the side chains of the constituent amino acids stick out perpendicularly above and below the sheet in an alternating fashion, which is often exploited in the design of β -sheet based materials and their resulting properties. Another group of secondary structures are the turns of which the β -turn is most prevalent. Normally, the β -turn consists of four residues and gains stability from hydrogen bonds between residues i and $i + 3$ and often residues at the turning points of β -sheets. The difference in interactions between the residues in the various secondary structures provides the opportunity to

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Jan C. M. van Hest

Jan C. M. van Hest conducted his doctoral research on molecular architectures based on dendrimers under supervision of Prof. Bert Meijer, for which the PhD title was granted in 1996. As a postdoctoral researcher, he investigated the possibilities of protein engineering for the preparation of materials under supervision of Prof. David Tirrell. In 1997 he then joined the chemical company DSM, where he worked as research scientist and later on as group leader on the

development of innovative material concepts. In 2000 he was appointed as a full professor at the Radboud University Nijmegen to set up a new group in bio-organic chemistry, which focuses on bio-inspired hybrid materials and processes.

Fig. 2 Schematic representation of a coiled coil structure using a helical wheel. The letters indicate the different positions.

create triggerable structures by inducing changes in these interactions.

In the following sections we describe recent developments and challenges in the design of peptides, which change properties upon application of an external stimulus. The different systems are classified in the first place according to the kind of stimulus that is employed. These stimuli in most cases exert a structural change in the peptides (secondary structure) which in turn changes the interaction between the peptides (tertiary structure) and hence the properties of the material the peptides constitute. We have attempted to arrange the examples according to these secondary structures involved in this change. Finally, we treated amphiphilic peptides separately because we think they constitute a category of their own with their own particularities. Of course such a strict separation of the peptide based materials cannot always hold up. Furthermore, in this review we only provide an introduction into the field and certainly do not give an exhaustive account on the variety of stimulus responsive peptides that have been designed. We have tried to present some illustrative examples of the great variety of strategies that have been reported recently.

2. pH-switches

A plethora of switchable peptides are triggered by manipulation of the pH of the environment. This is often a consequence of protonation and deprotonation of basic and acidic amino acids.

α -Helices

Apostolovic and Klok created a pH-dependent system using a heterodimeric coiled coil consisting of two pH-sensitive peptides with an IAAL E3/K3 motif (IAALEKE, IAALKEK).⁴ The oppositely charged lysine and glutamic acid residues provide electrostatic interactions, and can therefore be used to manipulate the stability of the heterodimeric coiled coil. At pH 7 a heterodimeric coiled coil was formed, which upon lowering the pH to 5 led to unfolding of the E3/K3 coiled coils. The authors suggested that this system could be attractive for the development of non-covalently bound polymer therapeutics, especially since association and dissociation took place in the near-physiological range. Zhang and co-workers designed a series of peptides which undergo a reversible α -helix to β -sheet transition in a pH dependent manner.⁵ For this purpose several peptides with alternating hydrophobic/hydrophilic residues were examined in the search for peptides that form stable β -sheets. To impose pH sensitivity and propensity to form α -helices the peptides also had a negative charge clustered towards the N-terminus and a positive charge clustered towards the C-terminus. Their EAK-12d peptide (Table 1, entry 1) was found to form a stable β -sheet at pH 1–3. Increasing the pH to 5 led to a complete structural transition to a new conformation with 30% helical content. No conformational change was detected following a further increase in pH (up to 10). Conversion into an α -helix was a result of the negative charge clustered towards the N-terminus, due to deprotonation of the glutamic acid residues. The resulting dipole is one of the driving forces of the helix to form. In contrast other peptides like DAR16-IV (Table 1, entry 2) have been shown to undergo the opposite conformational change, *i.e.* the peptide forms a helical structure at pH 1–2 and a β -sheet at a pH of 5 or higher. In addition to pH, temperature could also be used to trigger the transformation for both these peptides. The transition from a β -sheet at 25 °C to an α -helix was detected for the EAK-12d peptide after increasing the temperature up to 85 °C. Conversely, the DAR16-IV peptide formed a β -structure at 25 °C and showed an abrupt conversion into an α -helix increasing the temperature up to 70 °C. Another example on peptides adopting both β -sheets and α -helices is provided by the group of Koksche. As a part of their research in Alzheimer's disease they designed a

Table 1 Peptides changing states depending on pH

Entry	Peptide	Sequence	Comment
1	EAK-12d	AEAEAEAEAKAK	α -Helix to β -sheet
2	DAR16-IV	ADADADADARARAR	β -Sheet to α -helix
3	VW19	LKVELKELKKELVVLKSELKELKKEL	Amino benzoyl N-capped
4	Unnamed	Ac-QATNTDGSTDYGLQINSR-NH ₂	Hydrogel formation below pH 12
5	P ₁₁ -2	Ac-QQRFQWQFEQQ-NH ₂	Gelation below pH 5
6	P ₁₁ -3	Ac-QQRFQWQFEQQ-NH ₂	Gelation below pH 10
7	P ₁₁ -4	Ac-QQRFWEFEQQ-NH ₂	Three different states, gelation below pH 3.2
8	P ₁₁ -5	Ac-QQXFXWFEQQ-NH ₂	Gelation above pH 7.8, X denotes an ornithine residue
9	RATEA16	RATRAEARATARA	Hydrogel at neutral pH, at low concentrations
10	C16-RGD	C ₁₅ H ₃₁ C(O)-CCCCGGGS(PO ₄ ²⁻)-RGD	Forms cylindrical micelles
11	C16-VEVE	C ₁₅ H ₃₁ C(O)-VEVE	Forms nanobelts
12	P1	Fmoc-VRGDV-COOH	Forms fibres and micelles
13	P2	C ₁₁ H ₁₉ O-NH-VRGDV-COOH	Forms fibres and micelles
14	P3	C ₁₂ H ₂₃ O-NH-VRGDV-COOH	Forms fibres
15	P4	C ₁₂ H ₂₃ O-NH-ERGDE-COOH	Forms micelles

model peptide VW19 (Table 1, entry 3), which was able to adopt three different secondary structures depending on pH and concentration of the peptide.⁶ At pH 4 or 7.4 and peptide concentrations below 250 μM only random coil could be detected by CD. However, at pH 4 increasing the peptide concentration promoted a conformational transformation that led to the formation of helically twisted β -sheet ribbons as shown with cryo-TEM. If the secondary structure of VW19 was studied at pH 7.4 rather than pH 4 (same concentration *i.e.* above 250 μM) the morphology of the assemblies changed into straight tubes with an α -helical coiled coil structure as determined with CD (Fig. 3). Presumably, protonation of the lysine residues disfavours formation of α -helices due to charge repulsion between *intra*-helical lysine residues, whereas greater spatial charge separation is possible by adoption of β -sheet structures. Unfortunately, the authors did not report whether a switch between preformed α -helices and β -sheets could be accomplished by changing pH. Also histidine can be employed in the design of pH-triggered peptide switches as the group of Conticello showed in their work. In their approach they introduced three histidine residues in a 41-residue peptide, TZ1H, containing (quasi-)heptad repeats of a trimeric coiled coil motif.⁷ Conticello and co-workers observed that below pH 5.8 the peptides existed as random coils, which upon a raise in pH changed conformation and associated into three-stranded helical bundles. This transition was reversible and protonation/deprotonation of the histidine residues is believed to provide the driving force. Furthermore, these bundles assembled into long fibres, which could be visualized by TEM.

Nature also exploits conformational peptide switches induced by changes in pH; the influenza virus protein hemagglutinin (HA) is one example on such a natural occurring pH switch. When an influenza virus infects target cells, the virus is first trapped in cytosolic endosomes, in which the virus would be degraded if it was not for the HA peptide in the virus envelope. At endosomal pH the HA peptide undergoes a conformational rearrangement of the N-terminus transforming a buried triple stranded coiled coil into an exposed hydrophobic loop-helix structure. This N-terminal loop-helix structure is known as the fusion peptide, since it is able to insert itself into the endosomal membrane inducing a fusion of the endosomal membrane with the virus envelope, leading to release of the viral RNA. Inspired by the HA fusion peptide Szoka and colleagues developed a synthetic 30-mer peptide called GALA containing four EALA repeats.⁸ The peptide was shown to adopt a random coil at pH 7 whereas at pH 5 the peptide

formed an amphipathic α -helix bearing a clear resemblance to the influenza fusion peptide. This α -helix is long enough to span a lipid bilayer and liposome studies showed that GALA could indeed insert into lipid membranes where it formed oligomeric pores of defined size causing leakage of the liposome contents. Also red blood cells could be lysed by GALA at pH 5.5 notwithstanding that GALA was part of a larger fusion-protein construct.⁹ These findings emphasize the great potential of GALA within development of drug delivery systems. That one is not restricted to this type of sequences was shown by Kashiwada *et al.*, who constructed another set of membrane fusion stimulating peptide responsive to pH.¹⁰

Coiled coils have also found their way into research on metallic nanoparticle materials as shown by Kokschi and co-workers. They employed a coiled coil forming peptide containing three LKELERK repeats and showed that binding of negatively charged gold nanoparticles to the coiled coils could be controlled by varying pH between 9 and 12.¹¹ At pH 12 the peptides are negatively charged disfavoring binding of the nanoparticles while a decrease in pH to 9 leaves a positive charge on the coiled coils triggering a formation of coiled coil-nanoparticle assemblies (Fig. 4).

