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(54) Title: HYDROGEL PARTICLE

(57) Abstract: Hydrogel particles comprise protease recognition means, which is adapted to be digested by a protease and cause a variation in the net charge of the particle. This variation in net charge produces a change in the molecular accessibility of the particle. Embodiments of the invention include positively or negatively charged particles. These particles may comprise charged amino acids bonded to the hydrogel polymer via the recognition means. The charged amino acids repel each other and cause swelling of the particle. Digestion of the recognition means causes loss of the charged amino acid and "collapse" of the particle. The "swollen" form of the particle may take-up a large molecular which is then entrapped on "collapse" of the particle. In further embodiments of the invention, the particle comprises oppositely charged amino acid residues flanking the protease recognition means to give an overall neutral particle. Digestion of the protease recognition means causes loss of one of the charged species to leave an overall charged particle which swells and is capable of releasing its contents.

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HYDROGEL PARTICLE

The present invention relates to hydrogel particles, and in particular to hydrogel particles that are responsive to biological stimuli. In particular, the invention relates to hydrogel particles, which respond to the catalytic action of enzymes. The invention extends to the preparation of such hydrogel particles, and to uses thereof, for example, for the detection, purification, encapsulation, entrapment, and/or delivery of sensitive biological molecules.

Polymer hydrogel particles, which are able to respond to applied stimuli by changes in their physical properties are known. Usually, the application of the appropriate stimulus, such as temperature, ionic strength, solvent polarity, electric/magnetic field or light, to the hydrogel results in the swelling or collapse of the macroscopic structure of the hydrogel. In addition, changes in the molecular accessibility of the hydrogel are also observed.

One use of such hydrogel particles is in biomedical applications, for example, in drug delivery, wound dressings or as implant coatings where the hydrogels selectively release or remove bioactive agents into or from the biological target site. However, each of the stimuli mentioned above are relatively non-selective, and in addition, fluctuating temperature, pH, ionic strength, and solvent polarity can each disrupt biological interactions in the hydrogel polymer. Accordingly, a significant problem with existing hydrogel particles is that it is not easy to tightly control their physical properties in the target biological environment. Hence, they are not ideally suited for applications in biomedical settings. Therefore, there is a requirement for hydrogel polymers that respond to stimuli that are compatible with biological conditions, and in addition, in which it is also easier to tightly control their physical properties.

One of the inventors of the present invention has previously demonstrated that it is possible to increase the molecular accessibility of a hydrogel polymer particle by introducing positively charged quaternary amine groups or negatively charged sulphonate groups into the backbone of a hydrogel polymer during the polymerization process, thereby resulting in a permanently charged cationic or anionic polymer. The inventors believe that the provision of a net charge throughout the hydrogel particle

causes electrostatic repulsion within the hydrogel network, thereby causing the hydrogel particle to swell, which in turn causes the mesh or pore size of the polymer network to increase. However, a disadvantage with such hydrogel particles is that it is not possible to selectively and dynamically control the degree of molecular accessibility in response to an enzyme.

It is therefore an aim of embodiments of the present invention to address the problems with prior art hydrogel particles *per se*, and to provide hydrogels, which exhibit improved molecular accessibility characteristics, and improved controllability thereof. It is also an aim to provide novel uses of such controllable hydrogel particles.

According to a first aspect of the present invention, there is provided a hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle.

According to a second aspect of the present invention, there is provided a method of preparing a hydrogel particle in accordance with the first aspect of the invention, the method comprising the steps of :-

- (i) preparing a hydrogel precursor; and
- (ii) incorporating protease recognition means into the precursor to produce a hydrogel particle, wherein the recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, and wherein the variation in net charge produces a change in the molecular accessibility of the particle.

According to a third aspect of the invention, there is provided a method of altering the molecular accessibility of a hydrogel particle, the method comprising contacting a hydrogel particle according to the first aspect with a protease, under suitable conditions such that the protease modifies the protease recognition means thereby causing a variation in the net charge of the hydrogel particle, and wherein the variation in net charge produces a change in the molecular accessibility of the particle.

The inventors of the present invention have devised a class of hydrogel polymer particle (referred to herein as enzyme responsive polymers - "ERPs"), in which the molecular accessibility of the particle may be selectively and tightly controlled by proteases present in the biological environment of the particle. The inventors have found that it is surprisingly possible to control the molecular accessibility of the hydrogel particle in accordance with the first aspect of the invention with high specificity. The molecular accessibility may be either reduced or increased, as desired, when the particle is contacted with a specific protease.

By the term "molecular accessibility of the particle" used herein, we mean the ability of the particle to absorb and retain molecules therein. Molecules may be able to diffuse and permeate into, and/or out of, the hydrogel particle, and this characteristic is largely determined by the average pore size or mesh size of the hydrogel particle. Hence, preferably, the hydrogel particle is adapted to either increase or decrease the average size of its pores, such that the size, type and concentration of molecules it can absorb or release may be varied.

The hydrogel particle of the invention incorporates a recognition means that is adapted to be modified by a protease. The modification is preferably cleavage.

Using the method according to the second aspect, the inventors have introduced a protease recognition means into a hydrogel precursor to produce the hydrogel particle according to the first aspect. Furthermore, when using the method of the third aspect, by choosing a specific protease enzyme that is able to cleave a specific peptide bond(s) present in the protease recognition means of the hydrogel particle, the inventors have found that it is possible to cause a change in the overall net charge of the particle.

By the term "net charge of the particle", we mean the overall charge when taking in to account any positively or negatively charged species in the particle under certain conditions. Hence, the net charge of the particle may be positive, negative, neutral and/or zwitterionic and may involve multiple charges.

The inventors were surprised to find that by changing the net charge of the hydrogel particle as a result of digesting the protease recognition means with the

protease, it was possible to induce a conformational change within the structure of the hydrogel particle, which in turn cause a change in the overall molecular accessibility of the hydrogel particle. This enables molecules to diffuse and/or permeate either into or out of the hydrogel particle. Accordingly, the hydrogel particle according to the first aspect of the invention is referred to as being 'programmable' by virtue of the protease recognition means, which is 'responsive' to, and may be modified by, a specific protease enzyme when using the method of the third aspect.

The inventors believe that the use of a protease enzyme in the method of the third aspect to digest the protease recognition means provides a number of key advantages to the mechanism and available uses of the hydrogel particle. Firstly, proteases are chemo-, regio-, and enantio-selective in the reactions that they catalyse. This means that these enzymes are uniquely capable of selecting between identical chemical bonds in different molecular structures. For example, proteases, which are peptide-hydrolysing enzymes, are known to selectively cleave specific amide bonds depending on their substrate specificity, i.e. the selectivity for amino acids flanking the scissile peptide bond. Secondly, proteases naturally work under mild conditions (aqueous, pH 5-8). This makes them suitable for most biological scenarios. Thirdly, many proteases catalyse reactions near surfaces *in vivo* and are therefore well equipped to catalyse reactions at interfaces. Fourthly, because many proteases play key roles in biological functions and disease states, the inventors believe that there is scope for the development of the hydrogel particle according to the invention, to be used in response to these disease markers in a highly selective manner.

The hydrogel particle according to the first aspect is prepared using the method of the second aspect, and this comprises combining two key components, namely:- (i) the hydrogel precursor; and (ii) the protease recognition means.

The hydrogel precursor may comprise an organic and/or inorganic polymer. However, it is preferred that the hydrogel precursor comprises substantially an organic polymer. Preferably, the hydrogel precursor is substantially hydrophilic. Preferably, the hydrogel particle has a net neutral charge. The hydrogel precursor may comprise a plurality of polymer chains that are inter-connected via cross-linkages. In the absence of cross-linkages, the polymer chains, which are preferably substantially

hydrophilic, tend to diffuse in water and the hydrogel particle would be completely solubilised. Hence, it is preferred that the polymer chains are connected together through physical and/or covalent cross-links. However, in a preferred embodiment, the polymer chains are interconnected by covalent cross-links.

5 The presence of cross-links within the polymer influences both the mechanical strength, and the permeability properties of the hydrogel precursor, and hence, resultant hydrogel particle. High cross-linking densities increase the hydrogel particle's elastic modulus, and decrease the particle's permeability. Hence, the cross-links determine the formation of a network of polymer chains in the hydrogel
10 precursor, whose average mesh size can cause a Molecular Weight Cut-Off (MWCO) or Molecular Size Cut-Off effect in the hydrogel's permeability.

 By the term "average mesh size", we mean the average size of pores in the hydrogel polymer precursor, and resultant polymer particle. Hence, the average mesh size provides a direct measure of the molecular accessibility of the hydrogel particle.
15 The molecular accessibility may also be defined in terms of its Molecular Weight Cut-Off (MWCO) value. By the term "Molecular Weight Cut-Off", we mean the maximum value of Molecular weight of molecule that may pass into/out of the particle, by molecular size cut-off we mean the maximum size of a molecule that may pass into/out of the particle.

20 It will be appreciated that the molecular accessibility of the polymer particle may be either increased or decreased as a result of the modification by the protease in the method of the third aspect, which in turn will determine the size and molecular weight of molecule which would be able to permeate into and diffuse through (either inwardly or outwardly) the hydrogel polymer. It will be appreciated that a molecule
25 having a molecular size (or molecular weight) that is larger than that of the average mesh size of the hydrogel polymer cannot diffuse through the hydrogel polymer. Conversely, a molecule with a molecular size (or molecular weight) that is smaller than, or substantially similar to, the average mesh size of the hydrogel polymer can permeate and diffuse through the polymer. Such diffusion may be in either direction,
30 i.e. either into the particle, or out of the particle.

Preferably, the hydrogel precursor comprises polymer chains resulting from a polymerisation reaction between one or more monomers, more preferably two or more monomers, and even more preferably three or more monomers, in which at least one monomer provides physical or chemical cross-links therebetween. Suitable monomers, which may be used to produce the hydrogel precursor will be known to the skilled technician.

Examples of preferred monomers used in the polymerisation reaction may include molecules (1) and (2) as shown in Figure 1.

It is preferred that these monomers react to form Polyethylene Glycol (PEG) macromonomers (3) and (4) as shown in Figure 2. The PEG macromonomers (3 & 4) produced may then react with an initiator, preferably in the presence of acrylamide to produce PEGA. Examples of suitable initiator include, for example, UV or a heat-activated initiator. An example of a heat-activated initiator includes a peroxy ester of organic acids or azo catalysts. This polymerisation reaction may be carried out in water. The mixture is preferably stirred at room temperature until it is visible that PEGA particles are forming fully.

It is preferred that the hydrogel polymer comprises polyethylene glycol acrylamide (PEGA). The formula of PEGA is defined by the formulae illustrated in Figure 3. The skilled technician will appreciate how to prepare a suitable hydrogel precursor molecule in accordance with the second aspect of the invention. PEGA particles may be prepared by an inverse suspension polymerization process, which will be known to the skilled technician. The beading process involves the dispersion of monomer molecules (e.g. compounds 1 and 2 and 3 and 4 shown in Figures 1 and 2, in a dispersed phase) by mechanical agitation in an inert oil phase (continuous phase), forming spherical particles. The PEGA product is naturally spherical.

For example, PEGA monomer in water and acrylamide may be added to each other in a continuous phase. Mixing (e.g. by stirring) may be conducted at room temperature. An initiator (e.g. Peroxy esters of organic acids or azo catalysts) for the polymerisation reaction may be added while stirring is carried out. Alternatively, UV light may be applied to initiate the polymerisation reaction. When droplets form fully the mixture may be purged with nitrogen for about 90 minutes, and then transferred to

awater bath, and preferably, heated to about 55°C. Preferably, the reaction is carried out with stirring, which may comprise using an impellor or stirrer.

The reaction may be conducted for sufficient time to allow polymerisation to occur, which may be about 90 minutes. The size of the polymer particles produced is dependent on the speed of the stirring. The oscillation shears the polymerisation mixture and “cuts the particles”. The greater the frequency of rotation of the stirrer, the smaller the size of the particles produced. The particle size is also controlled by the nature of the surfactant and its concentration.

Further disclosure relating to PEGA and the synthesis thereof is given in US-A-5 352 756, US-B-6,642,334 and WO-A-0018823.

Although PEGA is the preferred hydrogel for use in the invention, other materials may be used, for example poly(hydroxylethylmethacrylate) (pHema), n-isopropyl acrylamide (NIPAM) and PVA.

Following preparation of the hydrogel precursor in step (i) of the method of the second aspect, the precursor is then preferably ‘functionalised’ by incorporation of the protease recognition means, as defined in step (ii) of the method of the second aspect.

In step (ii) of the method of the second aspect, the protease recognition means may be incorporated into the hydrogel precursor by suitable means to form the hydrogel particle.

Preferably, the protein recognition means is attached via an amine group within the hydrogel polymer in the precursor, and this may be achieved using standard chemical techniques, which will be known to the skilled technician. Preferably, the method according the second aspect comprises incorporating a plurality of protease recognition means into the hydrogel precursor. Hence, it is preferred that the or each protease recognition means is attached to the hydrogel polymer via an amide bond. The protease recognition means may also be referred to as an enzyme cleavable linker.

By way of example, step (ii) of the method according to the second aspect may comprise adding a solution of protease recognition means to a solution of the hydrogel precursor prepared in step (i), and the resultant solution may be incubated for about 3 hours at about room temperature.

5 Preferably, the recognition means may be attached to the precursor using a coupling agent and, an activation agent. The coupling agent and the activation agent are used to link amino acids together stepwise to form peptides, and may be referred to as Fmoc peptide synthesis. Such Fmoc peptide synthesis is well known and numerous protocols, activation agents, and coupling agents are available, and are
10 described in for example NovaBiochem or Bachem catalogues for materials and methods.

Suitable coupling agents include N,N'-Diisopropylcarbodiimide (DIC) or Dicyclohexylcarbodiimide (DCC).

A suitable activation agent comprises 1-Hydroxybenzotriazole (HOBt) HOBt
15 (1), which is currently the most frequently used activating agent for the carboxyl group of amino acids. Advantageously, the procedure is fast and suppresses racemization. HOBt-DCC or -DIC methodology may be used in all peptide couplings. However, DIC may be preferred because its urea by-product is more soluble in organic solvents than that formed from DCC.

20 Coupling of the peptide chain may be carried out either in one step, or in a plurality of steps. Each step may be double coupled-addition reaction is carried out twice for each reaction. PEGA is preferably functionalised by the free amine groups present.

The protease recognition means serves essentially two functions. Furthermore,
25 preferably, the recognition means is adapted to provide the particle with a net charge (i.e. any one of either (a) positive, (b) negative, (c) neutral, and/or (d) zwitterionic). In addition, preferably, the recognition means comprises a protease hydrolysis sequence, or substrate sequence, which is specifically recognised and cleaved therefore by the protease enzyme. Proteases hydrolyse or cleave peptide bonds in proteins and
30 peptides. Hence, the protease recognition means is preferably capable of being

modified or hydrolysed by a protease enzyme, wherein such modification or hydrolysis causes a variation in the net charge of the hydrogel particle.

The overall net charge of the hydrogel particle prior to the hydrolytic action of the protease will be determined by the intended use of the particle. For example, the overall net charge of the particle prior to protease action may be positive, negative, or neutral. Then, following contacting of the hydrogel particle with the protease in step (ii) in the method of the third aspect, the overall net charge of the particle will be varied to cause a change in the molecular accessibility of the particle. Hence, following protease action, the net charge of the particle may be positive, negative, or neutral, depending on the charge of the particle prior to enzymatic hydrolysis.

Hence, in a first embodiment, the hydrogel particle may have a net positive charge before the hydrolytic action of the protease. Preferably, the net positive charge of the particle is provided by the protease recognition means. For example, the protease recognition means may comprise a chemical species having a net positive charge.

It will be appreciated that for biomedical uses, it is preferred to use particles which are active in a pH of about 7. Hence, preferably, the chemical species has a pKa of less than about 4 for an acidic moiety or greater than about 9 for a base, thus ensuring that the molecules are in their charged states when used at pH 7.

For example, the chemical species may comprise an amino acid having a net positive charge. Examples of suitable amino acids may include arginine, lysine, or histidine. The chemical species may comprise a non-natural amino acid. For example, the chemical species may comprise a charged group, such as quaternary amine. However, it is preferred that the protease recognition means comprises an arginine residue.

It will be appreciated that the net positive charge provided by the protease recognition means preferably causes the hydrogel particle to adopt a substantially 'swelled' configuration. By the term "swelled configuration", we mean the particle is substantially enlarged, and therefore has a greater average diameter compared to the size of the particle if it had a net neutral charge.

While the inventors do not wish to be bound by any hypothesis, they believe that this swelling effect is because the molecules of the hydrogel polymer to which the positively charged protease recognition means are attached are electrostatically repelled away from each other due to their positive charge. As a result, when the hydrogel particle adopts a swelled configuration, the pores (and hence, average mesh size, and hence, the Molecular Weight Cut-Off value) between the polymer molecules are increased in size, and so the molecular accessibility of the particle is referred to as being high. It will be appreciated that if the hydrogel comprises a higher number of protease recognition means comprising the positive charge, then there will be greater amount of electrostatic repulsion, and so the particle will swell to a greater extent, and accordingly, the particle will have an even higher molecular accessibility.

Purely by way of example, the average mesh size of the hydrogel polymer prior to modification of the protease recognition means with a protease may be approximately 0.1 nm - 50 nm. More preferably, the average mesh size may be approximately 0.5 nm - 20 nm and most preferably, approximately 1 nm - 10 nm. It is preferred that the average mesh size of the hydrogel matrix may be about 1nm, 2nm, or 3nm.

Purely by way of example, the average Molecular Weight Cut-Off value of the particle is approximately 35kDa for PEGA₁₉₀₀ (where the subscript refers to the molecular weight of the PEG crosslinks) and over 70kDa for PEGA₆₀₀₀.

In addition to providing a net positive charge to the particle, it is preferred that the protease recognition means comprises a protease hydrolysis substrate sequence for the protease, and preferably, a catalytic site thereof. The type of the substrate sequence in the protease recognition means incorporated into the hydrogel precursor will be determined by the specific protease chosen for use in the method of the third aspect, which will itself be determined by the intended ultimate use of the hydrogel particle, as will be described hereinafter.