Even more complex structures based on self-assembled α -helical peptides can be constructed as was shown by Aili *et al.*¹² They designed two helix-loop-helix 42-residue peptides that could after induced folding spontaneously form nanorings or fibrous structures. The monomers of the peptides do not display a particular fold but around neutral pH the peptides that are oppositely charged heterodimerize with a dissociation constant of 20 μM . The structure that is formed is a four-helix bundle that can further organize itself into fibres and rings. It is suggested such structures have potential applications in the areas of drug delivery and tissue engineering. In our opinion it is also indicative that this type of materials can be the starting point for the generation of much complex structures opening a path towards the generation of complexity that can be found in nature.

β -Sheets as secondary structures

In addition to α -helix based systems, β -sheet motifs have also been employed as the basis for pH triggered peptide switches. They have been studied extensively and can form hydrogels which are porous three-dimensional networks. The β -sheets give rise to fibrous assemblies (Fig. 5) that due to the presence of interactions between these fibres can cause crosslinks, which results in the formation of a gel. A characteristic and potentially useful property for these hydrogels is the breakdown of the networks under mechanical force and their subsequent recovery. Moreover, such gels provide good permeability and

Fig. 3 Cryo-TEM micrographs of the peptide, VW19. Three different conformations can be adopted by changing the pH and peptide concentration. (a) Peptide conc. 250 μM , pH 4.0—random coil; (b) Peptide conc. 600 μM , pH 4.0—helically twisted β -sheet ribbons; (c) Peptide conc. 600 μM , pH 7.4—helical fibres. Figure taken from ref. 6.

Fig. 4 Binding of gold particles upon acidification. At pH 12 the negatively charged coiled coils do not bind to the negatively charged gold nanoparticles. However, when the pH is lowered to 9, the charge of the coiled coils is reverted and the coiled coils bind to the gold particles. Figure taken from ref. 11.

Fig. 5 Formation of stable fibres starting from a monomer. Increasing the peptide concentration can result in formation of a hierarchy of supramolecular structures. Starting from helical tapes over twisted ribbons and fibrils, and finally the formation of fibres. Figure adapted from ref. 13.

mechanical support and therefore are often able to incorporate different molecules, which could be exploited in the design of triggerable drug release systems and self-healing materials.

Although proteins have also been shown to form functional hydrogels by self-assembly, research on hydrogel forming oligopeptides continues due to their synthetic accessibility, lower cost and higher stability. Pioneering research on this field was performed by the group of Boden. They found that by ensuring the presence of attractive forces between side-chains, lateral interaction between adjacent β -sheets and strong adhesion of solvent to the β -sheets, peptides could be designed to form hydrogels.¹⁴ They also discovered that some of their hydrogels were sensitive to extreme changes in pH, *e.g.* their peptide (Table 1, entry 4) formed a hydrogel below pH 12, however when pH was increased beyond 12 the hydrogel dissolved. Inspired by this pH sensitive hydrogel Boden and co-workers also developed a series of shorter peptides containing pH responsive residues (arginine, glutamic acid and ornithine; Table 1, entries 5–8). P₁₁-2 formed a stable nematic gel consisting of fibrils at pH values lower than 5, whereas above pH 5 flocculation occurred making the gel unstable.¹³ Presumably this pH sensitivity is a result of overall charge of the peptide; below pH 5 the glutamate residues become protonated leaving the peptides with a net positive charge (arginine), which serves to stabilize the fibrillar dispersion. In contrast, when P₁₁-2 is uncharged (above pH 5) the inter-strand interactions (hydrophobic interactions between Phe–Phe and Trp–Trp as well as hydrogen bonding between Gln–Gln) become too strong and flocculation takes place.

Due to the effect of the glutamate residues on the pH sensitivity of the hydrogel Boden and co-workers also made a peptide, in which glutamate was substituted with glutamine (P₁₁-3). This peptide formed nematic hydrogels almost independent of pH; only above pH 10 flocculation could be observed, apparently due to deprotonation of the arginine residues (albeit at 2.5 pH units lower than expected based on the pK_a value of free arginine). By incorporation of three glutamic acid residues (P₁₁-4) a peptide was developed, which could adopt four different phases depending on pH: below pH 3.2 this peptide formed a nematic gel, between pH 3.2 and 5.0 flocculation occurred, between pH 5.0 and 7.0 a nematic fluid prevailed and above pH 7.0 the peptides formed an isotropic fluid. The existence of four different phases could roughly be ascribed to four differently charged species (Arg^+ ; Arg^+ and Glu^- ; Arg^+ and 2Glu^- ; Arg^+ and 3Glu^-).

By incorporating three ornithine residues in their model peptide (P₁₁-5) Boden and co-workers were able to expand their repertoire of pH switching hydrogels with a peptide that forms nematic gels at high pH. Within the pH interval of 7.4–7.8, this peptide converted from an isotropic fluid into a nematic gel. Interestingly, by mixing the two oppositely charged peptides, P₁₁-4 and P₁₁-5 in equal amounts at pH 7.2 to 7.4 (both peptides are monomeric in this interval) a nematic hydrogel was obtained instantaneously.¹⁵ Once formed the gel turned out to be stable over a very wide pH-interval (pH 1–12); leaving the gel above pH 12 for several days, a gel of P₁₁-5 was detected while P₁₁-4 was present as a monomeric anionic peptide in a solution.

The group of Tan employed a strategy similar to the one described above. They showed that their RATEA16 peptide (Table 1, entry 9) formed a hydrogel with a very high water content (>99.5%) around neutral pH.¹⁶ Below pH 3.5 the RATEA16 peptide existed as monomers due to protonation of the glutamate residues leaving a net positive charge of +4 on the peptides leading to inter-strand repulsion. Above pH 12.5 deprotonation of the arginine residues started taking place leaving the peptides with no net charge. This led to aggregation, presumably promoted by hydrophobic interactions between alanine residues. However, at neutral pH the glutamate and arginine residues are all charged giving rise to electrostatic attraction between arginine and glutamate residues as well as inter-strand repulsion due to a net charge of +2. This combination of attractive and repulsive forces ensures that the peptides interact and disperse well respectively. To explore the potential of this reversible three-phase system (solution–hydrogel–precipitate) vitamin B1 was incorporated into the hydrogel. The fibrous network contained pores in which the vitamin was entrapped. By acidifying the medium vitamin B1 was released faster than at neutral pH. Schneider's group developed a different hydrogel self-assembly system based on β -hairpins.¹⁷ To ensure formation of a hairpin they integrated a tetrapeptide, known to adopt a type II' loop, into a series of alternating Lys and Val residues, which favour a β -sheet conformation (Fig. 6, top). This peptide, MAX1, existed as a random coil below pH 5.5, however upon alkalization to pH 9.0 a reversible transition into a β -sheet as well as gelation was observed. This transition is, most likely, a result of deprotonation of the lysine residues, which are protonated at low pH resulting in intra-strand repulsion; upon deprotonation the repulsion is relieved and intra-molecular hydrogen-bonds can form,

Fig. 6 Secondary structure of the MAX1 peptide and its self-assembly into hydrogels. Pathway a shows the self-assembly process if the hairpin contains a *trans*-prolyl amide geometry. Pathway b shows hetero-conformer self-assembly formed when the hairpin contains a *cis*-prolyl amide geometry. The β -branched valine at position nine (a normal α -amino acid is shown) is believed to force the proline into a *trans*-prolyl amide conformation. Figure adapted from ref. 17.

yielding a β -hairpin. Assembly of a gel from monomeric hairpins is probably, as suggested by the authors, due to formation of lateral hydrogen bonds and facial hydrophobic interactions. Importantly, a control peptide VKVKVKVK-NH₂ did not show any sign of gelation when pH was increased suggesting that formation of β -hairpins is essential for gelation to take place (Fig. 6, pathway a *versus* pathway b).

Besides change in pH, Schneider and co-workers also studied the effect of temperature on β -hairpin formation of MAX1 as well as the effect of altering the net charge of the peptide.¹⁸ For unmodified MAX1 they saw that at pH 9.0 folding was impeded at temperatures below 20 °C and favoured above 25 °C. In addition an increase in pH (pH 9.7) or decrease (pH 8.0) led to a decrease in the transition temperature or no folding at all, respectively. This implies that the net charge of MAX1 has a crucial role since an increase in pH (and thus a decrease of the overall charge) correlates with a decrease in transition temperature; *i.e.* it requires less energy to fold the hairpin when the overall charge is small and the electrostatic repulsion is minimal. To investigate this charge effect further, the lysine residues in MAX1 were point-mutated into glutamate one-by-one. All MAX1 (Lys $\rightarrow$ Glu) mutants—having a lower overall positive charge—folded into β -hairpins at lower pH relative to the parent MAX1 peptide confirming that the net charge has an impact on folding of the β -hairpin. Interestingly, the position of the mutation had a big effect on the pH value required for folding; positions 8 and 13 exhibited the smallest effect (pH 8–9), whereas position 4 (pH 7.0) caused a drop of almost two pH units relative to the parent MAX1. These findings demonstrate clearly how careful peptide design can be employed to tune the pH and temperature at which hydrogel gelation occurs.

In an application-oriented study by Banerjee *et al.* hydrogels were used to remove toxic dyes from wastewater.¹⁹ They found that Boc-protected tripeptides (Fig. 7) formed anti-parallel β -sheets and hydrogels at pH 11.5–13.5. Additionally, these hydrogels were shown to absorb toxic dyes such as

Fig. 7 Boc-protected tripeptides used for removal of dyes from wastewater. These peptides form a hydrogel at pH 11.5 to 13.5.

Rhodamine B, Reactive Blue 4 and Direct Red 80 from wastewater efficiently—even from highly diluted aqueous solutions. Presumably, the hydrophobic residues are responsible for sequestering the organic dyes *via* hydrophobic interactions. Subsequently, the peptides could be salvaged by adjusting the pH to 7.5, which precipitated the peptides selectively, *i.e.* free of any dye.

Peptide amphiphiles

A different group of pH responsive self-assembling peptides are the peptide amphiphiles. These peptides consist of a hydrophilic part (hydrophilic amino acids) and a hydrophobic part (hydrophobic amino acids or hydrophobic non-amino acid elements).²⁰ Pioneering research on this topic was performed by the Stupp group. In their research on mineralization of fibres to prepare bone-like assemblies they developed a lipidated peptide C16-RGD (Table 1, entry 10) that was able to

self-assemble in a pH dependent manner into cylindrical micelles forming self-supporting gels.²¹ Below pH 4.0 they showed that the peptides associate in long fibres resembling a cylindrical micelle, in which the C16 palmitoyl tails pack against each other forming the interior of the micelles. An increase in pH led to a reversible disassembly of the fibres, however upon oxidation this reversibility was removed and the fibres became unaffected by changes in pH, most likely due to formation of inter-strand disulfide bonds. Reduction restored the reversible pH controlled assembly/disassembly demonstrating the high degree of control this system allows over the assembly process. Stupp and co-workers also showed that the shape of nanostructures can be pH dependent.²² The peptide C16-VEVE (Table 1, entry 11) assembled into belt-like structures (termed nanobelts by the authors) at low pH with lengths of tens of micrometres and widths of 150 nm. Presumably this stacking was possible due to protonation of the glutamate residues alleviating electrostatic repulsion. When the pH was increased the width of the belt-like structures decreased from 150 nm to 50 nm and TEM revealed a grooved surface instead of a smooth surface as seen at low pH. The authors propose that deprotonation of the glutamate residues leads to separation of the multilayered structures and folding of the resulting bilayers (generating the grooves) due to inter-strand electrostatic repulsions.

Shorter peptide amphiphiles are also able to change morphology when pH is varied as demonstrated by Zhuo's group. They prepared four different peptide amphiphiles (Table 1, entries 12–15) based on the cell-adhesion RGD motif.²³ P1 and P2 formed interwoven fibres at pH 4; however, when the pH was increased from 4 to 7, the fibres transformed into large vesicular structures containing β -sheet structural elements making the solution turbid. Deprotonation of the carboxylic acid moieties is believed to cause too much inter-strand repulsion for the interwoven fibres to exist; whereas β -sheet packed vesicles are less affected by the repulsion. P3 and P4 on the other hand formed fibres and micelles,

respectively, over the entire pH range (pH 4–11), and did not show any sign of pH response. P4 with an additional glutamate residue is most likely too charged over the pH interval to form fibres, whereas the explanation for the lack of micelle formation of P3 is incomprehensible, since the structural similarity to P2 is so striking that a similar behaviour would be expected.

3. Temperature

Peptide folding is sensitive to temperature changes and therefore temperature is often utilized to trigger a change in peptide conformation. Increasing the temperature often leads to unfolding of the secondary structure and therefore disruption of function. Heat-induced denaturation can also be a powerful tool to obtain detailed information on the thermodynamic stability of proteins.

α -Helices as secondary structures

Elevated temperatures facilitate aggregation of many proteins. α -Helix to β -sheet transitions are often temperature triggered, mainly determined by the hydrophobic/hydrophilic pattern in the helical structure. It was shown for certain peptides that changing the non-interacting region (position *f*) of α -helical coiled coils can lower the thermal energy necessary for the transition into amyloid-like β -sheets.²⁴ The transition temperature was lowered from 76 °C to 55 °C by substituting a serine for a tyrosine at the *f* position (Table 2, entry 1). The change in hydrophobicity was the most important characteristic of the substituted amino acid. The amino acid at the *f* position is uniquely situated opposite the hydrophobic amino acids at positions *a* and *d* in an α -helix while in a β -sheet conformation these three hydrophobic amino acids cluster on one side. It should be noted, however, that the temperature drop might also partially be a result of the higher propensity of serine as compared to tyrosine to fold in an α -helix. Another temperature triggered structural change was the change in handedness of an α -helix from right handed to left handed as found by

Table 2 Peptides changing states depending on temperature

Entry	Peptide	Sequence	Comment
1	Change S for Y in a coiled coil	EIAQLESEISQLEQ $\rightarrow$ EIAQLEYEISQLEQ	Decrease melting temperature
2	Helical peptide	H- β -Ala- Δ^Z Phe-Aib- Δ^Z Phe-L-Phe-(Δ^Z Phe-Aib) ₂ -OCH ₃	From left- to right-handed helix upon heating
3	Synthetic polymers with coiled coil motive		From gel to collapsed gel upon heating
4	DARV16-IV	ADADADADARARARAR	From β -sheet to α -helix upon heating
5	ELP (VPGXG) _n	X either V, I, L or F, <i>n</i> between 1 and 5	From random coil to β -spiral upon heating
6	ELP with lysine	VPGKG(VPGVG) _n , <i>n</i> = 6 or 16	From gel to contracted gel upon heating
7	ELP VPGXG	SKGPG(VGVPGVGVPGGGVPGAGVPGVGVPGVGVPGVGVPGGGVPGAGVPGGGVPG) ₁₅ WP	Accumulates in tumor
8	L12-ELP-CPP	ELP (VPGXG) _n met X = V : G : A 5 : 3 : 2, <i>n</i> = 150. Tat as CPP	Delivery to cancer tissue
9	ELP (VPGXG) _n	X = V : A : G 5 : 2 : 3 or V : L : G 5 : 2 : 3, <i>n</i> = 90	Selective positioning on a surface
10	ELP (VPGXG) _n	X = V : A : G 5 : 2 : 3, <i>n</i> = varying	Purification
11	ELP block copolymer	E(VPGEG(IPGAG) ₄) ₁₄ -VPGEG-(VPGFG(IPGVG) ₄) ₁₆ -VPGFG	From random structure to micellar upon heating
12	ELP triblock copolymer	See Fig. 11	From micelles to compacter micelles upon heating
13	Beta sheet fibre	Pentacosadiynoic-GANPNAAG-OH Tricosadiynoic-GANPNAAG-OH	From β -sheet to random coil upon heating

Fig. 8 The helicity of this peptide changes upon adding Boc-D-amino acids and changing the temperature.

Komori and Inai.²⁵ The peptide used for these studies is depicted in Fig. 8. They demonstrated that temperature was an effective external stimulus to change the chirality of the helix. The nona-peptide preferred a right-handed helix with a small imbalance at room temperature in the presence of a Boc-D-amino acid that can interact at the N-terminus of this peptide. Cooling the solution to 5 °C induced a left-handed helix, while heating again resulted in the original right-handed helix (Table 2, entry 2). This system could be a good starting point in the design of peptide based 'chiroptical switches' driven by a chiral seed and temperature. Chiroptical switches, with their chirality change upon illumination with a defined wavelength, may be used both for data storage, but also, in supramolecular chemistry and nanotechnology to control structure and function.

Temperature can also be used to control the formation of hydrogels.²⁶ A hybrid hydrogel system was constructed from water soluble synthetic polymers combined with a well-defined coiled coil protein folding motif. Increasing the temperature from 25 °C to 70 °C induced a collapse of the gel to 10% of its initial equilibrium volume with a mid-point transition temperature of 39 °C (Table 2, entry 3). The temperature collapse occurred due to the cooperative conformational transition of the coiled coil protein domain. As the temperature increased the protein unfolded resulting in a decreased radius of the original molecules which caused the gel to collapse. This 'smart' system was proposed to be potentially useful for responsive biomaterials or drug-delivery systems.