However, preferably, the substrate sequence comprises a peptide bond, which attaches two or more amino acid residues together. It should be appreciated that the protease will be capable of cleaving or hydrolysing this specific peptide bond. The skilled technician will appreciate that there is a wide range of protease enzymes in

existence, and that generally each protease will recognise a very specific hydrolysis substrate sequence. For example, a list of suitable protease enzymes, which may be used in accordance with the invention may be found at www.brenda.uni-koeln.de.

5 The inventors investigated the protease action and selectivity against particles according to the invention of the three enzymes, thermolysin, chymotrypsin, and trypsin. Hence, by way of example, the inventors prepared a protease recognition means as shown in Figure 4. It can be seen that the recognition means comprises a net positive charge by virtue of the attachment of a positively charged arginine residue (R1) in the formula shown. Furthermore, this exemplary protease recognition means
10 comprises the two protease hydrolysis substrate sequences denoted as arrow A and arrow B as illustrated in Figure 4. The substrate sequence comprises a glycine residue (i.e. where R2 or R3 = H) and a phenylalanine residue (i.e. where the other of R2 or R3 = CH₂-C₆H₅).

As shown in Table 2 of Example 3, the protease, chymotrypsin, is known to
15 cleave preferentially at the carboxylic acid side of hydrophobic residues, whereas it is rather non-specific for the second amino acid. By contrast, thermolysin, preferentially cleaves hydrophobic residues at the amine end of the cleaved peptide bond. Finally, trypsin, cleaves selectively at the carboxylic acid side of positively charged residues.

Hence, the protease recognition means may comprise a suitable substrate
20 sequence for recognition and hydrolysis by any of the proteases chymotrypsin, thermolysin, or trypsin.

Preferably, the function of the protease used in conjunction with the particles according to the invention is to hydrolyse the substrate sequence of the protease recognition means in order to cause a change in the net charge of the particle. Hence,
25 preferably, the substrate sequence is positioned in between the charged species and the site of attachment to the hydrogel polymer, wherein following the hydrolytic action of the protease, the charged species is cleaved off the protease recognition means. Accordingly, following cleavage of the charged species, the net charge of the particle is preferably changed so that it becomes neutral. This is achieved by the
30 hydrolytic action of the protease, which is preferably adapted to remove the positive charge of the recognition means. This is illustrated in Figures 4 and 5 of the

Examples. As a consequence of the removal of the positive charge, the hydrogel particle collapses or adopts a de-swelled configuration. By the term “de-swelled configuration”, we mean the size of the particle is substantially reduced, and therefore has a lower average diameter compared to the size of the particle if it had a net positive (or negative) charge.

While the inventors do not wish to be bound by any hypothesis, they believe that this is because the repelling effect between the molecules of the hydrogel polymer has now been removed. As a result, when the hydrogel particle is de-swelled or collapsed, the pores between the polymer molecules decrease in size, and so the molecular accessibility of the particle is lowered.

By way of example, following modification of the protease recognition means with a protease, the average mesh size of the hydrogel polymer may be approximately 0.1 nm - 5nm. More preferably, the average mesh size may be approximately 0.2 nm - 4nm and most preferably, approximately 0.3 nm - 3nm. It is preferred that the average mesh size of the hydrogel matrix may be about 2nm, 1nm, 0.5nm, or 0.3nm.

Accordingly, suitably, the decrease in average mesh size of the hydrogel polymer following hydrolysis by the protease may be at least 1%, more suitably at least 2%, and even more suitably, at least 5%.

By way of example, following modification of the protease recognition means with a protease, the average Molecular Weight Cut-Off of the hydrogel polymer may be less than approximately 35kDa, and more preferably, less than about 20kD, and even more preferably, less than about 10kDa. Accordingly, suitably, the decrease in average Molecular Weight Cut-Off of the hydrogel polymer following hydrolysis by the protease may be at least 10%, more suitably at least 20%, and even more suitably, at least 50%.

The skilled technician will appreciate the different types of protease, which may be used. For example, the protease may be chymotrypsin, thermolysin, elastase, or trypsin.

In a second embodiment, the hydrogel particle may have a net negative charge before the action of the protease. For example, the protease recognition means may comprise a chemical species having a net negative charge.

5 Preferably, the chemical species has a pKa of less than 4 for an acidic moiety or greater than 9 for a base, thus ensuring that the molecules are in their charged states when used at pH 7 (for biomedical uses).

For example, the chemical species may comprise an amino acid having a net negative charge. For example, the protease recognition means may comprise an amino acid residue having a net negative charge. Examples of suitable amino acids include
10 glutamic acid or aspartic acid. The chemical species may comprise a non-natural amino acid. The chemical species may comprise for example, sulphonate. However, it is preferred that the protease recognition means comprises a glutamic acid residue.

It will be appreciated that the net negative charge provided by the protease recognition means causes the hydrogel particle to take on a swelled configuration, as
15 with the previous embodiment. This is because the molecules of the hydrogel polymer to which the protease recognition means are attached are repelled away from each other due to the net negative charge they carry. As a result, when the hydrogel particle is swelled, the pores between the polymer molecules are increased in size, and so the molecular accessibility of the particle is referred to as being high.

20 In addition to providing a net negative charge to the particle, it is preferred that the protease recognition means also comprises a protease hydrolysis substrate sequence for the protease. Preferably, the substrate sequence comprises a peptide bond, which attaches two amino acid residues together. Preferably, the function of the protease used in conjunction with the particles according to the invention is to
25 hydrolyse the substrate sequence of the protease recognition means in order to cause a change in the net charge of the particle. Hence, preferably, the substrate sequence is positioned in between the charged species and the site of attachment to the hydrogel polymer, wherein following the hydrolytic action of the protease, the charged species is cleaved off the protease recognition means. Accordingly, following cleavage of the
30 charged species, the net charge of the particle is preferably changed so that it becomes neutral. This is achieved by the hydrolytic action of the protease, which is preferably

adapted to remove the negative charge of the recognition means. As a consequence of the removal of the negative charge, the hydrogel particle collapses or adopts a de-swelled configuration. As a result, when the hydrogel particle is de-swelled or collapsed, the pores between the polymer molecules decrease in size, and so the molecular accessibility of the particle is lowered.

In a third embodiment, the hydrogel particle may have a net neutral charge prior to the hydrolytic action of the protease. For example, the protease recognition means may comprise a first chemical species having a net positive, and in addition preferably, a second chemical species have a net negative charge, which when taken together neutralise the overall charge of the protease recognition means. The first and second chemical species may be amino acid residues having positive and negative charges, respectively.

It will be appreciated that the net neutral charge provided by the protease recognition means causes the hydrogel particle to adopt a de-swelled or collapsed configuration before the action of the protease. This is because the molecules of the hydrogel polymer to which the protease recognition means are attached are attracted to each other due to the positive and negative charges they carry, i.e. electrostatic interactions cause the hydrogel molecules to attract each other. As a result, when the hydrogel particle is de-swelled, the pores between the polymer molecules are decreased in size, and so the molecular accessibility of the particle is referred to as being low.

As with the previous embodiments of the invention, in addition to providing a net neutral charge to the particle, it is preferred that the protease recognition means also comprises a protease hydrolysis substrate sequence for the protease. Preferably, the substrate sequence comprises a peptide bond, which attaches two or more amino acid residues together. Preferably, the function of the protease used in conjunction with the particles according to the invention is to hydrolyse the substrate sequence of the protease recognition means in order to cause a change in the net charge of the particle. Hence, preferably, the substrate sequence is positioned in between the two charged species, wherein following the hydrolytic action of the protease, one of the two charged species is cleaved off the protease recognition means.

By way of example, the substrate sequence may be –Ala-Ala- or –Gly-Gly-. Furthermore the charged residues flanking the substrate sequence may be Arg and Asp.

Following the hydrolytic action of the protease, the protease cleaves off either
5 the positively charged or negatively charged residue(s) from the protease recognition means. Accordingly, following the hydrolytic action of the protease, the net charge may be changed so that it is either positive or negative, depending on whether the cleaved residue is positive or negative. For example, if the protease is adapted to cleave off a negatively charged species, then the resultant net charge of the particle
10 will be less negatively charged, neutral (zwitterionic) or positive. Conversely, if the protease is adapted to cleave off a positively charged species, then the resultant net charge of the particle will be less positively charged, neutral (zwitterionic), or negative. Which chemical species is cleaved off by the protease will depend on the respective positioning of the positively and negatively charged species and the
15 substrate sequence in relation to the attachment site of the protease recognition means to the hydrogel polymer.

Accordingly, in the third embodiment, the result of the hydrolytic action of the protease is to produce a particle, which has either a net positive charge or a net negative charge. Therefore, it will be appreciated that following protease action, the
20 particle will swell in size due to electrostatic effects between either the positively charged or negatively charged hydrogel polymer molecules. As the particle swells, the pore size increases and so does the molecular accessibility. As a result, when the hydrogel particle is swelled, the pores between the polymer molecules increase in size, and so the molecular accessibility of the particle is increased.

25 For example, an increase in the average Molecular Weight Cut-Off may be from 35kDa to over 70kDa.

Hence, it will be appreciated that an important feature of the particle according to the invention is the ability to be able to control the swelling and de-swelling, and hence, increase and decrease the molecular accessibility of the hydrogel particle by
30 changing its net charge. This is achieved by the careful selection of the protease recognition means, and in particular the specific sequence of the protease hydrolysis

substrate sequence, which is determined by specific proteases used. In addition, the construction of the protease recognition means is important, as the respective positioning of the charged species (either positive, negative, or both) with respect to the substrate sequence within the protease recognition means is important to
5 determine how the net charge is changed following hydrolysis by the protease.

Accordingly, it is preferred that the particle comprises PEGA hydrogel, and a protease recognition means having either a net positive, negative, or neutral charge. Furthermore, preferably, the protease recognition means comprises a suitable protease hydrolysis substrate sequence, which is adapted to be hydrolysed by a specific
10 protease enzyme. Examples, of preferred embodiments of the hydrogel particle according to the invention will be described hereinafter in conjunction with a number of different examples of the uses to which the particle may be put.

The swelling/de-swelling effect of the particles in accordance with the invention under the control of the protease may be used in a wide variety of ways. For
15 example, the particle may be in the field of detection, encapsulation, entrapment, and/or delivery of sensitive biological molecules (payload molecules) to target environments. One preferred example is the use of the hydrogel particle according to the invention to entrap or purify target proteases from solutions of impure protease or other biomolecules. Hence, the hydrogel particle may be used in a separation or
20 purification column, either to supplement or replace existing biochemical purification methods. A second example is the use of the hydrogel particle in medicine. For example, the particle may be used to entrap and thereby remove deleterious protease enzymes, which are produced by a variety of different diseases, for example, inflammation. A third example is the use of the hydrogel particle for the effective
25 delivery and release of active payload molecules to a target site. Each of these preferred uses will now be described in detail below.

(1) Smart Column

The hydrogel particle may be used to purify or isolate a target protease enzyme, for example, from a solution containing a mixture of biomolecules, which
30 includes the target protease. The target protease to be isolated from the solution may be either known (i.e. the researcher knows what the protease is) or unknown (i.e. the

researcher does not know what the protease is). For example, the known target enzyme may be present in an impure sample, which may be contaminated with other unwanted enzymes or other biomolecules. Alternatively, the particle may be used to isolate or purify an unknown target protease, for example, from a solution containing a mixture of potentially active enzymes. In the field of biocatalysis, it is often the case that a certain catalytic reaction may be required, for example, the hydrolysis of a peptide bond flanked by a specific sequence. However, it may be the case that the specific protease which would catalyse that reaction has not been isolated. Accordingly, there is need to set up an assay wherein it is possible to isolate the protease which would carry out the desired reaction. The inventors believe that the hydrogel particles according to the invention may be used in such an assay.

The key to the invention is that the hydrogel particle may be ‘programmed’ by specifically choosing the substrate sequence in the protease recognition means such that *only* the target protease (whether known or unknown) is able to hydrolyse the peptide bond, and thereby cause the change in net charge of the particle.

Hence, accordingly, in a fourth aspect of the invention, there is provided use of a hydrogel particle in accordance with the first aspect for purifying a solution of target protease.

In a fifth aspect, there is provided a method of purifying a target protease from a solution, the method comprising the steps of:-

- (i) contacting a solution containing a target protease with a hydrogel particle according to the first aspect; and
- (ii) separating the hydrogel particle from the solution.

In a further aspect, there is provided use of a hydrogel particle according to the first aspect for isolating a target protease from a solution.

The solution may comprise either a low concentration of the target protease, or a solution containing the target protease in addition to at least one other non-target biomolecule (e.g. enzyme). By the term “target protease”, we mean the protease

enzyme, which is to be purified. By the term “non-target biomolecule”, we mean any other molecule, which is not to be purified.

The uses and method are carried out by initially designing the protease recognition means of the hydrogel particle such that it preferably comprises the substrate sequence that is catalytically recognised by the target protease to be purified. If the target protease is known, then the researcher can incorporate the corresponding substrate sequence into the protease recognition means. However, if the target protease is not known, then the researcher can incorporate the substrate sequence, for which he would like to isolate a protease adapted to hydrolyse that specific sequence.

It is preferred that the particle has either a net positive charge or a net negative charge prior to protease hydrolysis. Hence, preferably, the particle has a swelled configuration, such that the pore size is sufficiently large to enable enzymes to permeate into the particles, i.e. it has an increased molecular accessibility. The hydrogel particle is preferably added to the solution, and the resultant solution may then be mixed, by suitable means.

It will be appreciated that ‘non-target’ molecules (e.g. enzymes) will not recognise the protease recognition means because the latter will not have the correct substrate sequence for these non-target enzymes. Hence, such non-target enzymes will be unable to hydrolyse the protease recognition means, and accordingly, such non-target enzymes, will permeate out of the particle. However, when the target protease enzyme passes into the hydrogel particles, it will be able to recognise and therefore hydrolyse the protease recognition means, thereby cleaving off the charged species, which may be either a positive or negative. For example, the charged species may be either a positively charged or negatively charged amino acid residue.

As a result of such protease hydrolysis, the net charge of the particle will be altered, i.e. it will change from either a net positive or negative charge to a net neutral charge. Consequently, the electrostatic repelling effect of the net positive or negative charge is removed, such that the particle collapses to adopt a de-swelled configuration. The result of the collapse is that the target protease, which hydrolysed the protease recognition means, is entrapped within the collapsed particle. Because

the particle has collapsed, the pore size has decreased, and accordingly, the target enzyme is unable to permeate out of the particle.

The use or method may comprise a step of separating particles from the solution. It may be preferred to filter the particles on the basis of their size. For example, only a portion of the particles may have successfully entrapped the target protease, which will have adopted the collapsed configuration. Any other non-collapsed particles, will not have had their protease recognition means cleaved, and accordingly, will not have entrapped the target protease. Accordingly, by separating the collapsed particles from the non-collapsed particles, it will be possible to purify those particles, which have entrapped the target protease.

Preferably, the method comprises eluting the target protease from the collapsed particles by altering the net charge of the particle so that they swell, and thereby release the target protease. Accordingly, the target protease, whether known or unknown, will be purified.

In a sixth aspect, there is provided apparatus for purifying a protease, the apparatus comprising a hydrogel particle according to the invention, which particle is immobilised on a support surface.

The support surface may comprise a column. Conventional biochemical purification involves the use of a column, which may, for example, be loaded with Sephadex. Hence, preferably, the column comprises at least one particle, and preferably a plurality of particles, according to the first aspect. Preferably, the at least one particle is attached to the column using suitable means. The at least one particle may be attached to the column as a film. For example, a polymer film may be spincoated onto a glass slide, which may be suitably modified, for example with, epoxide. Initiator, such as photoinitiator may then be added, and polymerisation may then be initiated using suitable means, for example, by UV. Patterns of the film may be obtained by using photo masks, which will be known to the skilled technician.

A solution comprising a mixture of biomolecules, including the target protease and non-target biomolecules, may then be applied to the apparatus. Only the target protease will trigger collapse of the particles, due to the specificity of the protease

recognition means and the highly specific catalytic activity of the target protease. Hence, all of the target biomolecules will be entrapped within the particles. However, it will be appreciated that some non-target molecules may also be co-entrapped, and so will run through the column. The column may be vertically arranged, and the initial solution may be applied to the top of the column. Most of the non-target biomolecules should run through the column and exit at its base. Accordingly, only the target protease will be entrapped within the immobilised particles. The target protease may then be eluted from the column by passing a solution adapted to induce either a net positive or net negative charge to the particles.

(2) Medical Uses

The inventors have found that the swelling/de-swelling effect exhibited by the particle in response to the hydrolytic action of a protease may also be used in a variety of different medical uses. Hence, the particles in accordance with the invention may also be used in the treatment of certain disease states, and in therapies and/or diagnostics relating to certain disease conditions.

Hence, according to a seventh aspect of the invention, there is provided a hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle, for use as a medicament.

It is preferred that the hydrogel particle is particularly useful for treating conditions in which the individual to be treated suffers from inflammation or inappropriate wound healing. Examples of such pathological situations involved with inflammation and inappropriate wound healing include the process of scar formation (angiogenesis sites: where new blood vessels and capillaries are quickly created). Hence, the hydrogel particle according to the invention offers new biomedical applications for use in treating such conditions. Inflammation in the body may be characterized by the presence of an excessive amount of certain protease enzymes at the site of inflammation. Therefore, the inventors have found that is possible to alleviate inflammation by either reducing the activity of, or removing entirely, such proteases from the site of inflammation.

Hence, according to an eighth aspect of the invention, there is provide use of a hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle, for the manufacture of a medicament for the treatment of inflammation, or inappropriate wound healing.

According to a ninth aspect, there is provided a method of treating an individual suffering from inflammation or inappropriate wound healing, the method comprising administering to an individual in need of such treatment, a therapeutically effective amount of a hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle.

In one embodiment, the hydrogel particle according to the invention may be capable of entrapping, or encapsulating protease enzymes produced in a subject, which proteases may be produced at a wound or inflamed site on the body.

Two important families of proteases involved in wound healing are serine proteases (for example, neutrophil elastase) and Matrix Metalloproteineases (MMPs). The MMP proteases specifically involved in wound healing are the collagenases (which degrade the connective tissue collagen), gelatinases (degrade basement membrane), and stromelysins (degrade ECM proteoglycans).

Figure 10 provides examples of a small selection of MMPs, which are involved in wound healing. For example, Figure 10 provides details of:- (i) collagenases (MMP-1, MMP-8, MMP-13); (ii) gelatinases (MMP-2, MMP-9); (iii) stromelysin (MMP-3, MMP-10, MMP-11); and (iv) MMP-12. In addition, Figure 10 gives details of neutrophil elastase, thrombin and elastase. Figure 10 also provides details of the various substrates for each protease listed, and in addition, gives detail about the substrate specificity of each protease of interest (target protease), i.e. the sequence of amino acids, which each protease recognises and can hydrolyse.

For example, MMP-1 protease has catalytic specificity for a range of different collagens such as (I, II, III, VII, VIII, X, XI); gelatin; aggrecan; tenascin; L-selectin; IL-1Beta; proteoglycans; entactin; ovostatin; MMP-2; MMP-9. More specifically, MMP-1 recognises, and hydrolyses the following sequences:-

- 5 (i) Ac-Pro-Leu-Gly-Ser~Leu-Leu-Gly-OEt;
- (ii) Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH₂;
- (iii) Pro-Met-Ala~Leu-Trp-Ala-Thr;
- (iv) Leu-Pro-Met~Phe-Ser-Pro;
- (v) Ac-Pro-Leu-Ala-Ser~Nva-Trp- NH₂;
- 10 (vi) Arg-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr;
- (viii) Pro-Glu-Gly~Ile-Ala-Gly;
- (ix) Pro-Glu-Gly~Leu -Leu-Gly.

Hence, the inventors have created hydrogel particles in accordance with the invention, in which the protease recognition means comprises a substrate sequence for
 15 each of the proteases as summarised in Figure 10. By way of example, the hydrogel particle may comprise a protease recognition means comprising a substrate sequence independently selected from a group consisting of:-

- (i) Ac-Pro-Leu-Gly-Ser~Leu-Leu-Gly-OEt;
- (ii) Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH₂;
- 20 (iii) Pro-Met-Ala~Leu-Trp-Ala-Thr;
- (iv) Leu-Pro-Met~Phe-Ser-Pro;
- (v) Ac-Pro-Leu-Ala-Ser~Nva-Trp- NH₂;
- (vi) Arg-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr

(viii) Pro-Glu-Gly~Ile-Ala-Gly; and

(ix) Pro-Glu-Gly~Leu -Leu-Gly.

It will be appreciated that that each of these recognition sequences could be preferentially recognised and cleaved only by the protease enzyme, MMP-1. Hence,
5 by introducing into the patient, a particle according to the invention, which has a protease recognition means as defined above, it is possible to entrap or 'mop' up excess concentrations of MMP-1. Once the particle has entrapped the target protease, it may then leave the target environment, and be subsequently removed from the patient. Hence, the particles will effectively remove high concentrations of deleterious
10 enzymes from a patient, and thereby alleviate the condition.

It should be appreciated that it will be possible to create a range of hydrogel particles according to the invention, each of which comprise specific protease recognition means which would be recognised and hydrolysed by any of the protease illustrated in Figure 10. In addition, it is possible to 'program' the particle to entrap
15 any protease, which causes a deleterious disease state.

In one application, the hydrogel particle may be administered in the bloodstream, and accumulate selectively in angiogenesis sites, for example, wounds and tumours.

In another application, the hydrogel particles may be administered
20 percutaneously in regions of limited or negligible lymphatic uptake and remain there to express their activity. Alternatively, they can be injected percutaneously in regions of good lymphatic uptake and express their activity in the lymphatic system for a period of up to 2-3 weeks, before being released in the bloodstream.

In another embodiment, the hydrogel particles may be administered directly to
25 wounded or inflamed site on the body. For example, the particles may be loaded on to a wound dressing (e.g a bandage or wipe), which may be placed over the wounded or inflamed area. The particles may then be exposed to the injured area, and are preferably adapted to permeate therethrough. Upon sufficient exposure to the particles, the protease enzymes produced in the wound will be absorbed into the

particles due to the result of the hydrolysis of the protease recognition means followed by the change in net charge, and consequential de-swelling.

In a preferred embodiment, the hydrogel particle comprises an average diameter of approximately 500 nm. The particle comprises a hydrogel matrix comprising polyethylene glycol acrylamide PEGA, in a ratio of PEGA monomer:Acrylamide of about 30:1. This composition ensures good mechanical stability, appropriate mesh size (in the range of about 2-3 nm), and suitable resistance to protein adsorption.

It will be appreciated that the hydrogel particle according to the present invention may be used in a monotherapy (i.e. use of the hydrogel particle according to the invention alone to prevent and/or treat inflammation or diseases characterised by inappropriate wound healing). Alternatively, the hydrogel particle according to the invention may be used as an adjunct, or in combination with known therapies.

Hydrogel particles according to the invention may be formulated in a composition. The composition may have a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, transdermal patch, or any other suitable form that may be administered to a person or animal in a hydrated or moist form. It will be appreciated that the vehicle of the composition should be one which is well tolerated by the subject to whom it is given, and preferably enables delivery of the carrier particle to a target tissue.

Compositions comprising the hydrogel particle according to the invention may be used in a number of ways. For instance, systemic administration may be required in which case the carrier particle may be contained within a composition that may, for example, be ingested orally in the form of a capsule or liquid. Preferably, the composition may be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion). The composition may also be administered by inhalation (e.g. intranasally).

The hydrogel particle may also be incorporated within a slow or delayed release device. Such devices may, for example, be inserted on or under the skin, and the composition may be released over weeks or even months. Such devices may be particularly advantageous when long-term treatment with a hydrogel particle according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

It will be appreciated that the amount or number of hydrogel particles that is required is influenced by the required amount of payload molecule encapsulated therein, and its biological activity and bioavailability, which in turn depends on the mode of administration, the physicochemical properties of the particle employed and whether the particle is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the above-mentioned factors and particularly the half-life of the encapsulated payload and hydrogel particle, within the subject being treated.

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the payload molecule and hydrogel particle in use, the strength of the preparation, the mode of administration, and the advancement of the disease condition. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to establish specific formulations of the hydrogel particle according to the invention and precise therapeutic regimes (such as daily doses of the carrier particle and the frequency of administration).

It will be appreciated that the dose of hydrogel particle is highly dependent on the specific payload molecule being carried, and the target cell, and the disease being treated. However, generally, a daily dose of between 0.01 $\mu\text{g/kg}$ of body weight and 0.5 g/kg of body weight of the particle according to the invention may be used for the prevention and/or treatment of inflammation or inappropriate wound healing, depending upon which specific hydrogel particle and payload molecule is used. More

preferably, the daily dose is between 0.01 mg/kg of body weight and 200 mg/kg of body weight, and most preferably, between approximately 1mg/kg and 100 mg/kg.

Daily doses may be given as a single administration (e.g. a single daily injection). Alternatively, the hydrogel particle used may require administration twice or more times during a day. As an example, the hydrogel particle according to the invention may be administered as two (or more depending upon the severity of the condition) daily doses of between 5 mg and 7000 mg (i.e. assuming a body weight of 70kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3 or 4 hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses to a patient without the need to administer repeated doses.

This invention provides a pharmaceutical composition comprising a therapeutically effective amount of a hydrogel particle according to the invention and optionally, a pharmaceutically acceptable vehicle. In one embodiment, the amount of the hydrogel particle is an amount from about 0.01 mg to about 800 mg. In another embodiment, the amount of the particle is an amount from about 0.01 mg to about 500 mg. In another embodiment, the amount of the particle is an amount from about 0.01 mg to about 250 mg. In another embodiment, the amount of the hydrogel particle is an amount from about 0.1 mg to about 60 mg. In another embodiment, the amount of the particle is an amount from about 0.1 mg to about 20 mg.

This invention provides a process for making a pharmaceutical composition comprising combining a therapeutically effective amount of a hydrogel particle according to the invention and a pharmaceutically acceptable vehicle. A “therapeutically effective amount” is any amount of a hydrogel particle according to the invention which, when administered to a subject provides prevention and/or treatment of inflammation or inappropriate wound healing. However, it will be appreciated that the type and amount of payload molecule in the hydrogel particle will contribute to the therapeutic efficacy of the particle. A “subject” is a vertebrate, mammal, domestic animal or human being.

A “pharmaceutically acceptable vehicle” as referred to herein is any physiological vehicle known to those of ordinary skill in the art useful in formulating

pharmaceutical compositions. In a preferred embodiment, the pharmaceutical vehicle is a liquid and the pharmaceutical composition is in the form of a solution. In a further embodiment, the pharmaceutical vehicle is a gel and the composition is in the form of a cream or the like.

5 Liquid vehicles are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The hydrogel particle may be dissolved or suspended in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can contain other suitable pharmaceutical additives such as solubilisers,
10 emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols
15 and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid vehicle for pressurized compositions can be halogenated hydrocarbon or other
20 pharmaceutically acceptable propellant.

 Liquid pharmaceutical compositions, which are sterile solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal, subcutaneous, and particularly, intravenous injection. The hydrogel particle may be prepared as a sterile solid composition that may be dissolved or
25 suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Vehicles are intended to include necessary and inert binders, suspending agents, lubricants, flavourants, sweeteners, preservatives, dyes, and coatings.

 The hydrogel particle according to the invention can be administered orally in
30 the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile

salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

The carrier particle according to the invention can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

The hydrogel particle according to the first aspect of the invention may be adapted to carry a payload molecule therein, and is preferably capable of carrying the payload molecule to a target biological environment. Hence, in this embodiment, the hydrogel particle according to the first aspect of the invention may also comprise a further component in addition to component (i) precursor, and component (ii) protease recognition means, as discussed above, i.e. (iii) a payload molecule. The particle comprising the payload molecule may be used in accordance with the eighth and ninth aspects. It is preferred that the payload molecule is substantially active when it is at least adjacent the target biological environment.

The inventors have found that the hydrogel particle according to the invention is very useful for targeting the payload molecule to a particular target biological environment, which may for example, be any body fluid, or a specific tissue. Hence, the target biological environment may be a body fluid with circulation extended to substantially all of the body, such as blood or lymph, or a body fluid with limited circulation, such as intraperitoneal or synovial fluids.

Hence, preferably, the hydrogel particle is sufficiently small such that it may be suspended in fluids in a body, for example, the bloodstream. Therefore, it is preferred that the particle is sufficiently small such that it can pass along the vasculature system of an individual's body, i.e. veins, arteries, and/or capillaries.

Suitable sizes of the carrier particle are approximately 1nm - 2000nm, preferably, 50 nm - 1000 nm, more preferably 50 - 500 nm, and most preferably, 50 - 100 nm. Preferably, the carrier particle is substantially spherical in shape. Hence, the

dimensions given above are the average diameter of the carrier particle. However, depending on environmental conditions a hydrogel particle may be modified to an elliptical or other shape. For example, an initial spherical geometry can become elliptical, due to the action of shear forces, or biconcave, due to higher external osmotic pressure.

In a specific tissue, the target biological environment to which the payload molecule may be targeted may be a cell, or a group of cells. It is preferred that the target biological environment is outside a cell, i.e. in the extracellular matrix, or at the surface of the cell. Hence, by the term "at least adjacent the target biological environment", it will be appreciated that the payload molecule is capable of being biochemically active when the hydrogel particle is sufficiently close to the target biological environment, for example, the cell or group of cells, and preferably outside the cell or cells.

The group of cells may constitute a tissue or, for example, a cancerous body. The carrier particle may be used for performing biochemical conversions, or for the *in vivo* production of pharmacologically active components. For example, the hydrogel particle may be used to perform chemical transformations of inert pro-drugs into corresponding active components (drugs) at the target environment, for example, in chemotherapeutics. Alternatively, the hydrogel particle may be used in the targeted delivery of an active compound towards a tumoral mass.

The payload molecule or particle, which is encapsulated within the hydrogel particle, may be any molecule or particle, which has suitable activity with, or against a target biological environment, for example, a target cell type. For example, the payload molecule may have catalytic activity. It is preferred that the compound has bioactive properties, i.e. the compound has a biological effect on reaching the target environment. Examples of the compounds bioactive properties are enzymatic reactions that hydrolyse protecting groups and transform pro-drugs into active drugs.

The payload molecule may remain in an active state while it is encapsulated within the hydrogel particle, preferably, as it is being carried to the target environment. However, preferably, once the hydrogel particle reaches the target environment, the payload molecule is released into the target environment. Therefore,

the payload molecule may be a sufficiently large such that it is unable to permeate through the hydrogel matrix as the hydrogel particle moves towards the target environment. Hence, it will be appreciated that the size of the encapsulated molecule used is dependent on the structure, and composition, and mesh size, and MWt cut-off value of the hydrogel layer so that it is retained therein. Preferably, the encapsulated molecule has a molecular size greater than 0.5 nm across, more preferably, greater than 1 nm, and even more preferably, greater than 2 nm across.

The hydrogel particle preferably has a net neutral charge prior to the hydrolytic action of the protease. For example, the particle may be designed as in the third embodiment described above, wherein the protease recognition means may comprise a first chemical species having a net positive, and in addition, a second chemical species having a net negative charge, which when taken together neutralise the overall charge of the protease recognition means. It will be appreciated that the net neutral charge provided by the protease recognition means therefore causes the hydrogel particle to adopt a de-swelled or collapsed configuration before the action of the protease. As a result, when the hydrogel particle is de-swelled, the pores between the polymer molecules are decreased in size, and so the molecular accessibility of the particle is referred to as being low.

In preparing the particle, the payload molecule may be water-soluble and may be incorporated into the PEGA particle during the polymerisation process. For example, the payload molecule may be a dye, electrochemical mediator, peptide, protein, antibody, drug molecule, or enzyme. The payload molecule may be derivatised with oligo- or polymeric species. The derivatization may be necessary if any of the previously mentioned examples does not reach the required size for avoiding permeation through the hydrogel layer. Examples of suitable enzymes, which may be encapsulated in the hydrogel particle, may include hydrolases, such as proteases and esterases.

Alternatively the hydrogel particles may be loaded with the payload by applying a pH switch, where an increase or decrease of pH may change the charge on the particle from neutral to positive or negative, thus allowing the payload to diffuse

into the particle. Changing the pH back to neutral will ensure that the payload remains entrapped.

Hence, the payload molecule is actively trapped in the de-swelled hydrogel particle 2. As with the previous embodiments of the invention, in addition to providing a net neutral charge to the particle, it is preferred that the protease recognition means also comprises a protease hydrolysis substrate sequence for the protease. Preferably, the function of the protease used in conjunction with the particles according to the invention is to hydrolyse the substrate sequence of the protease recognition means in order to cause a change in the net charge of the particle.

Hence, preferably, the substrate sequence is positioned in between the two charged species, wherein following the hydrolytic action of the protease, one of the two charged species is cleaved off the protease recognition means.

The particle may then be introduced into the subject to be treated, wherein the particle then moves to the target environment. On reaching the target environment, the particle will then be exposed to a protease enzyme, which will hydrolyse the peptide bond in the hydrolysis substrate sequence. For example, in wound sites, there may be a high concentration of a certain protease that will hydrolyse the substrate sequence. Following the hydrolytic action of the protease, the protease cleaves off either the positively charged or negatively charged residue from the protease recognition means. Accordingly, following the hydrolytic action of the protease, the net charge of the particle is preferably changed so that it is either positive or negative, depending on whether the cleaved residue is positive or negative.

Accordingly, the result of the hydrolytic action of the protease is to produce a particle, which has either a net positive charge or a net negative charge. Therefore, it will be appreciated that following protease action, the particle will swell in size due to electrostatic effects between either the positively charged or negatively charged hydrogel polymer molecules. As the particle swells, the pore size increases and so does the molecular accessibility (Molecular Weight Cut-off). As a result, when the hydrogel particle is swelled, the pores between the polymer molecules increase in size, and so the molecular accessibility of the particle is increased. Hence, the payload molecule is preferably released into the target environment, where it can act on the

subject. Advantageously, the use of the hydrogel particle is a way of encapsulating a payload molecule and delivering it to a target environment where it can be released.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Although the invention has been described with particular reference to hydrogel particles that comprise protease recognition means it will be appreciated that variations are possible. Thus, for example, where the end use application permits the hydrogel may be in a physical form other than a particle. Thus the hydrogel may be provided as a film or layer (e.g. on a support surface). A film may be formed, for example, by spin-coating, e.g. on to a support surface. In a further modification (which may be applied to hydrogels either as particles or in another physical form) the hydrogel may incorporate any enzyme recognition means (rather than necessarily a protease recognition means). Thus, for example, the hydrogel may incorporate a polysaccharide for recognition by a glycosidase or an amylase, a polynucleotide for recognition by a DNAase or a RNAase, or an ester for recognition by an esterase or a lipase.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

Figure 1 shows chemical formulae of embodiments of monomers, which may be used in accordance with the invention;

Figure 2 shows chemical formulae of embodiments of monomers, which may be used in accordance with the invention;

Figure 3 shows embodiments of hydrogel polymer polyethylene glycol acrylamide (PEGA);

Figure 4 is a schematic diagram of a first embodiment of an enzyme responsive polymer hydrogel particle in accordance with the invention. The polymer particles 1 consist of acrylamide backbones cross-linked with polyethylene glycol (PEGA₈₀₀). Polymer particles are functionalised through their amine groups with enzyme cleavable peptide linkers using standard Fmoc-peptide synthesis methods (for example, see http://www.emdbiosciences.com/SharedImages/TechnicalLiterature/6_Fmoccleavage.pdf). Peptides are composed of an enzyme cleavable section (consisting of Gly (i.e. R=H) or Phe (i.e. R=CH₂-C₆H₅) and carry a positive charge through an Arginine (i.e. side chain is R1) residues causing them to swell to produce Enzyme Reponsive Polymer (ERP) particle 2. Upon exposure to proteases that are able to cleave (at enzyme cleavage sites A or B) the positive charge is removed, resulting in collapse of the bead structure to form particle 3;

Figure 5 is a schematic diagram showing the swelling and collapse (de-swelling) of the hydrogel particle in accordance with the invention;

Figure 6 represents data from HPLC analysis of enzyme modifications of the peptides Fmoc-Gly-Phe, and Fmoc-Phe-Gly with thermolysin and chymotrypsin. The upper line represents the action of chymotrypsin, and the lower line represents the action of thermolysin;

Figure 7 represents data from HPLC analysis of an enzyme modification of the peptide Fmoc-Gly-Phe with thermolysin and chymotrypsin. The upper line represents the action of chymotrypsin, and the lower line represents the action of thermolysin;

Figure 8 represents data from HPLC analysis of an enzyme modification of the peptide, Fmoc-Arg⁺-Phe-Gly, with thermolysin and chymotrypsin;

Figure 9 represents on the left hand image two photon microscopy images of single representative particles show diffusion of fluorescein labelled 77kDa dextran into the centre of the PEGA bead after 1, 2, 5, 10 minutes. The right hand image shows the pixel intensity after 10 minutes (from top to bottom the lines represent: control 4 (no enzyme), 4b, 4c, 4a, control 2 (no Arginine). These images clearly show that

treatment of bead 4 with trypsin and thermolysin decreases the accessibility of the labeled dextran;

Figure 10 is a table providing examples of proteases involved in wound healing and their substrate specificity;

5 **Figure 11** is a schematic diagram of a second embodiment of enzyme responsive polymer hydrogel particle in accordance with the invention;

Figure 12 illustrates the results of Example 4;

Figure 13 is a schematic diagram of a column in accordance with the invention; and

10 **Figure 14** is a schematic diagram of a use of the hydrogel particle for delivery of a drug payload.