β-Sheets as secondary structure

The conversion of a β-sheet into an α-helix upon increasing the temperature has been demonstrated by Zhang and Rich.²⁷ Their peptide, with alternating hydrophobic and hydrophilic residues, was initially shown to be a peptide that changes its conformation upon variation in the pH, however, this peptide can also change its conformation upon temperature changes (Table 2, entry 4). At room temperature the peptide displayed a β-sheet conformation. After increasing the temperature to 75 °C for 32 minutes a transformation into an α-helix conformation took place without going through a detectable random-coil transition state. The 32 minutes period for the transition to take place was thought to be rather long, indicating a thermal disruption of the β-sheet lattice in order to free the peptides took place over time. Moreover, once the α-helix was formed it took weeks at room temperature before the β-sheet conformation was formed again. The transformation from β-sheet to α-helix was accompanied with a 50% reduction in length from 5 nm to 2.5 nm in this 16 residue peptide. Therefore this system might be useful for biomaterials designed as an actuator set off by environmental temperature changes.

Frequently used temperature responsive structures are the elastin-like polypeptides (ELPs), composed of oligomeric repeats of the pentapeptide sequence Val-Pro-Gly-X-Gly, with X being any amino acid except Pro. Such peptides are inspired by the amino acid sequence of natural elastin and display a so-called lower critical solution temperature (LCST) behaviour.²⁸ Below the LCST ELPs are random coil structures soluble in aqueous media whereas above the LCST a transition takes place into a β-spiral conformation resulting in contraction, desolvation and aggregation of the polypeptide, leading to precipitation eventually. This process is reversible and can take place in a very small temperature window, often within 3 °C. The LCST is dependent on the amino acid sequence, molecular weight, ELP concentration, ionic strength and pH. In one study peptides were used with a Val at the X position with varying lengths from one to five pentapeptide repeats.²⁹ Three other peptide groups were synthesized as well, in which some of the valine residues at position X were substituted with Ile, Leu or Phe. All these peptides were composed of three pentapeptide repeats with a varying number of substituted valine residues at the X position. Almost all these structures showed a transition from random coil to β-spiral upon increasing the temperature (Table 2, entry 5). The trend that was observed indicated that the LCST was found to decrease with increasing size of the R group of the substituted amino acid and upon increasing number of valine substitutions for isoleucine, leucine or phenylalanine. Yet, a clear correlation between the secondary structure and apparent LCST with increasing number of substitutions was not found. It was implied that within one family of homologues the LCST is mainly determined by the hydrophobicity of the peptides. In contrast, the LCST between the different families with identical chain length showed no correlation. This indicated that hydrophobicity is probably not the only factor determining the LCST behaviour, and other factors like conformational preference of the substituted amino acids also play a role. A temperature dependent hydrogel constructed from ELPs was prepared by Chilkoti *et al.*, who investigated this kind of systems elaborately.³⁰ For their experiments 'residue X' was chosen to be valine, except for every 7th or 17th pentapeptide, in which position a lysine was built in (Table 2, entry 6). It was shown that the size and the stiffness of this kind of hydrogel can change upon changes in the temperature. At low temperature the hydrogel was elastic. Intermolecular cross-links between the lysine residues were formed upon reaction with trisuccinimidyl aminotriacetate. At higher temperatures the gel contracted losing water due to intermolecular interactions between the condensed polypeptide segments. When crosslinked at this temperature, more intramolecular and non-functional cross-links were formed which weakened the gel (Fig. 9).

At low ELP concentrations with low molecular weight polymers and a low lysine content (low possible amount of crosslinks), a significant loss of viscous energy upon increasing the temperature was detected. These kinds of well-defined gels were suggested to be applicable in tissue engineering and micro actuation.

One application of this kind of materials was demonstrated in thermally targeted drug delivery to solid tumours, by using ELPs with an LCST between body temperature and the temperature in the locally heated tumour region.³¹ A thermally responsive ELP with a mixture of glycines, alanines and valines at the X position was designed to have an LCST around 41 °C. It was shown that this oligopeptide was accumulated twice as much in heated tumours as compared to the same ELP without hyperthermia (Table 2, entry 7). There are two reasons why a higher temperature (41 °C) for delivery is more efficient. Firstly, the ELP will undergo a reversible phase transition, and secondly, hyperthermia itself increases delivery to the tumour due to an increased permeability and a higher perfusion of tumour vasculature. Also the delivery of systematically injected polymer–rhodamine conjugates to human ovarian tumours implanted in the dorsal skin of nude mice was shown to be possible *via* this system. In another recent application a macromolecular carrier was found that was able to deliver a lactoferrin-based peptide (L12) in a thermally responsive manner.³² L12 induces apoptosis in cancer and as such inhibits cell growth of a tumour. Three functional moieties can be distinguished in the designed system: L12, an ELP and a cell penetrating peptide. Cell penetrating peptides (CPP) are short peptides that can aid in the transport of various cargos across the cell membrane. For these experiments the HIV-derived Tat peptide was chosen as CPP. The ELPs consisted of a polypeptide of 150 pentapeptide

repeats with at the X position Val, Gly or Ala at a ratio of 5 : 3 : 2. It was shown that thermally activated Tat-ELP-L12 can target cancer cells selectively *in vitro* and induce apoptosis and necrosis in that place (Table 2, entry 8).

ELPs are not only useful for drug delivery, but these peptides can also be employed in assembly and immobilization experiments, *e.g.* in microreactors. Positional functionalization inside microreactors can be temperature controlled with the help of these kinds of peptides using a temperature gradient to selectively deposit two ELP-fusion proteins (ELPs fused with EGFP and DsRed2).³³ The peptides used in these studies consisted of 90 pentad repeats. X was chosen to be a combination of Val, Ala and Gly in a ratio of 5 : 2 : 3 for one ELP (ELP1). For another (ELP2) a more hydrophobic sequence was chosen, namely with Val, Leu and Gly in the same ratio (Table 2, entry 9). The reactor included a glass slide functionalized with ELP2 to act as a hydrophobic base for the temperature dependent positioning of the ELP fusion proteins. When a temperature gradient was applied over the glass slide, local precipitation took place depending on the LCST of the ELP connected to the protein. ELP2–DsRed2 precipitated at 40 °C in the beginning of the micro-channel, while ELP1–EGFP precipitated at 55 °C at the end (Fig. 10). The transition behaviour (LCST) could be further controlled by varying salt concentrations (NaCl) and the amino acid composition, making this system even more flexible and hence more applicable.

ELPs are also efficiently applied in the purification of proteins (Table 2, entry 10). Using protein engineering the desired protein was covalently coupled to an ELP tag, leading to a so-called fusion protein. As described before ELPs aggregate at the LCST and give precipitates that might have sufficient mass to be isolated by centrifugation. Since aggregation due to temperature transition is reversible for ELPs, these

Fig. 9 ELP hydrogel at different temperatures in the presence and absence of applied strain. At lower temperature (7 °C) more functional cross-links were formed and so the gel was more elastic. At higher temperature (37 °C) the functional cross-links were weakened and more non-functional cross-links were formed. This resulted in the loss of viscous energy and water which afforded again a less elastic gel. Figure taken from ref. 30.

Fig. 10 Positional assembly of two different ELP fusion proteins due to their LCST. The light grey arrow indicates the flow direction. (A) The glass slide in the micro-channel is functionalized with ELP2. (B) ELP2–DsRed2 fusion protein was transferred through the micro-channel. At a temperature of 40 °C it precipitated, and so positional functionalization took place. (C) ELP1–EGFP fusion protein was transferred through the micro-channel. This protein precipitated at 55 °C making positional functionalization possible. Figure taken from ref. 33.

complexes can be resolubilized once the temperature is lowered again. This purification method was termed inverse transition cycling (ITC) by Meyer *et al.*³⁴ The ELP fusion protein aggregated by either increasing the temperature above the LCST or by depressing the LCST *via* the addition of NaCl, which facilitates purification by centrifugation. After resolving the protein, site specific proteolysis afforded the free target protein *via* another round of ITC. This system was shown to be effective for the purification of thioredoxin–ELP fusion protein. Reducing the molecular weight of ELP resulted in a higher thioredoxin–ELP fusion protein expression by *E. coli*. Decreasing the ELP tag to 9 kDa resulted in expression levels comparable to the expression level of free thioredoxin by *E. coli*. As a result, a more complex phase transition was observed for the fusion proteins with these small ELP tags. The selection of an appropriate combination of salt concentration and temperature was necessary to favour the formation of large aggregates.

Peptide amphiphiles

A commonly used structure for encapsulation of small molecules and proteins for drug delivery are polymer nanoparticles.³⁵

An amphiphilic block copolymer was derived from ELPs that can undergo thermo-reversible segregation of the hydrophobic block in aqueous solution resulting in biocompatible nanoparticles. At a temperature above the LCST, a spontaneous phase separation took place (Table 2, entry 11). The amphiphilic block copolymer used for these studies contained at position X of the ELPs either a Val, Glu, Phe or Ala residue. In contrast to synthetic homopolymers and random copolymers of ELPs, this peptide did not precipitate in aqueous solution above the LCST. It was shown that entropy-driven dehydration of the hydrophobic block in aqueous solution at temperatures higher than the LCST took place, allowing the reversible formation of micellar structured nanoparticles. Due to the formation of these micelles at different pH and temperature conditions and their biocompatibility, these micelles are attractive for controlled delivery and release applications. In another study Sallach *et al.* prepared triblock copolymers consisting of three different ELP forms (Fig. 11A).³⁶ Normally this type of ELP consists of 10% α -helix, 35% β -strand and 55% disordered conformations. In this case the helix was a polyproline II (PPII) conformation. The ratio of the secondary structures was found to be largely dependent on the temperature, concentration and time. The PPII conformation is preferred at a lower temperature and concentrations while the β -sheet conformation is more abundant at a higher temperature and concentrations (Table 2, entry 12). The obtained micellar nanoparticles displayed a core–corona structure. Upon increasing the temperature a transition from helix to sheet took place which led to an abrupt increase in micelle compactness with a reduction in particle size (Fig. 11B).

The assembly and disassembly of amphiphilic peptides can be dependent on relatively small structural features of the peptide.³⁷ A relatively big difference in assembly was detected between two peptide amphiphiles consisting of an alkyl tail comprising 23 or 25 carbon atoms both with diacetylene functionality in the tail, while the peptide sequence was the same for both, *i.e.* GANPNAAG. It was shown that the transition temperature for the peptide with the two carbons shorter tail was approximately 50 °C, while the peptide with the longer tail showed a transition at approximately 80 °C. The longer peptide reassembled upon cooling in contradistinction to the peptide with the shorter alkyl tail, which only reassembled if it was allowed to stand overnight at temperatures lower than 40 °C. Since the only difference is at the end of the alkyl tail, which is more than 10 Å away from the β -sheet forming peptide, this result was found to be remarkable and said to be indicative of how intricate the behaviour of such molecules can be (Table 2, entry 13).

4. Metal ions

In nature metals often play a crucial role in proteins catalyzing important processes like photosynthesis and oxidation. Also in smaller tertiary peptide motifs metals are important, as can be seen for example in so-called zinc fingers. In this motif, that is part of over 1000 human transcription factors, zinc stabilizes an α -helix that binds to the major groove of DNA. Nearly one-fourth of the characterized proteins from nature contain metal ion binding sites. Moreover, metal ions are ubiquitous in

Fig. 11 Peptides inside micelles can change their secondary structure upon temperature changes and thereby the micelles' density. (A) The triblock copolymer sequence used for these experiments. (B) Schematic representation of the folding of the different micelles structures. Figure adapted from ref. 36.

body fluids and cell culture media and therefore metal ions have great potential as a chemical trigger in drug delivery systems. The function of these metal ions is often to induce a conformational transition from an unfolded to a folded state, which is possible because of their interactions with the side chains of different amino acid ligands within a polypeptide. The majority of metals bind to N-heterocycles, like histidine, since these cyclic structures easily coordinate to the metals *via* their lone pair on the nitrogen.

α -Helices as secondary structures

In an attempt to conformationally switch from a random coil to an α -helix the effect of a palladium compound on an eight-residue peptide (HAAHHELH) was investigated (Table 4, entry 1).³⁸ The peptide had its histidine residues methylated at the N3 position to avoid interference by binding to this less preferred position. It was shown that upon addition of the metal a transition took place changing the secondary structure from random coil into a turn. In time the secondary structure gradually changed into the kinetically more preferred α -helix. It was suggested that the turn might lower the activation energy and so assist folding into the thermodynamically stable helical structure. The rate and extent of α -helix folding could be regulated by changing the solvent and metal coordination sites. Of the sequences HXXXH and HXXXH, common metal binding domains in proteins, it was found that HXXXH favoured a helix, while HXXXH gave metalocycles. Methionines can bind similarly to metals, as described by Ma *et al.*,³⁹ reporting a stronger helix for MARAM as compared to HAAH (Table 4, entry 2).

Cerasoli *et al.* showed that an elegantly designed coiled coil peptide displayed a structural duality depending on the presence of metal ions (Table 4, entry 3). In a metal free medium a coiled coil was formed while after the addition of metal ions a classical zinc finger motif was adopted as tertiary structure (helix-loop-helix).⁴⁰ Also Rossi *et al.* designed a system that folds into a helix-loop-helix motif.⁴¹ Typically, now in the absence of zinc ions an helical conformation was found to be present to afford a trimeric α -helical coiled coil, while in the presence of zinc the less structured conformations were detected (Table 4, entry 4). This time it was not histidine responsible for the metal binding but the unnatural amino acid

ATANP (Fig. 12). 42-mer peptides were examined with either two or four ATANP residues at different positions. The ATANP residues were placed in positions *i* and *i* + 4 to allow the lateral arms bearing the ligand to face each other at the same side of the helix. The peptides with a helix-loop-helix tertiary structure dimerized in solution at higher concentrations (200 μ M) to form a four-helix bundle. The system was pH dependent as well because the amines of the ATANP can be protonated, occupying the lone pairs and thus impairing metal complexation. Hence below pH 7 a disruption of the helix-loop-helix structure and thus of helical content was observed, even in the presence of zinc.

α -Helical peptides can also be triggered to form self-assembled helical fibres upon the addition of silver(I) as was shown for the *de novo* designed peptide TZ1H (Table 4, entry 5).⁴² Three sets of proximal histidine residues were responsible for the interaction with the metal ion affording a trimeric coiled coil structure (Fig. 13). The peptide could accommodate silver ions within its binding sites. Although silver ions effectively induced this conformational transition, other ions isovalent with silver such as zinc(II), copper(II) and nickel(II) did not, regardless of the concentration used. This is probably due to the different coordination geometry these ions prefer, hampering a good fit in the trimeric coiled coil structure.

Pires and Chmielewski were able to assemble a triple helical peptide reversibly employing metal ion stimuli to form a collagen-like macromolecular structure.⁴³ The triple collagen helix was provided with distinct metal binding ligands at both termini and was shown to assemble in the presence of transition metal ions resulting in microfloretes of significant

Fig. 12 The zinc binding amino acid ATANP.

Fig. 13 A schematic representation of the tertiary trimeric coiled coil that the TH1Z peptides form upon adding silver(i) ions. The lateral layer of histidine residues, that can be formed between these three helices in this structure, is probably responsible for the self-assembly into trimeric coiled coils. Figure taken from ref. 42.

size and distinct shapes (Table 4, entry 6). Microflorettes are flower-like shapes that some peptides tend to form after self-assembly. The central collagen-based core consisted of a nona-repeat of the tripeptide Pro-Hyp-Gly, with Hyp being hydroxyproline. Each terminus was functionalized with a distinct metal binding ligand: two histidines at the C-terminus and a nitrilotriacetic acid (NTA) group at the N-terminus. The differentiation was necessary to ensure a head-to-tail coupling in order to maintain a correctly ordered Pro-Hyp-Gly repeat, although the authors could not completely exclude interference from head-to-head or tail-to-tail couplings. The triple helix formation of the individual strands led to clustering of six histidine residues at one end, and three NTA groups at the other end. The formation of microflorettes was detected for the metals Zn(II), Co(II), Ni(II), and Cu(II) and was reversed upon adding EDTA. The molar ratio between the individual helices and the metal ions had a significant effect on the assembly process and the morphology of the structures that formed (Fig. 14). The assembly process went *via* multiple intermediates, starting in an amorphous state, going through a curved sheet and finally yielding the microflorettes. Since this process was controlled precisely and took place at room temperature in a neutral solution, it was suggested by the authors that it might be useful for a range of biomedical and drug delivery systems.

Peptide-based materials that mimic some aspects of three-dimensional matrices of cells, enabling cellular encapsulation and cell growth, can be useful in the field of tissue engineering

and regenerative medicine. In this context, Pires *et al.* showed that these metal triggered self-assembling collagen peptides could be decorated with multiple functionalities affording a hybrid three-dimensional scaffold.⁴⁴ A peptide, named NHbipy satisfied three important aspects that are necessary in such three-dimensional matrices (Table 4, entry 7). First, the core was composed of monomers capable of self-assembling upon the addition of metals. Second, the trigger was compatible with living cells and finally the system was reversible under mild conditions. The peptide used for these experiments consisted again of nine repeats of Pro-Hyp-Gly and three distinct metal binding units, NTA at the N-terminus and a His₂ at the C-terminus to ensure that it had similar physical and biomechanical properties as natural collagen. However, this time a bipyridine moiety was incorporated in the side-chains in the fifth group of the tripeptide Pro-Hyp-Gly, affording another metal ion binding site in the middle. Several metals [Zn(II), Co(II), Ni(II), and Cu(II)] were able to cross-link the α -helices resulting in fibres. Specifically Zn(II) appeared to generate a less dense and more fibrous scaffold. Disassembly was once more promoted by the addition of EDTA. To demonstrate the modular nature of the system it was shown that the bipyridine moiety could be easily replaced with a biotin handle. Finally, it was shown the matrix did not display any cytotoxic effects towards human endothelial cells that were encapsulated. Another simultaneous change in conformation and aggregate morphologies upon adding transition metal ions has been reported by Koksche *et al.*⁴⁵ Two different kinds of α -helical peptides were used for these studies, with histidine residues again responsible for metal binding (Table 4, entry 8). In the first peptide (1), the histidine residues were placed two residues ($i, i + 2$) apart from each other, while in the second peptide (2) the histidine residues were placed two more residues ($i, i + 4$) apart. The positions *b*, *c* and *f* were solvent exposed and were thus expected to have no influence on the coiled coil formation. These residues were substituted by β -sheet preferring amino acids (three valine residues), resulting in a competition between an α -helical coiled coil structure and a β -sheet rich amyloid-like structure.