The inventors have devised a class of enzyme responsive polymer hydrogel particles (ERPs), in which the molecular accessibility of the hydrogel polymer can be selectively controlled by enzymes present in the biological environment. The inventors envisage that these enzyme responsive polymers (ERPs) may be put to a
15 variety of therapeutic, diagnostic, and analytical uses. For example, the ERPs may be used for selectively entrapping proteases, which may cause disease, or for delivering drugs to a target tissue, and so on.

Embodiments of the invention are described with reference to the following non-limiting examples in which Examples 1-3 relate to the first embodiment of the
20 invention illustrated in Fig 4 of the drawings and Examples 4 relate to the second embodiment of the invention illustrated in Fig 11 of the drawings.

First Embodiment

Example 1A schematic of the overall design of Enzyme Responsive Polymers (ERPs) 2 in accordance with a first embodiment of the invention is shown in Figure 4.
25 As the starting point, commercially available PEGA₈₀₀ polymer particles 1 (Polymer Labs, UK; co-polymers of polyethylene glycol and acrylamide), were used, as shown on the left-hand side of Figure 4. The PEGA polymer particles 1 are converted into

ERPs 2, by the incorporation of a linker sequence 4, which is responsive to the hydrolytic action of at least one specific protease enzyme 5. The result of enzymatic hydrolysis by the protease is to digest the linker sequence 4, and to produce a hydrolysed particle 3.

5 Referring in more detail to Figure 4, the PEGA polymer particles 1 are made of inter-connected chains of the polymer, polyethylene glycol and acrylamide PEGA (PEGA - A flow stable polyethylene-glycol dimethylacrylamide copolymer for solid-phase synthesis, Meldal M., Tetrahedron Letters 33 (21): 3077-3080 May 19 1992.)

10 The monomers used are shown in Figures 1 and 2, to produce the polymer shown in Figure 3. The PEGA particles 1 are highly hydrated (consisting of more than about 90% water), and their high polyethylene glycol content prevents non-specific protein adsorption (i.e. they are 'non-fouling'). It is known that PEGA polymers are compatible with biological molecules such as enzymes, as it has been shown that enzymes are able to remain active and also selective inside the polymer material
15 whilst being able to access the interior of the particles 1. Therefore, the inventors of the present invention believe that the PEGA hydrogel would form a suitable substance for the entrapment or delivery of different biomolecules, depending on the ultimate use of the particles according to the invention. Example 6 describes the use of the particles 2 for the delivery of active payload molecules 20.

20 The inventors of the present invention have previously demonstrated that that PEGA₁₉₀₀ molecular accessibility may be improved by the introduction of permanently charged quaternary amine groups in the backbone of the polymer during the polymerization process, resulting in cationic PEGA (Basso *et al.*, 2003, Chem. Commun., 1296-1297). However, the inventors of the invention, have now found that,
25 by separating the positive charge from the hydrogel polymer backbone via an enzyme cleavable linker 4, it is surprisingly possible to achieve controlled hydrogel polymer accessibility that is *only* reduced in the presence of a protease that is able to cleave the specific peptide bond(s) present in the enzyme cleavable linker 2, as shown in Figure 4. Hence, the inventors believe that the hydrogel particle (ERPs) 2 according to the
30 invention are essentially 'programmable', and their molecular accessibility may be varied as and when required.

In order to convert the hydrogel particles 1 into the enzyme responsive ERPs 2 in accordance with the invention, the particles 1 are functionalised with a range of specific enzyme cleavable linkers 4. This functionalisation step is achieved using standard peptide synthesis methods known to the skilled technician (Meldal discussed *supra*).

The peptide linker 4 shown in Figure 4 has two peptide cleavable recognition sites, denoted A and B. Each of these recognition sites A and B may be hydrolysed by a specific protease 5 having specificity for either A or B. By way of example, the peptide linkers 4 shown in Figure 4 are composed of an enzyme cleavable section consisting of a glycine residue (i.e. where R2 or R3 = H) or a phenylalanine residue (i.e. where the other of R2 or R3 = CH₂-C₆H₅). As will be described in the following Examples, the inventors choose these specific amino acid residues for inclusion in the linker 4 shown in Figure 4, as they knew that they would be selectively modified by different proteases, i.e. either thermolysin, chymotrypsin or trypsin.

The enzyme cleavable linkers 4 are capped with a positively charged Fmoc-Arg⁺ residue at the position R1, as shown in Figure 4. The purpose of the Fmoc-Arg⁺ residue in the linker 4 is to induce the hydrogel polymer of the ERP 2 to swell, and this was caused by electrostatic repulsion between the polymer chains. As can be seen in Figure 4, because of the positive charge of the arginine residue on each linker 4, the ERP 2 has considerably swelled in size compared to that of the initial uncharged bead 1. The result of this swelling is to cause an increase in the pore or mesh size of the hydrogel component of the resultant ERP 2. Hence, as the mesh size of the ERP 2 increases, so does the molecular accessibility of the ERP 2, i.e. the ability for molecules to diffuse inter and permeate through the ERP 2. Therefore, the provision of the Fmoc-Arg⁺ residue is to cause swelling of the ERP 2, which increases its ability to absorb molecules.

When the ERP 2 is in use, it is possible to harness the ability of the ERP 2 to swell and de-swell, and to absorb molecules in the surrounding environment, as illustrated in Figure 4. The ERP 2 particle is exposed to an environment containing a protease enzyme 5, which has catalytic specificity for either enzyme cleavage site A or B of the linker 4. Upon enzymatic hydrolysis of the enzyme cleavable linker 4 in

the ERP 2, the positive charge of the Fmoc-Arg⁺ residue is removed by hydrolysis either at recognition site A or B. As a result of the loss of the positive charge from the linker 4, the ERP 2 collapses or de-swells, thereby producing particle 3. As a result of the collapse in size from the ERP 2 to the particle 3, there is a concomitant decrease in the pore size of the hydrogel polymer, which thereby decreases its molecular accessibility. Accordingly, the protease enzyme 5, which caused the removal of the linker 4, is absorbed into and entrapped within the hydrogel polymer matrix.

This mechanism is described in further detail with respect to Figure 4. Referring to Figure 5, there is shown an embodiment of the hydrogel particle according to the invention in use. The hydrogel bead 1 is initially functionalised by the integration of a number of positively charged linkers 4, for example, arginine capped enzyme cleavage site as discussed above in relation to Figure 4. The provision of the positive charge on the linker 4 causes the particle 1 to swell, thereby forming an ERP 2, having an overall net positive charge.

The ERP 2 is then added to an environment containing a protease 5, which has catalytic specificity for the enzyme cleavage site. Due to the swelling of the ERP 2, the protease 5 is able to diffuse into and permeate through the ERP 2. The protease comes into contact with the positively charged linker 4, and then hydrolyses the enzyme cleavage site, thereby causing the positive charge to be released from the ERP 2. As shown in Figure 5, the result of the removal of the net positive charge, is that the ERP 2 then has an overall net neutral charge. Accordingly, the ERP 2 now de-swells or collapses producing particle 3, and the molecular accessibility of the particle 3 decreases. Because the molecular accessibility has decreased, the protease 5 is now entrapped within the resultant particle 3.

Example 2 - The effect of coupling charged residues on the ERP, and swelling and molecular accessibility of PEGA

The effect of coupling charged residues into the ERP 2 on the swelling and molecular accessibility of PEGA was studied. It is known that PEGA particles have a strict molecular weight cut off. For example, for PEGA₁₉₀₀, the cut-off value has consistently been estimated at 35kDa for globular proteins. However, the introduction

of charged residues in to the polymer backbone resulted in a significant increase of molecular accessibility up to >88kDa.

The inventors determined the molecular accessibility of PEGA₈₀₀ particles 1, as shown in Figure 5, by using a ladder of fluorescein labelled dextrans with molecular weights of 4, 10, 20, 40 and 77 kDa. Solutions of each of these dextran markers were exposed to the particles 1 and the penetration of the fluorophores to the centre of the bead 2 was observed using two-photon fluorescence microscopy. This technique is a useful tool to analyse fluorescence events inside PEGA particles 1 (Bosma et al., 2003, Chem. Commun. 2790-2791). The inventors also determined the molecular accessibility of a number of proteins, with those of 35 kDa Mw or less able to diffuse into the particles (trypsin (22 kDa), elastase (25 kDa), thermolysin (35 kDa) and those above 35 kDa not able to diffuse in (albumin (55 kDa), penicillin G acylase (88 kDa))

When dissolved in purified water, all of the dextran markers used were able to access the non-charged particles 1. This was thought to be due to protonation of amine groups present in the particles 1 resulting in electrostatic repulsion between the polymer chains in a pH dependant manner. The pKa value of these amines groups is believed to be between about 7-8, and swelling of the particles 1 due to the amine charge is expected to be minimized slightly in basic media. Indeed, in 0.1 M K/Pi buffer of pH 8, both PEGA₈₀₀ and PEGA₁₉₀₀ particles 1 allowed exclusively access the 4, 10, 20kDa markers, but not of the 40 and 70kDa makers. Thus, the molecular weight cut-off for labelled dextrans in the particles 1 was found to be <40kDa in aqueous buffer.

Next, the inventors investigated the molecular weight cut-off of the ERP particles 2, following functionalisation with Fmoc-Arg⁺, which has a net positive charge, as described in Example 1 with reference to Figures 4 and 5. It was found that in both purified water and also in buffer solutions, that all dextrans could enter the ERPs 2. The inventors believe that this was made possible due to increased swelling of the hydrogel particle structure 2. As described above, the positive charge causes the ERP 2 to swell, and the mesh or pore size of the hydrogel to increase, such that each of the ladder of fluorescein labelled dextrans with molecular weights of 4, 10, 20, 40

and 77 kDa, were able to pass through the hydrogel's pores and enter the ERP 2. Hence, the molecular weight cut-off had increased from <40 kDa (i.e. in the non-swelled, non-charged particles 1) to >77kDa (i.e. in the swelled, positively charged ERPs 2).

5 Whilst the hydrogel polymers described herein are not mono-disperse (i.e. the hydrogel diameters vary thus creating a size distribution), it is possible to measure the swelling of the ERPs 2 as an average increase in bead diameter, by using image analysis of a large number of particles, and then calculating the average. Accordingly, the inventors found that the average diameter of the positively charged ERPs 2
10 increased (swelled) by 10% compared to the initial uncharged, non-swelled particles 1. Furthermore, this suggests that the volume of the charged ERP 2 increases by approximately 33% when compared to the uncharged bead 1.

Example 3 - Selectivity of proteases on the ERP

15 The inventors then investigated the enzyme (protease) action and selectivity inside modified ERP particles 2. Peptide linkers 4 were coupled to the vacant amine groups of the hydrogel, and designed to respond in different ways to three different protease enzymes, each with different selectivities for the amino acids flanking the cleaved amide bond (i.e. P1 and P'1, see Table 1).

20 Table 1 - Primary substrate specificities and molecular weights of four different proteases. P1 and P'1 refer to the amino acids directly adjacent to the cleaved amide bond.

Enzyme	Size (kDa)	P1	P'1
thermolysin	35	Any	hydrophobic
chymotrypsin	25	hydrophobic	any
Trypsin	22	charged (+)	any
Elastase	25	Small hydrophobic	Small hydrophobic

Chymotrypsin, from bovine pancreas, is well known to cleave preferentially at the carboxylic acid side of hydrophobic residues, whereas it is rather non-specific for the second amino acid. By contrast, thermolysin from *thermoproteolyticus rokko*, prefers hydrophobic residues at the amine end of the cleaved peptide bond. Finally, hog pancreatic, trypsin, cleaves selectively at the carboxylic acid side of positively charged residues.

Referring to Figure 6, there is shown the results of HPLC analysis demonstrating the enzyme selectivity of the protease enzymes, thermolysin and chymotrypsin, on two different peptides, Fmoc-Gly-Phe, and Fmoc-Phe-Gly. It can be seen that both enzymes are able to selectively remove the expected amino acid. The upper line of Figure 6 represents the action of chymotrypsin, and it can be seen the chymotrypsin removes the glycine residue leaving Fmoc-Phe, which forms a peak at about 14.0. The lower line of Figure 3 represents the action of thermolysin, and it can be seen that thermolysin removes the phenylalanine residue leaving Fmoc-Gly, which forms a peak at about 10.4.

Referring to Figure 7, there is shown the results of HPLC analysis demonstrating the enzyme selectivity of the proteases, thermolysin and chymotrypsin, on only the peptide Fmoc-Gly-Phe. The upper line represents the action of chymotrypsin, and the lower line represents the action of thermolysin. It can be seen that thermolysin is able to cleave off the Phenylalanine residue, thereby producing a significant peak at about 10.4. It will also be seen that chymotrypsin is unable to remove the phenylalanine residue, and so does not produce a peak.

Referring to Figure 8, there is shown data from HPLC analysis of an enzyme modification with thermolysin and chymotrypsin of the peptide, Fmoc-Arg⁺-Phe-Gly, i.e. this peptide has a net positive charge due to the presence of the arginine residue. As shown in Figure 8a, the application of the positive charge to PEGA₁₉₀₀ and PEGA₈₀₀ shows higher removal of the amino acid glycine residue from the peptide. This is the opposite to uncharged particles. Furthermore, PEGA 800 accessibility is improved by the presence of the positive charge.

Furthermore, as shown in Table 2 below, peptides 1 and 2 demonstrate the expected selectivity for the cleaved bond B (as shown in Figure 1) with thermolysin being less selective than chymotrypsin for this combination of amino acids. Peptides 3 and 4 carry the Fmoc-Arg⁺ group, and were cleaved in a similar way, with thermolysin now also cleaving the Arg-Phe bond. Trypsin cleaved the Fmoc-Arg from both peptides 3 and 4.

Table 2 - Enzymatic cleavage of modified particles by different enzymes. A and B refer to the possible enzyme cleavage sites (see figure 1) and give % conversion based on HPLC analysis.

Entry	Peptide linker	Enzyme	A	B
1a	Phe-Gly	thermolysin	n.a.	66
1b	Phe-Gly	chymotrypsin	n.a.	15
2a	Gly-Phe	thermolysin	n.a.	98
2b	Gly-Phe	chymotrypsin	n.a.	6
3a	Arg(+)-Phe-Gly	thermolysin	68	32
3b	Arg(+)-Phe-Gly	chymotrypsin	7	14
3c	Arg(+)-Phe-Gly	Trypsin	99	<0.1
4a	Arg(+)-Gly-Phe	thermolysin	28	43
4b	Arg(+)-Gly-Phe	chymotrypsin	3	2
4c	Arg(+)-Gly-Phe	Trypsin	53	<0.1

The inventors then assessed whether enzymatic cleavage had any effect on the molecular accessibility of the ERPs. To this end, hydrogel particles 1 were functionalised with peptide 4 as referred to in Table 2 to produce ERP 2 particles, and were treated with the three different enzymes, thermolysin, chymotrypsin, and trypsin. The ERPs 21 were then exposed to the 77kDa fluorescein labeled dextran. The molecular accessibility of the ERP 2 by the dextran was assessed by two photon microscopy (Bosma *et al.*, 2003, Chem. Commun. 2790-2791).

Referring to Figure 9, there are shown cross-sections through the centre of individual positively charged, and swelled ERP particles 2 at intervals of 1 minute, 2 minutes, 5 minutes and 10 minutes (from left to right). Referring to the row corresponding to the control ERP 2 (i.e. no protease enzyme added), it will be seen that as time proceeds, the fluorescence increases from left to right. Hence, there is a steady increase in the concentration of dextran, is absorbed by and permeates into the ERP particle 2. However, there is reduced fluorescence for ERPs 2 in the presence of thermolysin (4a) and trypsin (4c) for each time-frame compared to the control. Accordingly, less dextran is able to permeate into the ERP 2, i.e. a decrease in molecular accessibility observed. However, for the ERP 2 treated with chymotrypsin (4b), similar fluorescence is observed as for the control ERP 2 (i.e. no enzyme).