Upon the addition of either Co(II) or Zn(II) a difference between the two metal ions was detected in the conformation of peptide 1. In the absence of metal ions peptide 1 formed fibrils. In the presence of Co(II) the peptide unfolded, while in the presence of Zn(II) no change was observed. On the other hand, for peptide 2 no discrimination between Co(II) or Zn(II) ions was observed. In the absence of metal ions peptide 2 formed twisted fibrils that contained four or more protofilaments as can be expected for such amyloid-like structures. In the

Fig. 14 Scanning electron microscopy images of a helical peptide showing the effect of metal ion concentration on the morphology of microflorettes (1 mM). Different concentrations of ZnCl₂ give different morphologies: (A) 200 μ M, (B) 400 μ M, (C) 600 μ M, (D) 800 μ M, and (E) 1 mM. The scale bar is 5 μ m. Figure adapted from ref. 43.

presence of either of the two metal ions helix stabilization took place, leading to inhibition of amyloid formation. Another system that displays two interfacial states that could reversibly and actively be switched has been reported by Dexter *et al.*⁴⁶ A first peptide (**3**) contained two histidine residues responsible for the metal binding, while a second peptide (**4**) contained four metal binding residues (Table 4, entry 9). The peptides were designed to form an α -helix with one hydrophobic and one hydrophilic face with the potential to assemble into a coiled coil in an aqueous environment. Upon the addition of Zn(II) to either peptide cross-linking took place between the peptides affording a mechanically strong interfacial film. In contrast to AM1, the histidine residues of AFD4 had an appropriate spacing permitting metal ion chelation to occur, resulting in intramolecular chelation. This led to the formation of a 'metal clip' structure that stabilized the amphipathic helix while permitting cross-linking to neighbouring peptides as well. The film formed from this peptide was found to be very strong for the transition metals employed [Co(II), Ni(II) or Zn(II)]. The systems were shown to be reversible upon the addition of a chelator like EDTA, or acidification.

β -Sheets as secondary structures

β -Sheet structures can be influenced through metal ion binding as well. β -Sheet growth and sheet/sheet packing were shown to be influenced by the addition of Zn(II) metal ions.⁴⁷ While the relative arrangement of β -sheet packing (parallel or anti-parallel) within a fibre and the assembly process are often not well defined, metal binding can be exploited to control the packing mode and the rate of self-assembly. This was shown for a peptide based on the amyloid A β sequence (Table 4, entry 10). The side chains of the histidine residues, once bound in a parallel fashion, provided a binding site for metal ions which made the self-assembly kinetics and fibril morphology dependent on the metal ion concentration. A mixture of fibres and ribbons was for example observed at low zinc concentrations, while increasing the concentration resulted in long homogeneous ribbons and at even higher concentration only short ribbons were found that had the tendency to aggregate.

Peptide amphiphiles

Also for peptide amphiphiles structural changes can be triggered by metal ions as was shown by Stupp *et al.* They prepared peptide amphiphiles that self-assemble into a three-dimensional network at physiological pH after adding polyvalent metal ions (Table 4, entry 11).⁴⁸ The peptide

amphiphiles (PA) that were examined in this study all contained a C₁₈ alkyl chain and the integrin binding RGD sequence as shown in Table 3.

Self-assembly of negatively charged peptide amphiphiles, except PA 7, was detected after the addition of positively charged metal ions, although the addition of monovalent potassium ions did not result in a self-supporting gel (Table 3). Apparently, polyvalent ions are more effective initiators for self-assembly. For PAs 3 and 5 not containing cell adhesion or signalling sequences it was shown that cells can internalize the nanofibres they form *via* endocytosis after which these fibres accumulate in membrane compartments, most likely lysosomes. This indicated that these nanofibres can be degraded by natural mechanisms without being cytotoxic. Further, it was shown that MC3T3-E1 cells were entrapped in the matrix that had formed. The cells were able to survive for prolonged periods of time, and cell proliferation was not hampered, suggesting these structures could be useful in applications such as cell transplantation and tissue engineering. To establish the factors that contribute to the self-assembly, PA 1 was further scrutinized.⁴⁹ It was shown that the self-assembly upon the addition of metal ions was effected by counter-ion screening, stabilized by van der Waals interactions, hydrophobic forces, ionic bridging, coordination and hydrogen bonding. The electronic structure, concentration and hydration of counter-ions influence the self-assembly process and the mechanical properties of the gel. The wedge-like structure of PA 1 (Fig. 15) allows packing into a cylindrical arrangement, since the bulky side chains can then be aligned making a β -sheet-like hydrogen bonding interaction possible. Due to the hydrophobic forces in the core and the ionic interactions between side chains, intermolecular spacing is probably slightly greater in the radial dimension.

5. Enzymes

Enzymes play key roles as selective catalysts in cell pathways and disease states including the synthesis and assembly of structural protein scaffolds, and hence have served as a source of inspiration in the design of enzyme switchable structures. Using enzymes as a trigger has several advantages; enzymes are chemo-, regio-, and enantioselective and they work under mild aqueous conditions. Post translational modification principles in cells have great potential to provide regulative mechanisms for peptide switches. Often phosphorylation and/or dephosphorylation of serine, threonine or tyrosine residues

Table 3 The structure of the amphiphilic peptides tested for the self-assembly after adding different metals. 10 mM aqueous PA solutions were mixed with cations and the consequent gelation behaviour was observed for all polyvalent cations⁴⁸

PA	Sequence	Charge	K ⁺ ^a	Mg ²⁺ ^b	Ca ²⁺	BA ²⁺	Cu ²⁺	Zn ²⁺	Gd ³⁺
1	Alkyl-C ₄ G ₃ S ^(P) RGD-COOH	-3	Viscous liquid	Gel	Gel	Gel	N/A	Gel	Gel
2	Alkyl-A ₄ G ₃ S ^(P) RGD-COOH	-3	Viscous liquid	Gel	Gel	Gel	Gel	Gel	Gel
3	Alkyl-A ₄ G ₃ S ^(P) KGE-COOH	-3	Viscous liquid	Gel	Gel	Gel	Gel	Gel	Gel
4	Alkyl-C ₄ G ₃ SRGD-COOH	-1	Viscous liquid	Viscous liquid	Gel	Gel	N/A	Gel	Gel
5	Alkyl-A ₄ G ₃ EQS-COOH	-2	Viscous liquid	Gel	Gel	Gel	Gel	Gel	Gel
6	Alkyl-A ₄ G ₃ ERGDS-COOH	-2	Viscous liquid	Viscous liquid	Viscous liquid	Viscous liquid	N/A	Gel	Gel
7	Alkyl-C ₄ G ₃ EIKVAV-COOH	-1	Gel	Gel	Gel	N/A	N/A	Gel	Gel
8	Alkyl-C ₄ G ₃ KIKVAV-NH ₂	+2	Gel	Viscous liquid	Viscous liquid	Viscous liquid	N/A	Viscous liquid	Viscous liquid

^a 200 mM KCl. ^b 20 mM.