The results shown in Figure 9 confirm that the peptide selectivity given in Table 2 causes reduced accessibility in 4a and 4c, and this can be explained in terms of the enzymatic cleavage of the peptide linker from the ERP 2, i.e. thermolysin (4a) and trypsin (4c) are able to cleave off the positively charged residue, Arg(+)-Gly-Phe, at sites A and B (as shown in Figure 4). As a result of the loss of the net positive charge, the ERP particle 2 becomes neutral and so collapses or de-swells, such that the pore size of the ERP particle 2 decreases. Accordingly, the dextran is unable to permeate in to the collapsed ERP particle 2, and so fluorescence levels are lower than in the control, which in the absence of any protease, stays in a swelled condition. Because chymotrypsin is unable to cleave site A or B to any extent compared to the control, the ERP 2 maintains its positive charge on the arginine residue, and has a comparable fluorescence to that of the control.

Second Embodiment

Figure 11 is a schematic of the overall design of Enzyme Responsive Polymers (ERPs) 100 in accordance with the invention.

As shown in Fig 11A, PEGA hydrogel beads containing free amine groups can be readily functionalised with peptides incorporating an enzyme cleavable linker (ECL) incorporating peptides with oppositely charged amino acids on opposite sides of the ECL to create zwitterionic peptide linkers with no overall charge. Examples of oppositely charged amino acids that may be used are Asp and Arg. In the illustrated

embodiment of the particle, the negatively charged amino acid is on the side of the ECL remote from the particle.

Enzyme catalysed hydrolysis of the ECL results in release of the negatively charged amino acid/carboxylic acid fragment to leave an amine terminated, cationic amino acid fragment attached to the hydrogel.

As a result, electrostatic repulsion causes the hydrogel bead to swell as depicted by the transition from (ii) to (iii) in Fig 11A.

Fig 11B depicts how swelling of the bead may be demonstrated. More particularly, the hydrogel beads incorporating the zwitterionic peptide linkers may be loaded with fluorescently labelled dextrans by lowering the pH of a dextran solution to 3, as depicted by the arrow going from Fig 11A (ii) to Fig 11B (i). This results in protonation of the aspartic acid carboxylic acid side chains, leaving a net positive charge and resulting in swelling of the hydrogel bead thus allowing the dextran to diffuse into the bead. A subsequent increase in solution pH regenerates the zwitterion and causes the hydrogel to collapse, as depicted by the arrow going from (i) to (ii) in Fig 11B. The dextran has thus been captured.

On enzymatic hydrolysis of the ECL, the negatively charged amino acid residue is released and (as described above) leaves the amine terminated, cationic amino acid fragment attached to the polymer so that the bead swells to result in release of the dextran (see transition from Fig 11B(ii) to (iii)).

Specific examples of zwitterionic peptide linkers are illustrated in Fig 11C together with cleavage of these specific linkers which results in release of a doubly-negatively charged carboxylic acid fragment to leave a double cationic amine fragment tethered to the polymer.

Designing Resposive Sequences

The choice of enzyme cleavable linker (ECL) is determined by the substrate preference of the target enzyme, and the degree of specificity required. Shorter sequences may result in slower or less selective hydrolysis as the peptides are a poor

fit for the enzymes active site. Conversely, longer sequences more closely related to the enzymes natural substrate may result in more rapid or more selective hydrolysis. Substrate efficiency data are readily available for a variety of relevant enzymes. The following discussion uses matrix-metalloprotease 2 (MMP-2) as an example target enzyme.

MMP-2 is an enzyme used to dissolve the connective extracellular matrix (ECM) fibres that anchor cells in place. It is continuously secreted into ECM, but its action is normally regulated by inhibitors released at the same time. In some disease states, notably carcinomas, MMP-2 is up-regulated beyond its inhibitor. This results in local ECM degradation and the consequent cell mobility results in malignant carcinomas.

Table 3 summarises some known MMP-2 substrate sequences.

Table 3: A sample of known MMP-2 substrate sequences:

<i>P4</i>	<i>P3</i>	<i>P2</i>	<i>P1</i>	<i>P1'</i>	<i>P2'</i>	<i>P3'</i>	<i>P4'</i>
Gly	Pro	Tyr	Glu	Leu	Lys	Ala	Leu
Gly	Pro	Glu	Gly	Leu	Arg	Gly	Ala
Gly	Pro	Glu	Gly	Val	Arg	Ser	Ser
Asp	Val	Gly	Glu	Try	Asn	Val	Xxx
His	Pro	Val	Glu	Leu	Leu	Ala	Arg

The enzyme active site has been shown to be most sensitive to up to four residues either side of the cleavage site between the amino acid residues designated *P1* and *P1'*. Examination of these sequences, and others available in the literature, reveal trends characteristic to this enzyme. Glutamic acid residues are well tolerated at the *P1* position, which is ideal for balancing a positively charged residue in the C-terminal half of the substrate. Polar amino acids are well tolerated at the *P2'* position. Thus, in order to create a very short substrate sequence either glutamic acid or glycine may be included as a *P1* residue, leucine as a *P1'* and arginine as a *P2'* (Table 4).

Table 4: Minimal MMP-2 substrate sequences:

<i>Capture</i>	Gly	Leu	Arg
<i>Release</i>	Glu	Leu	Arg

In order to create a longer sequence which more closely resembles the natural substrate, sections from known sequences containing the desired amino acids at the *P1* and *P2'* positions may be fused to result in hexapeptide sequences (Table 5).

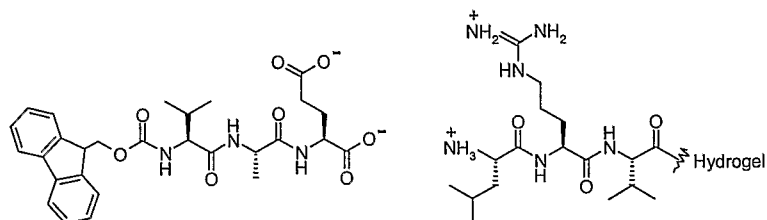
5

Table 5: Extended MMP-2 substrate sequences:

<i>Capture</i>	Val	Ala	Glu	Leu	Val	Val
<i>Release</i>	Val	Ala	Glu	Leu	Arg	Val

Cleavage of this substrate by MMP2 at physiological pH should result in the formation of a doubly negatively charged C-terminal fragment and a doubly positively charged N-terminal fragment as shown below.

10



Example 4

This Example relates to hydrogel particles in accordance with the second embodiment of the invention described with reference to Fig 11 and containing Asp and Arg as the charge modifying agents and Gly-Gly or Ala-Ala as the enzyme recognition peptides.

15

Production of PEGA micro particles

3.14 g of the PEGA 800 macromonomer was dissolved in 10 ml of distilled water and purged for 30 minutes with N₂ gas. 50 ml of Isopar M was added to the reactor and was also purged for 30 minutes. The reactor was heated to 70°C by means of a thermostated water bath on a hot plate and mounted above this was an overhead mixer (Yellowline OST basic, overhead mixer). After 20 minutes of purging 0.16 ml of TEMED was added to the oil phase, and 0.156 g of Acrylamide to the dissolved macromonomer. 3 minutes later 0.328 g Span 20 was dissolved in the oil, which was stirred at 500 rpm for 30 seconds to ensure the surfactant was fully dispersed in the oil phase. 0.070g of APS was dissolved in the macromonomer solution and the reactor

25

was set to the required stirring speed (2000 rpm), after 30 seconds the macromonomer solution was added to the oil phase in the reactor which was stirred for a further 30 minutes.

5 The particles were collected from the oil phase via a sintered glass filter funnel with porosity 4 (maximum pore size 10-16 μm) and were washed with (2 x 50ml) DCM, (2 x 50ml) THF, (3 x 50ml) methanol and (4 x 50ml) distilled water.

10 The peptides (incorporating ECL) were synthesised directly on to the PEGA particles using standard Fmoc solid phase synthesis chemistry as detailed in the next paragraph.

Peptide Synthesis

15 The particles were weighed into plastic syringe bodies fitted with frits to retain the particles. The fluorenylmethyloxycarbonyl (Fmoc) amino acid (3 molar equivalents) was then added with di-*iso*-propylethylamine (DIPEA, 3 molar equivalents) and hydroxybenzotriazole (HOBt, 2.95 molar equivalents) in DMF (2 mL). The tube was then capped and agitated on a blood rotator or roller mixer for 1 hour. The supernatant was then drained from the tube and the particles washed with methanol (3 $\times$ 2 mL) to remove coupling by-products and then DMF (3 $\times$ 2 mL) to remove the methanol. The particles were then exposed to piperidine in DMF solution
20 (20%, 2 mL) and returned to the agitating apparatus for 30 minutes. These coupling and deprotection steps were repeated until all the constituent residues of the desired peptide had been added. The sidechains of the peptide are then unmasked by reaction with trifluoroacetic acid (95% in water) for 30 minutes to 1 hour and the particles washed with methanol (3 $\times$ 2 mL) then DMF (3 $\times$ 2 mL) before the particles are
25 stored under water (HPLC grade, 1 mL).

Testing of Particles

30 The effects of molecular accessibility following enzyme reaction were studied *via* the accessibility of the hydrogel to a fluorescently labelled dextran marker (molecular weight 40kDa), combined with HPLC analysis of released fragments and optical microscopy analysis to obtain bead diameters.

The average bead diameter as measured by optical microscopy (random sample of 200 particles) was found to be 0.25 mm for the zwitterionic particles.

Enzymes with different specificities were chosen to highlight how the complementary selectivity of the protease for the amino acids present in the peptide chain can control the hydrogel swelling. The enzymes selected, and the results obtained, are shown in Table 7 below.

Table 7

Cleavage of peptide linkers on PEGA particles by three different enzymes, and the various mean bead diameters and volumes following each reaction.

Entry	Enzyme	% cleavage	Mean diameter (mm)	Mean Volume $\times 10^{-3}$ (mm ³)
1a	None	<0.1	0.26 ± 0.022	8.7 ± 1.6
1b	Chymotrypsin	7.7	0.26 ± 0.016	9.2 ± 1.1
1c	Elastase	>99	0.35 ± 0.031	21.9 ± 2.2
1d	Thermolysin	>99	0.34 ± 0.030	20.6 ± 2.1
2a	None	<0.1	0.26 ± 0.025	9.2 ± 1.7
2b	Chymotrypsin	1.5	0.26 ± 0.019	9.0 ± 1.3
2c	Elastase	4	0.25 ± 0.022	8.6 ± 1.5
2d	Thermolysin	53	0.32 ± 0.030	17.2 ± 2.1

Entries 1a-1d consist of the di-Ala ECL and 2a-2d consist of the di-Gly ECL.

Significant cleavage (determined by HPLC) results in an increase in bead diameter and volume following the generation of net positive hydrogels.

As expected, the Gly-Gly linker was cleaved preferentially by thermolysin, while the Ala-Ala linker was cleaved by thermolysin and elastase. Chymotrypsin, which is known to have a preference for bulky amino acids in the P1 position, was found to leave both sequences largely untouched.

Fig 12A shows two-photon microscopy images of cross sections of individual particles 1a-d and 2a-d (as identified in Table 6 above) and demonstrates the increase in molecular accessibility following successful enzyme hydrolysis (a: control, b: chymotrypsin, c: elastase, d: thermolysin).

Fig 12B shows a comparison between the mean bead diameter and the percentage of hydrolysis (HPLC) for the Ala-Ala ECL. A higher extent in swelling was observed for a high degree of cleavage compared to low swelling seen at low peptide cleavage.

Fig 12C shows a comparison between the mean bead diameter and the percentage of cleaved residue for the Gly-Gly ECL.

PEGA₈₀₀ particles functionalised with Asp(-)-Ala-Ala-Arg(+) were studied to determine entrapment of 40kDa fluorescently labelled dextran and release thereof when triggered by thermolysin.

Initially it was determined that, at pH 7.4, the dextran was excluded (Fig 12D (3a), this being due to the fact that there is no overall charge present on the hydrogel.

The hydrogel particles were then loaded with the fluorescent dextran by applying a pH switch (see Figure 11B) to provide a pH of 3 which results in the protonation of the aspartic acid, allowing penetration of the 40kDa dextran – see Fig 12D (3b).

Once the dextran had entered the bead a subsequent increase in hydrogel pH back to pH 7.4 caused the hydrogel to collapse with physical entrapment of the dextran due to the re-introduction of zwitterion.

After 30 minutes the hydrogel was replaced in water with the fluorescent marker entrapped therein. Fig 11D (3c) was taken 30 minutes after the fluorescently labelled dextran was replaced in water and demonstrates no significant loss of fluorescence during this time.

Thermolysin was then added to the solution and the progress of the fluorescent marker diffusing out of the hydrogel was monitored. Fig 12D (3d) was taken 60 minutes into the reaction. This demonstrate significant diffusion of fluorescence out of the bead.

The release of the dextran was also monitored using a total pixel intensity plot (Fig 12E) of a cross-section of a di-Ala ECL bead during the reaction with thermolysin (black diamonds). The presence of fluorescence at a distance of 50 microns outside the bead (white diamonds) shows the release profile. It will be seen that Fig 12E demonstrates significant diffusion of fluorescence out of the bead during the enzyme reaction.

Therefore the molecular accessibility of PEGA₈₀₀ can be tightly controlled by the selective catalytic action of proteases. The hydrogel responds by swelling specifically to target enzymes based on the enzyme cleavable linker employed in the PEGA coupled peptide chain. Increased swelling causes the release of an encapsulated molecule which will only occur if the hydrogel encounters the target protease in a complex mixture that may contain other enzymes. This technique has applications in the selective release of therapeutic agents at specific sites in which the target enzyme is found, creating a highly selective drug release system without some of the drawbacks of existing systems for enzyme triggered drug release.

Summary

In summary, the inventors have demonstrated that the molecular accessibility of PEGA₈₀₀ polymer hydrogel particles can be controlled selectively using different specific enzymes. By taking advantage of the selective enzymatic cleavage of only certain peptides, and by selecting the appropriate removal of permanently charged groups from peptide functionalised hydrogels, these ERP particles may be considered to be 'programmable'. Since proteases play key roles in various diseased states, the inventors believe that this approach has potential for selective removal of harmful macromolecules in response to disease specific enzymes.

Hence, the inventors have found that the swelling/de-swelling effect of the particles 2 under the control of the protease 5 may be used in a wide variety of ways. For example, the particle 2 may be in the field of detection, entrapment, and encapsulation, and/or delivery of sensitive biological molecules 20 to target environments. One example is the use of the hydrogel particle 2 to entrap or purify

target proteases from solutions of impure protease or other biomolecules. Hence, the hydrogel particle may be used in a separation or purification column, either to supplement or replace existing biochemical purification methods (see Example 6). A second example is the use of the hydrogel particle in medicine. For example, the particle may be used to entrap and thereby remove deleterious protease enzymes, which are produced by a variety of different diseases, for example, inflammation (see Example 5). A third example is the use of the hydrogel particle for the effective delivery and release of active payload molecules to a target site. Each of these preferred uses will now be described in detail below (see Example 7).

Example 5 - Use of ERPs in a wound healing formulation

The inventors envisage that the ERP hydrogel particles in accordance with the invention can be used to treat angiogenesis, and tumor cell metastasis, and also to improve wound healing in patients. Two important families of proteases involved in wound healing are serine proteases (neutrophil elastase) and Matrix Metalloproteinases (MMPs). The MMP proteases specifically involved in wound healing are the collagenases (which degrade the connective tissue collagen), gelatinases (degrade basement membrane), and stromelysins (degrade ECM proteoglycans).

Matrix metalloproteinases are a group of enzymes that can break down proteins, such as collagen, that are normally found in the spaces between cells in tissues (ie, extracellular matrix proteins). Because these enzymes need zinc or calcium atoms to work properly, they are called metalloproteinases. Matrix metalloproteinases are known to be involved in wound healing, angiogenesis, and tumor cell metastasis.

Referring to Figure 10, there are shown a range of MMPs, which are involved in wound healing. For example, Figure 10 provides details of:- (i) collagenases (MMP-1, MMP-8, MMP-13); (ii) gelatinases (MMP-2, MMP-9); (iii) stromelysin

(MMP-3, MMP-10, MMP-11); and (iv) MMP-12. In addition, Figure 10 gives details of neutrophil elastase, thrombin and elastase. Figure 10 also provides details of the various substrates for each protease listed, and in addition, gives detail about the substrate specificity of each protease, i.e. the sequence of amino acids, which each
 5 protease recognises and can hydrolyse.

For example, MMP-1 has catalytic specificity for a range of different collagens such as (I, II, III, VII, VIII, X, XI); gelatin; aggrecan; tenascin; L-selectin; IL-1Beta; proteoglycans; entactin; ovostatin; MMP-2; MMP-9. More specifically, MMP-1 recognises and hydrolyses the following sequences:-

- 10 (i) Ac-Pro-Leu-Gly-Ser~Leu-Leu-Gly-OEt;
- (ii) Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH₂;
- (iii) Pro-Met-Ala~Leu-Trp-Ala-Thr;
- (iv) Leu-Pro-Met~Phe-Ser-Pro;
- (v) Ac-Pro-Leu-Ala-Ser~Nva-Trp- NH₂;
- 15 (vi) Arg-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr
- (viii) Pro-Glu-Gly~Ile-Ala-Gly;
- (ix) Pro-Glu-Gly~Leu -Leu-Gly.

The data in Figure 10 is modified from McCrawley, L.J. et al., 2001; Sternlicht, M.D., et al., 2001 and Woessner, J.F. et al., 2002. The isoelectric point (pI)
 20 of all enzymes was calculated using 'Compute pI/MW' through the database provided by SwissProt (www.ebi.ac.uk/swissprot/) for the human species. Dnp (dinitrophenyl), Dpa (N³-dnp-L-2,3-diaminopropionic acid). McCrawley, L.J. et al. (2001). Matrix metalloproteinases: they're not just for matrix anymore! Curr. Opin. Cell Biol., v13, p534. Sternlicht, M.D., et al. (2001). How matrix metalloproteinases regulate cell
 25 behaviour. Annu. Rev. Cell Dev., v17, p463. Woessner, J.F. et al. (2002). Matrix metalloproteinases and TIMPs. Oxford University Press, Oxford, England. Order Ref 92 from this book.