Table 4 Metal ion responsive peptides

Entry	Metal	Peptide	Comment
1	Pd^{2+} ($[\text{Pd}({}^{15}\text{NH}_2(\text{CH}_2)_2{}^{15}\text{NH}_2)(\text{NO}_3)_2]$)	HAAHHELH HAAH HAAAH HARAH MARAH	Random coil to turn Random coil to metallocycle Random coil to α -helix With M a stronger helix is formed
2	Ru^{2+} , Pd^{2+}		
3	Zn^{2+}	YIHALHRKAFKAKIARLERHIRALEHAA	Zinc finger motive when Zn is present
4	Zn^{2+}	42-mer peptides with ATANP on i and $i + 4$	Trimeric α -helical coiled coil to less helical
5	Ag^+	EIAQHEKEIQAEKKIAQHEYKIQAE IEEKIAQHKEKIQAIK	Random coil to trimeric coiled coil
6	Zn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+}	Collagen with His and NTA at opposite sides	Formation of microfibrilles
7	Zn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+}	Collagen with His and NTA at opposite sides and bipy in the middle	Crosslinking helices to fibers
8	Co^{2+} , Zn^{2+}	1: LKVELEKLKSELVVLHSHLEKLKSEL 2: LKVELEKLKSELVVLHSELHKLKSEL	Fibrils to disassembled (Co^{2+}) Fibrils to helix
9	Zn^{2+}	3: MKQLADSLHQLARQVSRLEHA 4: MKQLADSLHQLAHKVSLEHA	Crosslinking to film formation
10	Zn^{2+}	HHQALVFFA	Fibres to ribbons
11	Polyvalent ions (see Table 3)	C18-X-RGD-Y (see Table 3)	Self-assembly to a gel for cell-growth

Fig. 15 The proposed self-assembly into nanofibres of PA 1. The wedge-like structure of PA 1 packs into nanofibres after adding metal ions. The hydrophobic and electrostatic interactions are shown in this picture. Also the β -sheet-like hydrogen bonding interactions are shown. Figure taken from ref. 49.

can function as trigger. *E.g.* matrixmetalloprotease-9 (MMP-9), an enzyme belonging to the family of metalloproteases, which play a role in physiological processes and have been related to the invasiveness and metastatic potency of human malignant tumours, was shown to instruct self-assembly of supramolecular hydrogelators to form nanofibres resulting in hydrogelation.⁵⁰ The enzyme cleaved off the residues Leu-Asp-Asp from the peptide Phe-Phe-Phe-Phe-Cys-Gly-Leu-Asp-Asp. This resulted in a change in the balance between hydrophobic-hydrophilic interactions that caused the formation of self-assembled nanofibres. Because MMPs are over-expressed in a variety of malignant cells, including different forms of tumours, these systems are suggested to be applicable as for example anti-cancer therapeutics. In another study Straley and Heilshorn

showed a three-dimensional hydrogel to be biodegradable at predictable and tuneable rates. For this purpose they prepared elastin-like based protein polymers containing peptide regions susceptible to cleavage by cell-secreted proteases.⁵¹ Degradation of the hydrogels was effected by cleavage with high concentrations of tissue plasminogen activator (tPA) or urokinase uPA proteases, involved in the regulation of physiologic and pathologic processes, such as angiogenesis and coagulation. In addition to triggering gelation, the group of Ulijn investigated responsive surfaces that present the cell adhesive RGD sequence mediated by enzymatic hydrolysis of a RGD precursor.⁵² This inactivated precursor of the cell adhesion peptide, Fmoc-AARGD, was attached to a bulky poly ethylene glycol (PEG) surface. Cleavage between the alanine residues with elastase allowed recognition and binding to the RGD moieties by cells *via* their surface integrins (Fig. 16). Because elastase is an enzyme associated with tissue remodelling related to chronic wounds and inflammation this system might be further developed to create responsive antimicrobial or anti-inflammatory biomaterial coatings or be useful for tissue engineering allowing cell binding and growth in response to a specific adhesion protein.

α -Helices as secondary structures

Tuchscherer *et al.* studied the transition from a random coil into an α -helix by the addition of dipeptidyl peptidase-4 (DDP4V).⁵³ The peptide used for this study was the C-terminal part of neuropeptide Y (NPY), having the minimal chain length required for binding to the NPY receptor Y2. This peptide was separated into two parts by a switch, which consisted of an *O*-acetyl connected derivative of Thr³² (Fig. 17A). The peptide adopts a random coil conformation, and therefore cannot bind to Y2. After cleavage by DDP4V, a transition to an α -helix took place as a consequence of *O* to *N*-acyl migration allowing the backbone to be connected through its native peptide bond (Fig. 17B). As a result of this migration the α -helical peptide was able to bind to the Y2 receptor.

Fig. 16 Schematic representation of an elastase enzyme responsive system. Elastase cleaves at the enzyme cleavable peptide (ECP), resulting in 'deprotected' ARGD. The cells can now recognize and bind the cell adhesion peptide *via* their integrins and therefore to the glass slide. Figure adapted from ref. 52.

In a different approach proteases were used for the reverse reaction, the coupling of peptides. Enzymes were used to produce peptide amphiphile hydrogelators that self-assembled to produce nanofibres.⁵⁴ It was shown that self-assembly of a peptide into a higher-order structure provided stabilization of the reaction product. This stabilization was the driving force for peptide synthesis instead of hydrolysis. It was shown that thermolysin was able to connect an Fmoc-amino acid and a dipeptide to form an amphiphilic Fmoc-tripeptide. Thermolysin has a preference for hydrophobic/aromatic residues on the amine side of the bond. No specificity is known for the amino acid at the carboxylic end. Phe-Phe was chosen as the dipeptide and for the Fmoc-amino acid several residues were tested (Gly, Ala, Val, Leu, Phe and Pro). It was found that the yield increased with increasing hydrophobicity of the Fmoc-amino acid of which Fmoc-Val gave the highest yield (~64%).

β -Sheets as secondary structures

The aggregation of amyloid peptides into core-shell nanofibres is kinetically controlled in nature, but can also be controlled by enzymatic dephosphorylation as was shown by Kühnle and Börner. They prepared a polymer-peptide consisting of poly-ethylene oxide attached to a penta-repeat of the dipeptide ThrVal.⁵⁵ This oligopeptide assembled into an anti-parallel β -sheet with all the side chains of the hydrophobic residues pointing to one face and the hydrophilic ones to the other.

p-Nitrophenylalanine was introduced C-terminally to the peptide as spectroscopic marker. Between this marker and the poly-ethylene oxide group methionine and glycine residues were positioned to ensure flexibility between the poly-ethylene oxide group and peptide. To prevent self-assembly of the conjugate three of the threonines were phosphorylated. Upon the hydrolysis of the threonine-phosphate moieties using a phosphatase, the peptide-polymer self-assembled to give a fibrillar structure.

Similarly, in a genetically engineered variant of spider dragline silk Winkler *et al.* showed how enzymatic phosphorylation and dephosphorylation reactions could be used to modulate assembly.⁵⁶ Their protein could be phosphorylated with a cyclic AMP dependent kinase and dephosphorylated with calf intestinal alkaline phosphatase, of which phosphorylation inhibited β -sheet assembly by controlling the structure of the protein. It was assumed that the insertion of a phosphorylation site would be a sufficiently small modification to avoid disrupting the normal silk assembly processes.

Peptide amphiphile

Also peptide amphiphiles have been shown to hydrogelate in a controlled manner employing enzyme mediated modifications as was shown by the Hartgerink group.⁵⁷ They constructed a peptide that can be divided into three functional regions; an enzyme cleavable site (GTAGLIGQ), a glutamic acid that assists calcium binding and improves solubility and finally the cell adhesion sequence RGDS. The enzyme that was used was metalloprotease 2, a protein whose production is stimulated by inflammatory cytokines and is known for its ability to encapsulate dental pulp cells into the elastic nanofibres it forms. The peptide was N-terminally acetylated providing a hydrophobic driving force for self-assembly. At neutral pH a net negative charge was present, preventing self-assembly. However, upon the addition of calcium shielding the negative charges, the peptide self-assembled into cylindrical micelles. Inside this network rat pulp cells were incorporated that play an important role in dental mineralization and dental-tissue development. The cells produce MMP-2 resulting in a breakdown of their matrix. It was shown that incorporation of these MMP-2 producing cells resulted in cell-mediated proteolytic

Fig. 17 *O,N*-Acyl migration as a result of DPPIV. (A) DPPIV cleaves off the dipeptide, resulting in a less constrained backbone. An α -helical structure can now be adopted. (B) The principle idea of *O,N*-acyl migration. Figure adapted from ref. 53.

degradation of the network. Again, such a system could be useful for tissue regeneration materials.

6. Photo-switchable molecules

In nature proteins exist that are influenced by the absorption of photons. These often play roles as biological catalysts and information and/or energy traffickers. The archetypal example of such a photo-controlled protein is Rhodopsin that enables vision at low light conditions. Inspired by this protein most synthetic light responsive peptides that have been created involve the incorporation at strategic positions of chromophoric small organic moieties that can change their conformation upon the absorption of light.⁵⁸

α -Helices as secondary structures

For the majority of the photoswitchable systems that have been described the azobenzene group was incorporated as a chromophoric linker. One derivative of this chromophore, BSBCA (Fig. 19), changes in length upon illumination, caused by the isomerization around the central double bond that can go back and forth between the *cis* and *trans* conformers. The popularity of this photo-switchable unit is caused by the fact that the isomerization is not very environment-sensitive, highly reversible and occurs with a high quantum yield. Because of the low wavelength regime in which the conformational transition is induced for unsubstituted azobenzenes a great variety of derivatives have been designed, in which ring substituents increase both the absorption wavelength of the *trans*-isomer and the rate of thermal back-isomerization.⁵⁹ The only serious drawback of these photo-switchable compounds is their sensitivity towards reducing agents often present in an intracellular environment, obviously hampering any possible *in vivo* applications. Woolley *et al.* prepared α -helical peptides containing cysteine residues placed on positions *i* and *i* + 7 of which the side chains were connected with an azobenzene unit using the iodoacetylated derivative of the compound shown in Fig. 18.⁶⁰ For one of the α -helical peptides that was examined (Table 5, entry 1) illumination resulted in a substantial increase in helix content due to the *trans*- to *cis*-isomerization of the chromophore.

Another peptide based on collagen was made to fold and unfold ultra fast after incorporation of the azobenzene chromophore.⁶¹ The collagen peptide was connected with the azobenzene moiety as shown in Fig. 19, which served as a clamp stabilizing a helical secondary structure. Complete isomerization to the *trans*-isomer was accomplished *via* thermal relaxation in the dark to allow the peptide to adopt its fully folded state, which induced formation of a self-assembled triple helix. Irradiation with light gave the azobenzene *cis*-isomer and led to the unfolding and disruption of this triple helix.

β -Sheets as secondary structures

Zhang *et al.* have modified an SH3 domain from fyn-tyrosine kinase to undergo a photo-controlled transformational switch.⁶² The protein domain was crosslinked with the earlier mentioned thiol reactive azobenzene chromophore, BSBCA (Fig. 18). For this purpose two different cysteine residues were engineered into the protein giving a L3C-L29C mutant. When the chromophore was brought into its *trans*-conformation, the protein existed as a mix of folded and unfolded structures. Irradiation of the diazo-containing protein produced the *cis*-isomer to recover the folded, active state of the protein. Peptides of the MAX family discussed earlier in this review were also modified to become photoswitchable. The photo-sensitive unit in this case was an α -carboxy-2-nitrobenzyl group attached to the side chain of cysteine. The peptide called MAX7CNB (Table 5, entry 2) was unfolded in an aqueous solution at ambient light.⁶³ Exposure to UV radiation resulted in the so-called uncaged free peptide that could fold

Table 5 Photo-triggerable peptides

Entry	Peptide	Sequence	Comment
1	Unnamed	EACARVXAA-CEAAARQ	X denotes aminoisobutyric acid
2	MAX7CNB	VKVKVKVKV ^D -PPTKVKCKVKV	C is a photo-caged cysteine
3	GP	GANPNAAG	Connected to a alkyl chain <i>via</i> an <i>o</i> -nitrobenzyl linker
4	Unnamed	GVVVAEEEE	Palmitoylated at N-terminus
5	Unnamed	GAAEERGDS	Palmitoylated at N-terminus

Fig. 18 A thiol reactive azobenzene derivative in its two conformers: (3,3'-bis(sulfonato)-4,4'-bis(chloroacetamide)azobenzene, BSBCA).

Fig. 19 A photo-controllable collagen derived peptide sequence.

into a β -hairpin conformation. This resulted in gelation of the solution due to the hydrophobic valine rich surface that formed and the lateral formation of intermolecular hydrogen bonds between neighbouring hairpins, as described earlier.

Peptide amphiphiles

Löwik *et al.* have shown how fibrous aggregates of peptide amphiphiles with a C_{12} or C_{18} tail connected to the N-terminus via a nitrobenzyl derivate to an octapeptide (GP, Table 5, entry 3) could be disassembled upon UV irradiation.⁶⁴ The irradiation caused the carbon tail to detach from the peptide resulting in a drastic change in the hydrophobic–hydrophilic balance of the peptide assembly which led to disassembly. No difference was detected between the C_{12} and C_{18} peptide amphiphiles. Another peptide amphiphile, containing a C_{16} tail and a 2-nitrobenzyl group (Table 5, entry 4) that forms a quadruple helical fibre was shown to be converted into single helical fibres upon irradiation due to cleavage of the 2-nitrobenzyl group.⁶⁵ The authors hypothesized that the poor internal packing of the caged peptide amphiphile stimulated the interactions among molecules in neighbouring nanofibres which were lost after cleavage of the 2-nitrobenzyl resulting in single nanofibres. With a slightly different peptide amphiphile containing the photocleavable 2-nitrobenzyl group, the same group was able to photoinduce gelation.⁶⁶ A bioactive RGD peptide (Table 5, entry 5) was again modified with a palmitoyl group. After irradiation three-dimensional nanofibres were formed due to the transition of the peptide into β -sheets. Moreover, it was shown that the light triggered gelation of the amphiphile peptide was able to increase the mRNA expression level of vinculin (a membrane cytoskeletal protein) in NIH 3T3 fibroblasts.

7. Dual stimulus response

Dual responsive systems are peptide systems that simultaneously respond to two different stimuli. A major benefit of multiple stimuli responsive systems is that they can be more precisely controlled. Most dual responsive systems consist of peptides that respond to a combination of temperature and pH. In one

study gold nanoparticles were functionalized with a polyhistidine peptide that has a pK_a around physiological pH.⁶⁷ For these particles temperature and pH worked cooperatively to trigger a conformational change of the polyhistidine peptide from a β -sheet to a mixture of random coil and β -turn (Fig. 20). Once β -sheets formed aggregation of the gold nanoparticles could take place. At pH 5.5 and at 4 °C only 5% β -sheet conformation was found, while increasing the temperature up to 75 °C gave approximately 45% β -sheet. Lowering the pH to 3.2 resulted in complete blocking of β -sheet formation that was independent of the temperature. This highly controllable system was shown to be reversible.

Li *et al.* designed peptide–polymer hybrids⁶⁸ consisting of a poly(*N*-isopropylacrylamide) (PNiPAM), a widely studied thermoresponsive polymer, block bound to a copolypeptide block consisting of glutamic acid and lysine residues, whose

Fig. 20 The assembly/disassembly process of poly histidine modified peptides. The poly histidine peptide is in a random coil at pH 3.2, while increasing the pH results in a β -sheet conformation. The gold particles assemble only if the temperature is above 75 °C. Due to the 'memory effect' the aggregation remains when the temperature is decreased to 4 °C. Figure taken from ref. 67.

Fig. 21 Schematic representation of the pH and temperature dependent vesicular formation. Only the two-dimensional monolayer is depicted, but consequently the same transitions will take place in the vesicle. The green structures represent the poly(L-lysine hydrochloride) block, while the red represents the poly(γ -benzyl-d7-L-glutamate) block. Figure taken from ref. 70.

ratio could be tuned by changing polymerization conditions.⁶⁹ The copolypeptide could be switched between random coil and α -helix by changing the pH. The PNiPAM block, introducing thermoresponsive behaviour, caused the hybrids to collapse around 33 °C at pH 3 due to the conversion from coil to globule as was studied with ¹H NMR spectroscopy and turbidity and DLS measurements. Finally, Iatrou *et al.* have shown a set of triblock copolypeptides of the poly(L-lysine hydrochloride)-*b*-poly(γ -benzyl-d7-L-glutamate)-*b*-poly(L-lysine hydrochloride) type to behave in a temperature and pH dependent manner.⁷⁰ The middle poly(γ -benzyl-d7-L-glutamate) block adopted an α -helical conformation, independent of the temperature and pH. At a temperature of 25 °C and at pH 7.4 the poly(L-lysine hydrochloride) block existed in a random coil, while at higher pH this block converted into an α -helix. However, when the temperature was increased up to 37 °C a β -sheet conformation was detected (Fig. 21), which led to formation of more elongated structures from the more compact assemblies that were present at 25 °C. Additionally, this peptide complex was shown to be able to act as a carrier for DNA. Efficient encapsulation of DNA was detected when the assembly formation of the triblock copolypeptide was performed in the presence of plasmid DNA.

Also the groups of Klok and Lecommandoux have performed a great amount of work on stimuli responsive polymer–polypeptide hybrid materials. *E.g.* Checot *et al.* have prepared polybutadiene–polyglutamic acid diblock copolymers that form well-defined vesicular morphologies in water.⁷¹ Changing the pH affected the morphology of the assemblies that formed. Moreover, the size of the aggregate could be manipulated reversibly by changing both pH value and ionic strength. It was claimed that the polypeptide block had the unique feature that it was capable of folding into a compact and well-defined secondary structure and that it might be suitable for applications like triggered release systems of both hydrophobic and hydrophilic compounds or as sensor nanodevices.

8. Conclusion

In this review a concise introduction has been given to peptide based systems that can be switched by common triggers such as pH, temperature, metal ion, light, enzymes, and ion

concentrations. These peptide switchable systems have developed into an emerging field of research with many potential applications. Notwithstanding the enormous effort that has been put into this area of research to our knowledge no large scale commercial application has emerged from it. This could in part be due to our limited knowledge on all aspects of intra and intermolecular protein interactions that determine folding and hence properties. Therefore, we think that only a further (fundamental) understanding of these stimuli dependent systems can bring the application of this type of compounds in new materials a step closer. Most of the described compounds in this review and their derived materials are said to be presently investigated for their use as drug delivery systems, tissue engineering and purification. Specifically pH and metal dependent materials seem to be very promising for the production of novel functional biomaterials and drug delivery systems. Both pH and metal ions are of course very interesting because they can be regarded as the archetypal parameters in the control of biological systems which nature exploits predominantly.

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