The inventors have therefore created hydrogel polymer particles 2 in accordance with the invention, in which the protease cleavable linker 4 consists of a substrate recognition sequence for each of the proteases as summarised in Figure 7. For example, with reference to Figure 1, an ERP 2 was made consisting of PEGA hydrogel, which was functionalised with peptide linkers 4 which individually included the following recognition sequences

- (i) Ac-Pro-Leu-Gly-Ser~Leu-Leu-Gly-OEt;
- (ii) Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH₂;
- (iii) Pro-Met-Ala~Leu-Trp-Ala-Thr;
- (iv) Leu-Pro-Met~Phe-Ser-Pro;
- (v) Ac-Pro-Leu-Ala-Ser~Nva-Trp- NH₂;
- (vi) Arg-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr
- (viii) Pro-Glu-Gly~Ile-Ala-Gly;
- (ix) Pro-Glu-Gly~Leu -Leu-Gly.

Hence, such ERPs 2 would be cleaved only by MMP-1.

Example 6 - Use of ERPs for isolating and purifying proteases

The hydrogel particles 2 described above can be used to purify or isolate a target protease enzyme, for example, from a solution containing a mixture of biomolecules, which includes the target protease 5. The target protease 5 to be isolated from the solution can be either known or unknown. For example, the known target enzyme may be present in an impure sample, which may be contaminated with other unwanted enzymes or other biomolecules. Alternatively, the particle may be used to isolate or purify an unknown target protease, for example, from a solution containing a mixture of potentially active enzymes.

The purification/isolated assay can be conducted in a column 10 as illustrated in Figure 13. The key to the invention is that the hydrogel particle 2 can be 'programmed' by specifically choosing the substrate sequence in the protease linker 4 such that *only* the target protease 5 (whether known or unknown) is able to hydrolyse the peptide bond, and thereby cause the change in net charge of the particle 2.

A solution 18 containing the target protease 5 (the protease enzyme, which is to be purified) may contain either a low concentration of the target protease 5, or a solution containing the target protease 5 in addition to at least one other non-target biomolecule (e.g. enzyme, which is not to be purified). The method is carried out by initially designing the protease linker 4 of the hydrogel particle 2 such that it comprises the substrate sequence that is catalytically recognised by the target protease 5 to be purified. If the target protease 5 is known, then the researcher can incorporate the corresponding substrate sequence into the linker 4. However, if the target protease is not known, then the researcher can incorporate the substrate sequence, for which he would like to isolate a protease 5 adapted to hydrolyse that specific sequence.

The particle 2 is designed so that it has either a net positive charge or a net negative charge prior to protease 5 hydrolysis. Hence, the particle 2 has a swelled configuration, such that the pore size (Molecular Weight Cut-Off) is sufficiently large to enable enzymes 5 to permeate into the particles 2, i.e. it has an increased molecular accessibility. The particles 2 are then supported on or within the column 10 as a matrix 12 as shown in Figure 13. The column can, for example, be loaded with Sephadex 12. Alternatively, the particles 2 can be attached to the column 10 as a film.

A solution 18 containing a mixture of biomolecules, including the target protease 5 and non-target biomolecules, is then be applied to the top 14 of the column 10. Only the target protease 5 will trigger collapse of the particles 2 in the matrix 12 or film, due to the specificity of the protease linker 4 and the highly specific catalytic activity of the target protease 5. Hence, all of the non-target biomolecules will not be entrapped within the particles 2, and so will run through the column and exit at the base 16. Most of the non-target biomolecules should run through the column 10 and exit at its base 16. Accordingly, only the target protease 5 will be entrapped within the immobilised particles 2. The target protease 5 can then be eluted from the column 10

by passing a solution adapted to induce either a net positive or net negative charge to the particles 2, and thereby release the target protease 5. Accordingly, the target protease 5, whether known or unknown, will be purified.

It will be appreciated that in some circumstances, there is no need to immobilise the particles 2 onto a support surface, such as the inside of the column 10. Particles could be added to solution 18, left to allow the entrapment of the target protease 5, and allow the change in particle 2 size to occur. The solution can then be filtered, where the smaller particles 2 contain the entrapped target protease 5, and the larger particles 2 do not contain any entrapped enzyme 5. The smaller filtered particles 2 can then be washed with a suitable washing buffer, and then separated off. Then, the entrapped target protease 5 can be eluted out of the particles 2, using a suitable buffer which causes the particles size to increase.

Example 7 - Use of ERPs for delivery of a drug payload

The hydrogel particles 2 described above can also be used to entrap and deliver active payload molecules 20 to a target site, for example, in the body of a subject in need of therapeutic treatment. For example, the payload 20 could be a drug molecule, which exhibits activity at a site in the patient's body. Alternatively, the payload molecule 20 could be a dye, electrochemical mediator, peptide, protein, antibody, drug molecule, or enzyme etc. The payload molecule 20 could be derivatised with oligo- or polymeric species. Derivatization could be necessary in order to prevent the payload 20 from permeating through the hydrogel layer 2.

Referring to Figure 14, there is shown a schematic drawing illustrating a mechanism for using the hydrogel particles 2 to deliver such a payload 20. The hydrogel particle 2 is designed such that it has a net neutral charge prior to the hydrolytic action of a target protease. By way of example, the particle 2 is designed such that the linker 4 comprises a first chemical species having a net positive, and also a second chemical species having a net negative charge, which when taken together neutralise the overall charge of the protease linker 4. It will be appreciated that the net neutral charge provided by the protease recognition means 4 causes the hydrogel particle 2 to adopt a de-swelled or collapsed configuration before the action of a protease as shown on the left hand side of Figure 14. As a result, when the hydrogel

particle 2 is de-swelled, the pores between the polymer molecules are decreased in size, and so the molecular accessibility of the particle or Molecular Weight Cut-off is low.

5 In preparing the particle 2, a payload molecule 20 (drug compound), which is water-soluble, is incorporated into the PEGA particle during the polymerisation process. As shown in Figure 14, the payload molecule 20 is actively trapped or encapsulated within the de-swelled hydrogel particle 2. In addition to providing a net neutral charge to the particle 2, the linker 4 also comprises a protease hydrolysis substrate sequence, which can be hydrolysed or modified by a target protease 5. 10 Hence, during the design of the linker 4, a specific amino acid sequence is used which would only be modified by a target protease 5 present in the target site in the patient.

Accordingly, the function of the protease 5 used in conjunction with the particles 2 according to the invention is to hydrolyse the substrate sequence of the linker 4 in order to cause a change in the net charge of the particle 2. The substrate 15 sequence is positioned in between the two charged species, wherein following the hydrolytic action of the protease 5, one of the two charged species is cleaved off the linker 4.

As shown in Figure 14, the particle is introduced into the subject to be treated, wherein the particle 2 then moves to the target environment. The target environment 20 could be any tissue or organ in the body or a damaged site, for example, a tumor. On reaching the target environment, the particle 2 is then exposed to a target protease enzyme 5. The enzyme 5 has specificity for and therefore hydrolyses the peptide bond in the linker 4. For example, in wound sites, there may be a high concentration of a certain protease 5 that will hydrolyse the substrate sequence. Following the hydrolytic 25 action of the protease 5, the protease 5 cleaves off either the positively charged or negatively charged residue from the linker 4 as shown in Figure 14. Following the hydrolytic action of the protease 5, the net charge of the particle 2 is then changed so that it is either positive or negative, depending on whether the cleaved residue from the linker 4 was positive or negative.

30 Accordingly, the result of the hydrolytic action of the protease 5 is to produce a particle 2, which has either a net positive charge or a net negative charge. As a

result, the particle 2 swells in size due to electrostatic effects between either the positively charged or negatively charged hydrogel polymer molecules. As the particle 2 swells, the pore size increases and so does the molecular accessibility (Molecular Weight Cut-off). As a result, when the hydrogel particle 2 is swelled, the pores
5 between the polymer molecules increase in size, and so the molecular accessibility of the particle 2 is increased. As the molecular accessibility increases, the payload molecule 2 is released into the target environment, where it can act on the subject. Therefore, the use of the hydrogel particle 2 in this manner is a very effective way of encapsulating a payload molecule 20 and delivering it to a target environment where
10 it can be released.

CLAIMS

1. A hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle.
5
2. A hydrogel particle according to claim 1, wherein the protease recognition means comprises a chemical species having a net positive charge prior to modification by the protease.
3. A hydrogel particle according to claim 2, wherein the chemical species comprises an amino acid (e.g. a natural or non-natural amino acid) having a net positive charge.
10
4. A hydrogel particle according to claim 3, wherein the amino acid is arginine, lysine, or histidine.
5. A hydrogel particle according to claim 1, wherein the protease recognition means comprises a chemical species having a net negative charge prior to modification by the protease.
15
6. A hydrogel particle according to claim 5, wherein the chemical species comprises an amino acid (e.g. a natural or non-natural amino acid) having a net negative charge.
7. A hydrogel particle according to claim 6, wherein the amino acid is glutamic acid or aspartic acid.
8. A hydrogel particle according to any one of claims 5-7, wherein the net positive or negative charge provided by the protease recognition means causes the hydrogel particle to adopt a substantially 'swelled' configuration prior to modification by the protease.
20
9. A hydrogel particle according to any preceding claim, wherein the protease recognition means comprises a protease hydrolysis substrate sequence, which is adapted to be hydrolysed by the protease.
25

10. A hydrogel particle according to any preceding claim, wherein the protease recognition means comprises a suitable substrate sequence for recognition and hydrolysis by any of the proteases chymotrypsin, thermolysin, or trypsin.

5 11. A hydrogel particle according to either claim 9 or claim 10, wherein the substrate sequence is positioned in between the charged species and the site of attachment to the hydrogel polymer, wherein following the hydrolytic action of the protease, the charged species is cleaved off the protease recognition means.

10 12. A hydrogel particle according to claim 11, wherein as a consequence of the removal of the positive or negative charge, the hydrogel particle collapses and adopts a de-swelled configuration.

13. A hydrogel particle according to claim 12, wherein the collapsed hydrogel particle has a decreased Molecular Weight Cut-off value after modification with the protease.

14. A hydrogel particle according to claim 1, wherein the hydrogel particle has a net neutral charge prior to the hydrolytic action of the protease.

15 15. A hydrogel particle according to claim 14, wherein the protease recognition means comprises a first chemical species having a net positive, and in addition, a second chemical species have a net negative charge, which when taken together neutralise the overall charge of the protease recognition means.

20 16. A hydrogel particle according to claim 15 wherein the first chemical species is arginine and the second chemical species is aspartic acid.

17. A hydrogel particle as claimed in any one of claims 14 to 16, wherein the net neutral charge provided by the protease recognition means causes the hydrogel particle to adopt a de-swelled or collapsed configuration before the action of the protease.

25 18. A hydrogel particle according to any one of claims 14-17, wherein the protease recognition means comprises a protease hydrolysis substrate sequence for the protease.

19. A hydrogel particle according to claim 18, wherein the substrate sequence is positioned in between the two charged species, wherein following the hydrolytic action of the protease, one of the two charged species is cleaved off the protease recognition means.

5 20. A hydrogel particle according to claim 18 or 19 wherein the substrate sequence is –Ala-Ala- or –Gly-Gly-.

21. A hydrogel particle according to claim 19 or 20, wherein following the hydrolytic action of the protease, the net charge of the particle is changed so that it is either positive or negative, depending on whether the cleaved species is positive or negative.

10 22. A hydrogel particle according to claim 21, wherein following protease action, the particle will swell in size due to electrostatic effects between either the positively charged or negatively charged hydrogel polymer molecules.

23. A hydrogel particle according to claim 22, wherein the swelled hydrogel particle has an increased Molecular Weight Cut-off value after modification with the protease.

15 24. A hydrogel particle according to any preceding claim, which particle is adapted to carry a payload molecule therein, and is capable of carrying the payload molecule to a target biological environment.

25. A hydrogel particle according to claim 24, wherein the payload molecule is encapsulated within the hydrogel particle, and is any molecule, which has suitable activity with, or against a target biological environment, for example, a target cell type.

20

26. A hydrogel particle according to either claim 24 or claim 25, wherein the payload molecule has catalytic activity.

27. A hydrogel particle according to either claim 25 or claim 26, wherein the payload molecule remains in an active state while it is encapsulated within the hydrogel particle, and once the hydrogel particle reaches the target environment, the payload molecule is released into the target environment.

25

28. A method of altering the molecular accessibility of a hydrogel particle, the method comprising contacting a hydrogel particle according to any one of claims 1 to 27 with a protease, under suitable conditions such that the protease digests the protease recognition means thereby causing a variation in the net charge of the hydrogel particle, and wherein the variation in net charge produces a change in the molecular accessibility of the particle.

29. A method of preparing a hydrogel particle according to any one of claims 1-27, the method comprising the steps of :-

(i) preparing a hydrogel precursor; and

(ii) incorporating protease recognition means into the precursor to produce a hydrogel particle, wherein the recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, and wherein the variation in net charge produces a change in the molecular accessibility of the particle.

30. A method according to claim 29, wherein the hydrogel precursor comprises a substantially organic polymer.

31. A method according to either claim 29 or claim 30, wherein the hydrogel precursor comprises polymer chains resulting from a polymerisation reaction between one or more monomers, in which at least one monomer provides physical or chemical cross-links therebetween.

32. A method according to any one of claims 29 to 31, wherein in step (ii), the or each protease recognition means is attached to the hydrogel polymer via an amide bond.

33. A method according to any one of claims 29 to 32, wherein the protease recognition means is attached to the precursor using a coupling agent and an activation agent.

34. A method according to claim 33, wherein the coupling agent includes N,N'-Diisopropylcarbodiimide (DIC) or Dicyclohexylcarbodiimide (DCC).

35. A method according to either claim 33 or claim 34, wherein the activation agent comprises 1-Hydroxybenzotriazole (HOBt).

36. A method of purifying a target protease from a solution, the method comprising the steps of:-

- 5 (i) contacting a solution containing a target protease with a hydrogel particle according to any one of claims 1-27; and
- (ii) separating the hydrogel particle from the solution.

37. A method according to claim 36, wherein the solution comprises either a low concentration of the target protease, or a solution containing the target protease in
10 addition to at least one other non-target biomolecule.

38. A method according to either claim 36 or claim 37, wherein the particle has either a net positive charge or a net negative charge prior to protease hydrolysis, and hence, has a swelled configuration.

39. A method according to claim 38, wherein as a result of such protease hydrolysis,
15 the net charge of the particle will be altered, thereby causing the particle to collapse, thereby entrapping the target protease, which hydrolysed the protease recognition means, within the collapsed particle.

40. A method according to claim 39, wherein the method comprises a step of separating particles from the solution.

20 41. A method according to any one of claims 36 to 40, wherein the step of separating comprises filtering the particles on the basis of their size and selectivity.

42. A method according to claim 41, wherein the method comprises eluting the target protease from the collapsed particles by altering the net charge of the particle so that they swell, and thereby release the target protease.

25 43. Apparatus for purifying a protease, the apparatus comprising a hydrogel particle according to any one of claims 1 to 27, which particle is immobilised on a support surface.

44. Apparatus according to claim 43, wherein the support surface comprises a column.

45. A hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle, for use as a medicament.

46. Use of a hydrogel particle comprising protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle, for the manufacture of a medicament for the treatment of inflammation, or inappropriate wound healing.

47. A use according to claim 46, wherein the hydrogel particle comprises a protease recognition means comprising a substrate sequence independently selected from a group consisting of:-

- (i) Ac-Pro-Leu-Gly-Ser~Leu-Leu-Gly-OEt;
- (ii) Mca-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH₂;
- (iii) Pro-Met-Ala~Leu-Trp-Ala-Thr;
- (iv) Leu-Pro-Met~Phe-Ser-Pro;
- (v) Ac-Pro-Leu-Ala-S~Nva-Trp- NH₂;
- (vi) Arg-Trp-Thr-Asn-Asn-Phe-Arg-Glu-Tyr
- (viii) Pro-Glu-Gly~Ile-Ala-Gly; and
- (ix) Pro-Glu-Gly~Leu -Leu-Gly.

48. A method of treating an individual suffering from inflammation or inappropriate wound healing, the method comprising administering to an individual in need of such treatment, a therapeutically effective amount of a hydrogel particle comprising

protease recognition means, which recognition means is adapted to be modified by a protease and thereby cause a variation in the net charge of the particle, wherein the variation in net charge produces a change in the molecular accessibility of the particle.

49. A hydrogel comprising enzyme recognition means, which recognition means is adapted to be modified by an enzyme and thereby cause a variation in the net charge of the hydrogel wherein the variation in net charge produces a change in the molecular accessibility of the hydrogel.

50. A method of altering the molecular accessibility of a hydrogel, the method comprising contacting a hydrogel according to claim 49 with an enzyme under suitable conditions such that the enzyme modifies the enzyme recognition means thereby causing a variation in the net charge of the hydrogel, and wherein the variation in net charge produces a change in the molecular accessibility of the particle.

51. A method of preparing a hydrogel according to claims 48, the method comprising the steps of :-

- (iii) preparing a hydrogel precursor; and
- (iv) incorporating enzyme recognition means into the precursor to produce a hydrogel, wherein the recognition means is adapted to be modified by an enzyme and thereby cause a variation in the net charge of the hydrogel, and wherein the variation in net charge produces a change in the molecular accessibility of the hydrogel.

52 A method of purifying a target enzyme from a solution, the method comprising the steps of:-

- (iii) contacting a solution containing a target enzyme with a hydrogel according to claim 49; and
- (iv) separating the hydrogel from the solution.

53. Apparatus for purifying an enzyme the apparatus comprising a hydrogel according to claim 49 which hydrogel is immobilised on a support surface.

54. A hydrogel comprising enzyme recognition means, which recognition means is adapted to be modified by an enzyme and thereby cause a variation in the net charge of the hydrogel, wherein the variation in net charge produces a change in the molecular accessibility of the hydrogel, for use as a medicament.

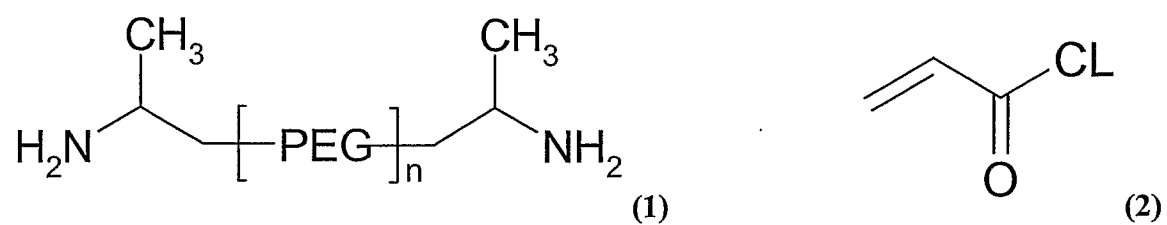
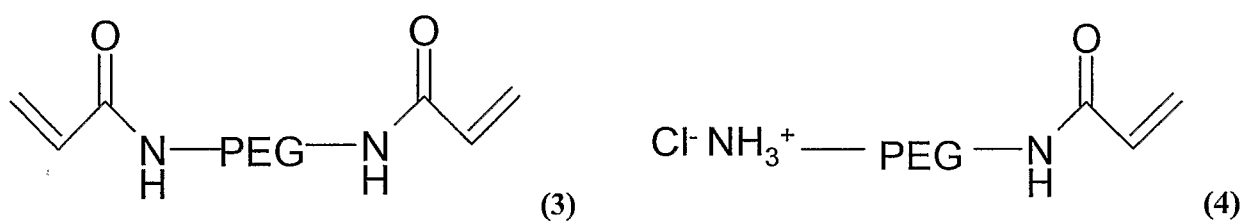
5 55. Use of a hydrogel comprising enzyme recognition means, which recognition means is adapted to be modified by an enzyme and thereby cause a variation in the net charge of the hydrogel, wherein the variation in net charge produces a change in the molecular accessibility of the hydrogel, for the manufacture of a medicament for the treatment of inflammation, or inappropriate wound healing.

10 56. A method of treating an individual suffering from inflammation or inappropriate wound healing, the method comprising administering to an individual in need of such treatment, a therapeutically effective amount of a hydrogel comprising enzyme recognition means, which recognition means is adapted to be modified by an enzyme and thereby cause a variation in the net charge of the hydrogel, wherein the variation
15 in net charge produces a change in the molecular accessibility of the hydrogel.

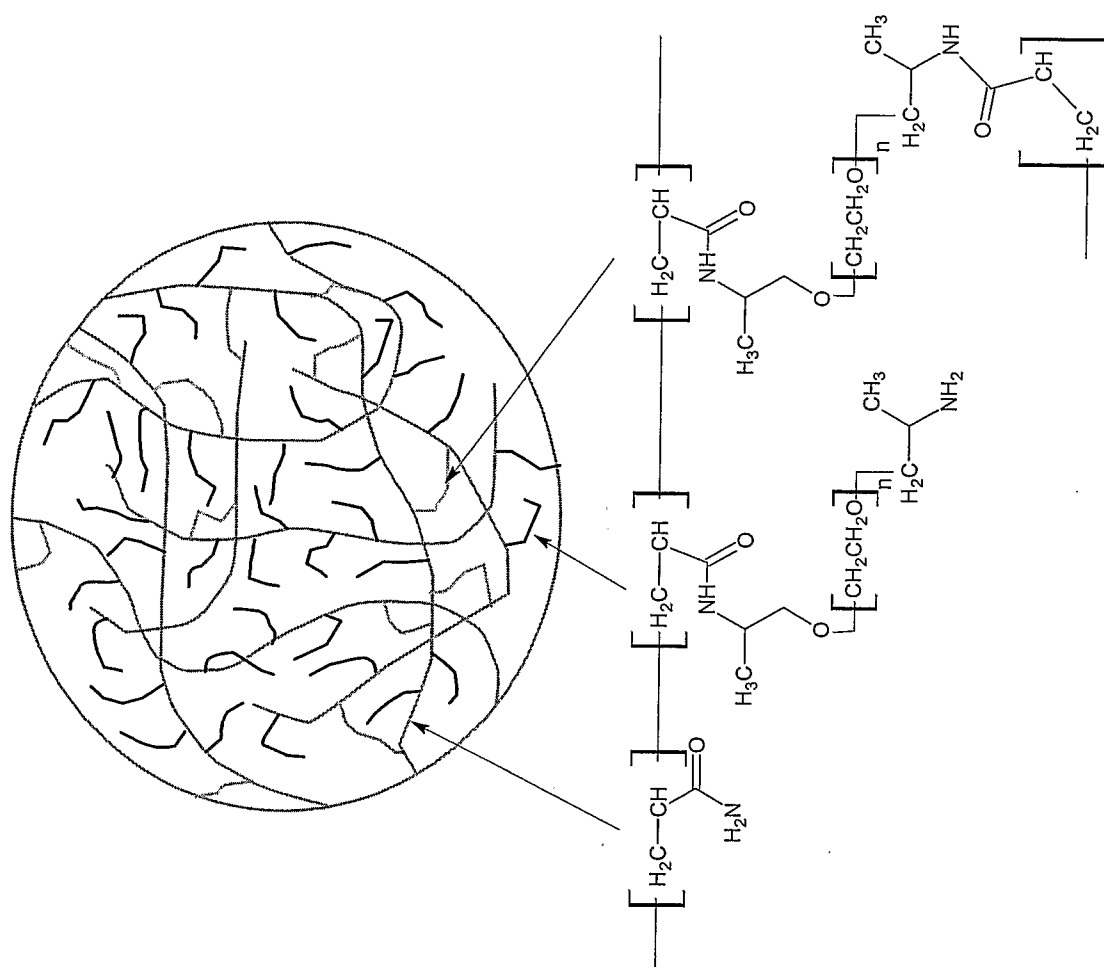
57. A method according to claim 46, wherein the hydrogel particle is administered in the bloodstream, and accumulates selectively in angiogenesis sites, for example, wounds and tumours.

20 58. A method according to claim 47, wherein the hydrogel particle is administered percutaneously in regions of limited or negligible lymphatic uptake and remain there to express their activity.

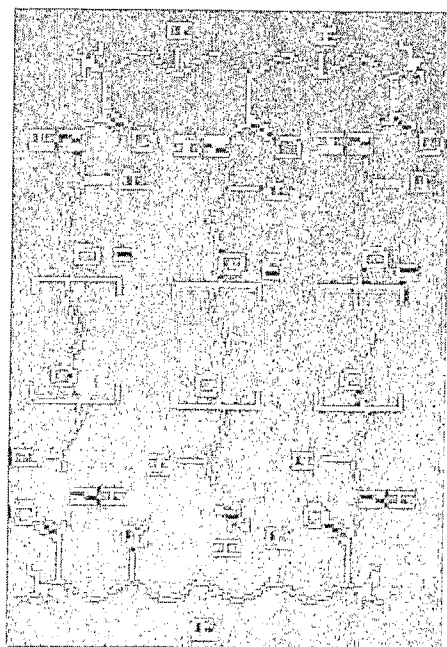
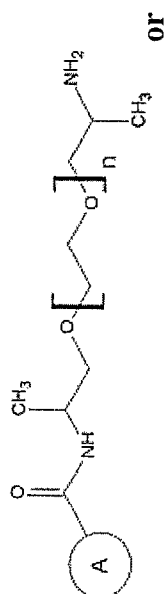
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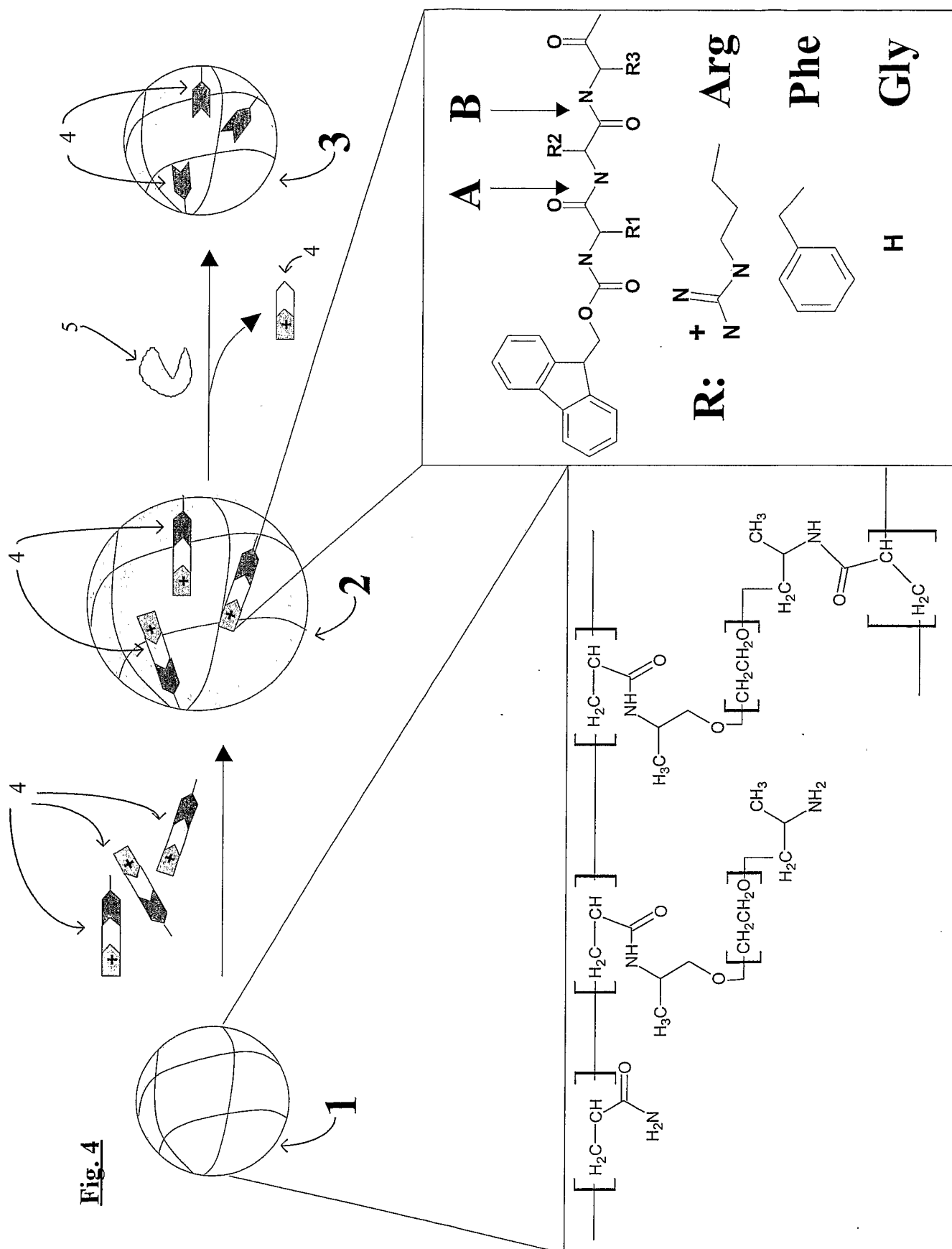
Fig. 1**Fig. 2**

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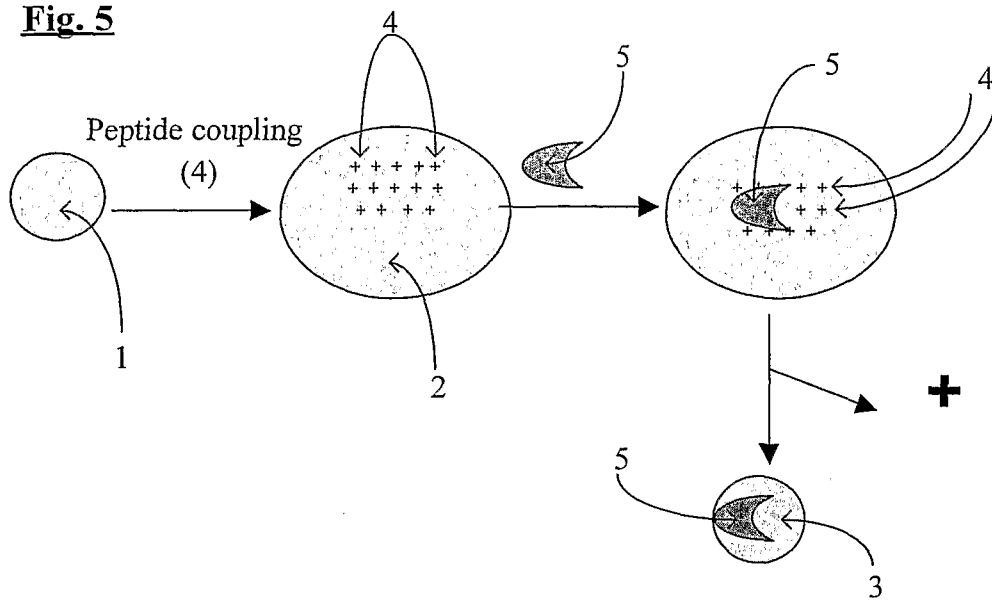


or

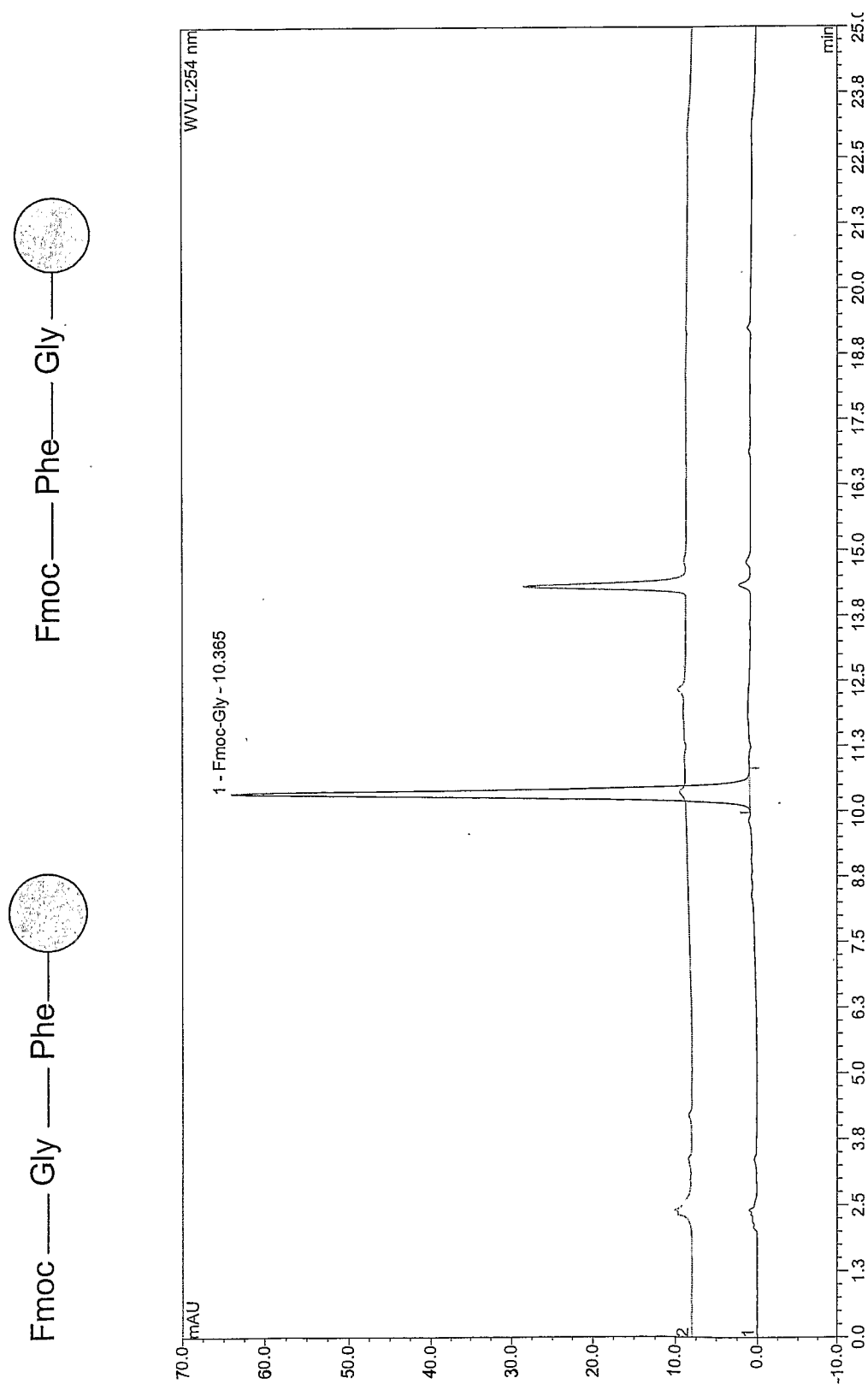
**Fig. 3**



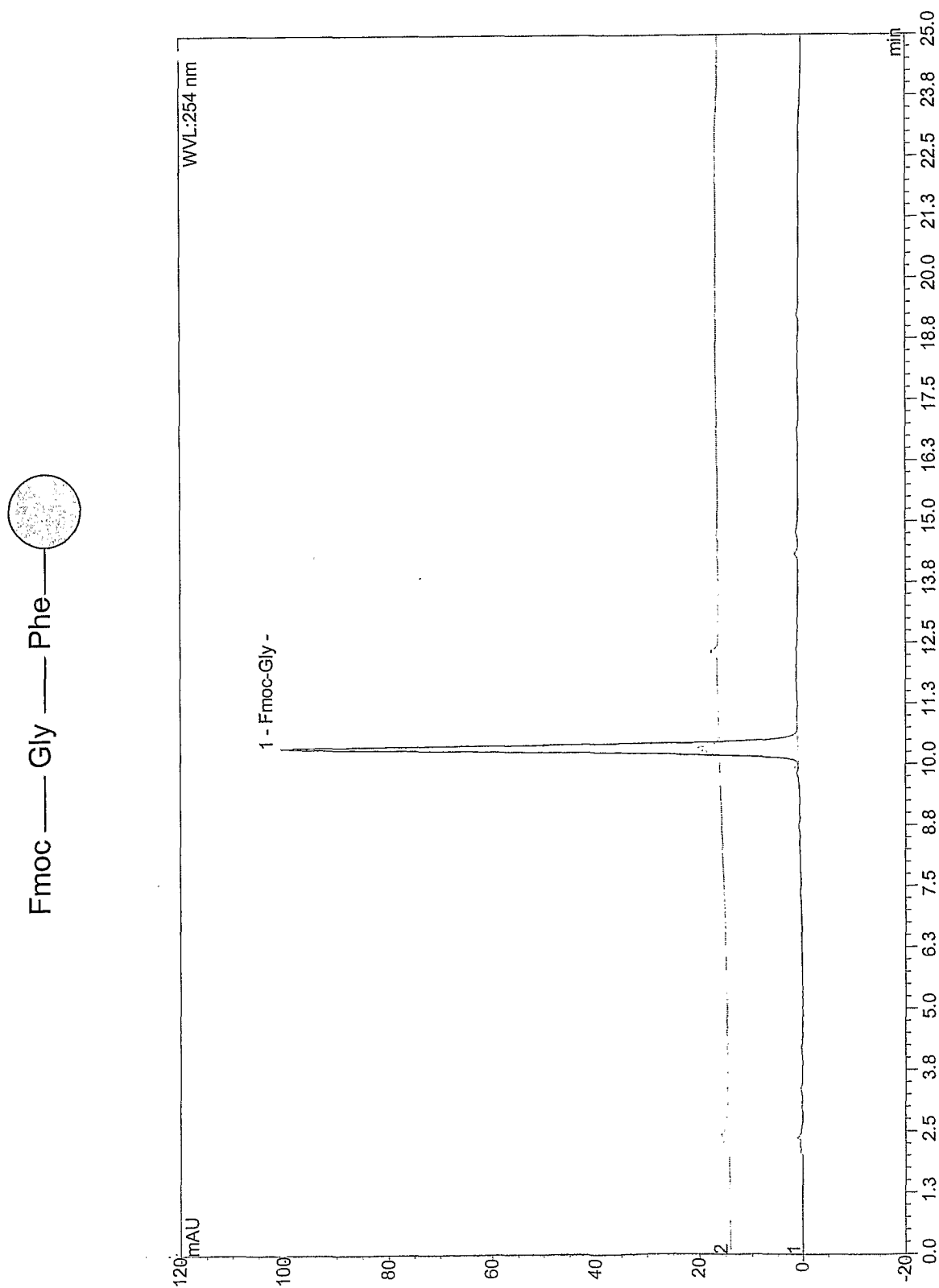
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Fig. 5

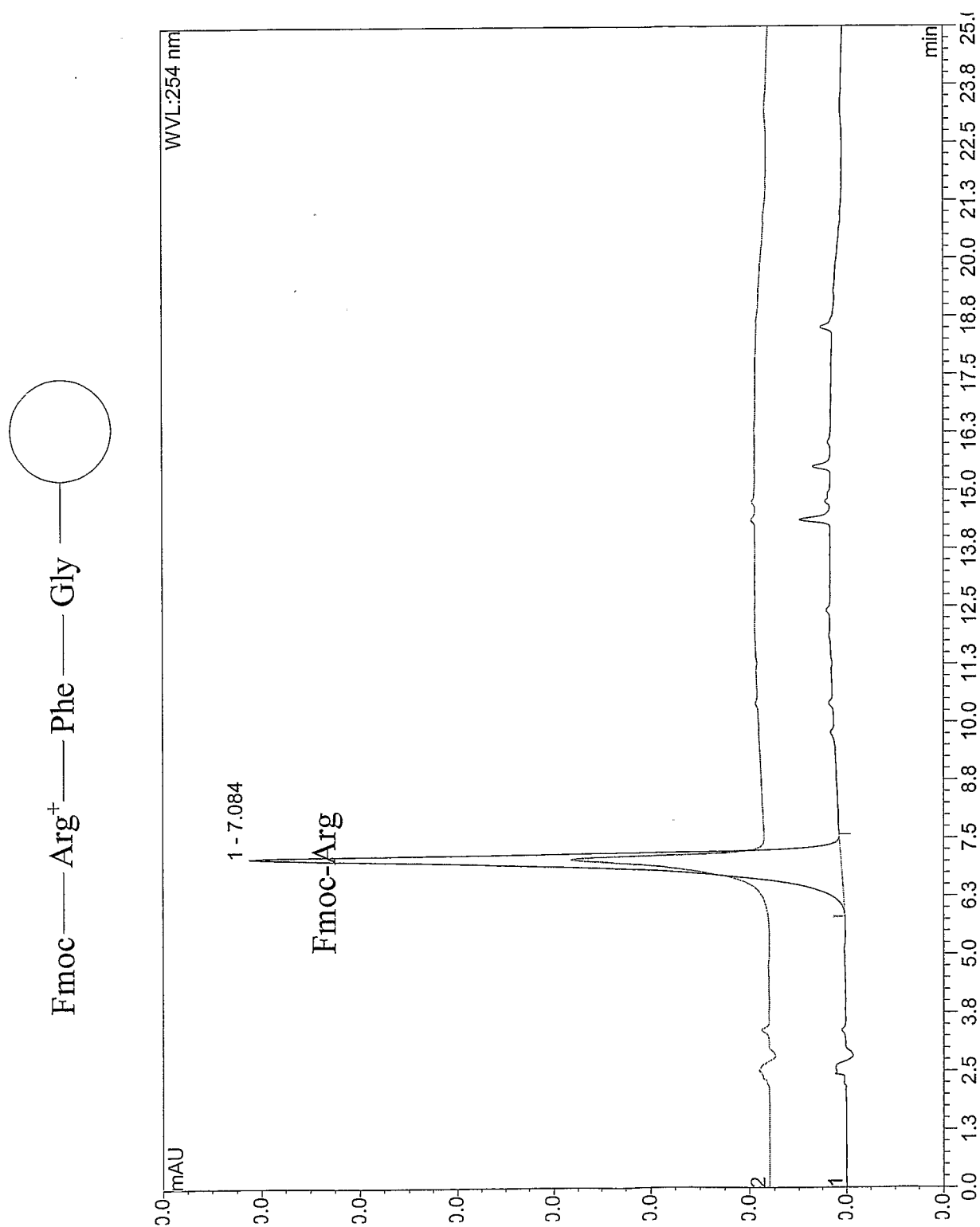
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Fig. 6

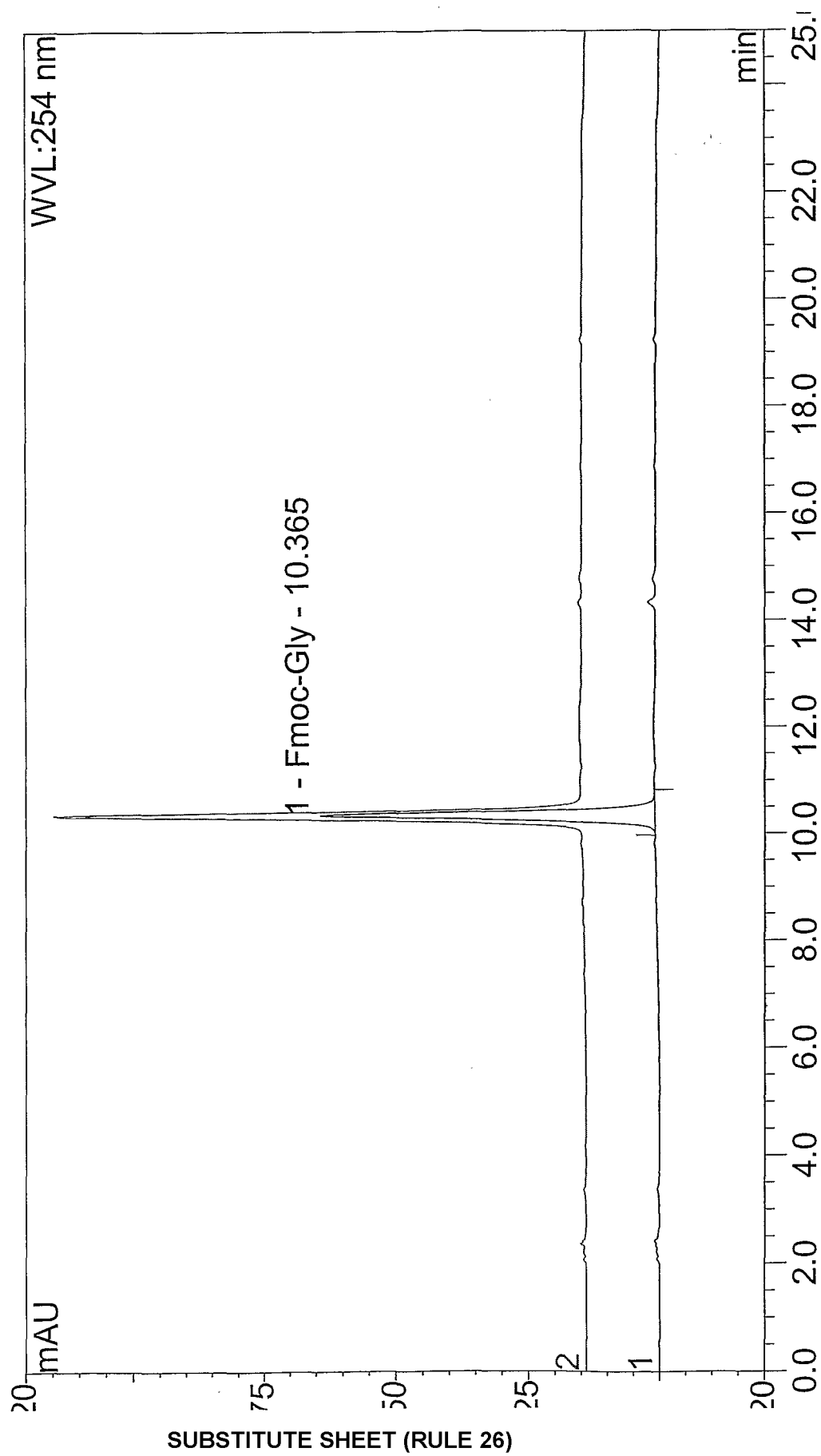
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Fig. 7

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Fig. 8a

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Fig. 8b

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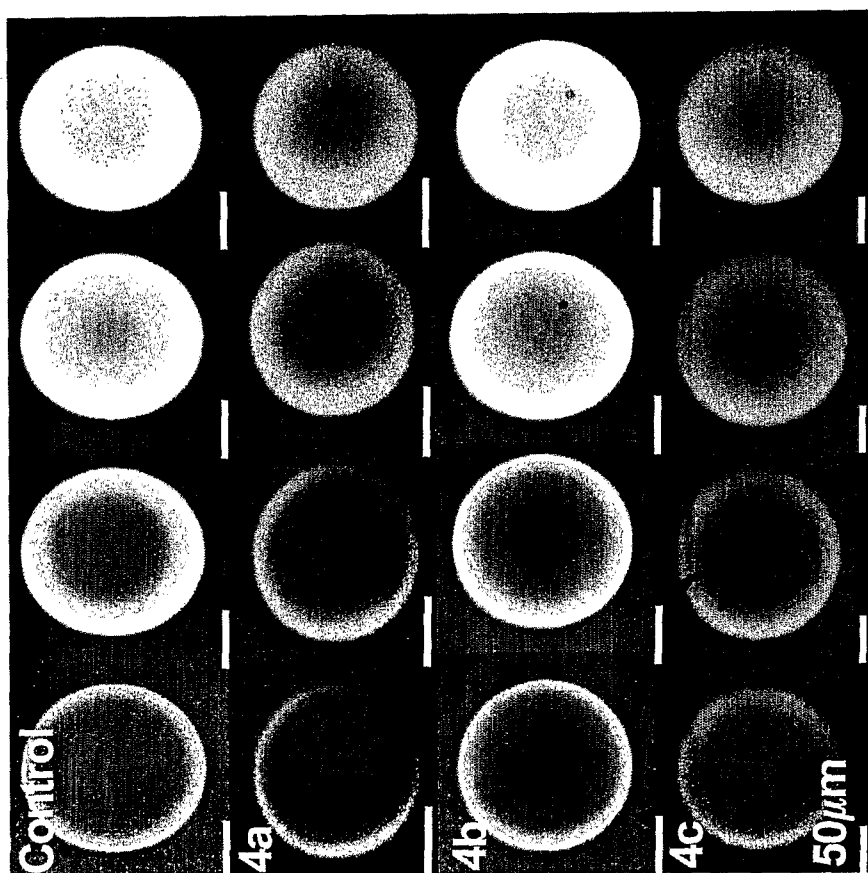
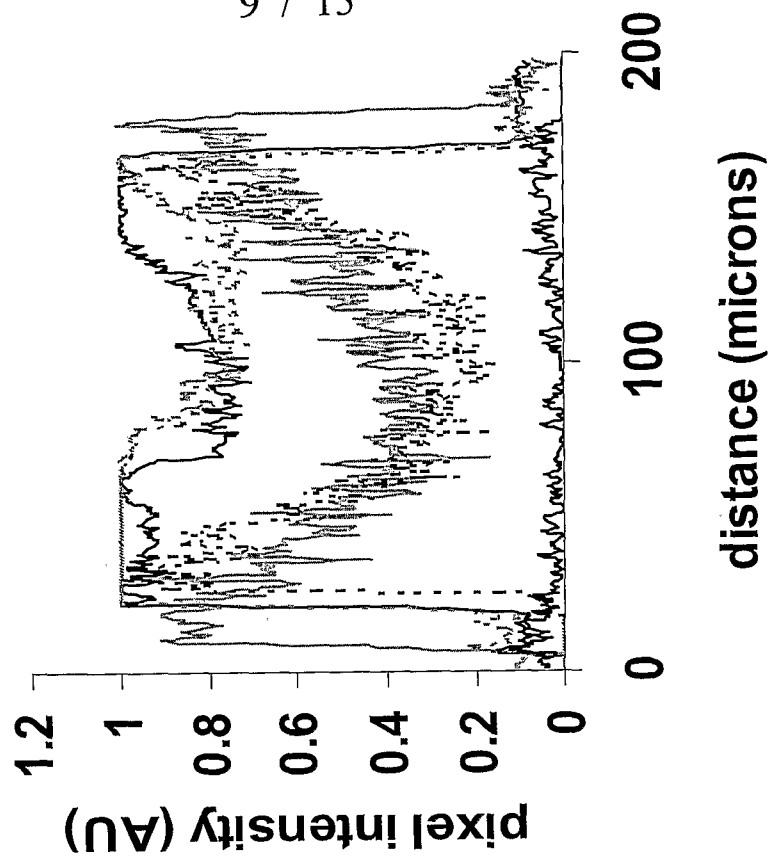


Fig. 9

Fig. 10 - Proteases involved in wound healing and their substrate specificity

Proteases	Enzyme Name(s)		MW (kDa)		Substrate	Substrate specificity	pI
	MMP	Other names	latent	active			
MMP Families	Collagenases						
	MMP -1	Collagenase-1, interstitial collagenase	52	43	Collagens (I, II, III, VII, VIII, X, XI); gelatin; aggrecan; tenascin, L-selectin; IL-1 β ; proteoglycans; entactin; ovostatin; MMP-2; MMP-9	Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OEt Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH ₂ Pro-Met-Ala-Leu-Trp-Ala-Thr Leu-Pro-Met-Phe-Ser-Pro Ac-Pro-Leu-Ala-S-Nva-Trp-NH ₂ ¹⁸³ R-W-T-N-N-F-R-E-Y ¹⁹¹ Pro-Glu-Gly-Ile-Ala-Gly Pro-Glu-Gly-Leu-Leu-Gly	6.47
	MMP -8	Collagenase-2, neutrophil collagenase	75	58	Collagens (I, II, III, V, VII, VIII, and X); gelatin; entactin; aggrecan; tenascin, fibronectin, ProMMP-1,2	GPQG-IWGQ Pro-Leu-Glu/Ala-Tyr-Trp-Ser 2,4-DNP-Pro-Gln-Gly-Ile-Ala-Gly-D-Arg-OH	6.38
MMP Families							
	MMP -13	Collagenase-3, Rat interstitial collagenase	52	42	Collagens (I, II, III, IV, IX, X, XIV); gelatin; entactin; aggrecan; tenascin, plasminogen; aggrecan; perlecan; fibronectin; fibrinogen/fibrin; osteonectin; MMP-9, ProMMP-9,13	Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH ₂ Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln779 Asp-Val-Gly-Glu-Tyr-Asn-Val-Phe88	5.32
MMP Families	Gelatinases	Gelatinase A, 72-kDa gelatinase, Type IV collagenase	71	62	Gelatinins; fibronectin; elastin; Collagens (I, IV, V, VII, X, XI); laminin; aggrecan; vitronectin; decorin; IGFBP-3/5	GPQG-IFGQ Mca-Pro-Leu-Gly-Leu-Trp-Ala-Arg-NH ₂ Ac-Pro-Leu-Ala-S-Nva-Trp-NH ₂ Pro-Gln-Gly-Ile-Ala-Gly-Gln	5.26

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Proteases	Enzyme Name(s)		MW (kDa)		Substrate	Substrate specificity	pI
	MMP	Other names	latent	active			
Stromelysins	MMP-9	Gelatinase B, 92-kDa gelatinase, Type V collagenase	76	67	Collagens (IV, V, VII, X, XIV, XVII); gelatin; entactin; aggrecan; elastin; fibronectin; fibrinogen/fibrin; osteonectin; plasminogen; MBP; IL-1beta	GPLG-IAGQ Pro-Leu-Gly-Met-Leu-Ser-His	5.69
	MMP-3	Stromelysin-1, Transin, CAP, proteoglycanase	52	43	Collagens (III, IV, V, and IX); gelatin; aggrecan; perlecan; decorin; laminin; elastin; caesin; osteonectin; ovostatin; entactin; plasminogen; MBP; IL-1beta; MMP-2/TIMP-2; MMP-7; MMP-8; MMP-9; MMP-13	Mca-RPKPVE-ZWRK(dnp)-NH ₂ Dnp-RPLA-XWRS, when X equals: Leu Phe Tyr Trp	5.77
	MMP-10	Stromelysin-2, Transin-2	52	44	Collagens (II-V); gelatin; casein; aggrecan; entactin; elastin; vitronectin; fibrinogen/fibrin; laminin; MMP-1; MMP-8	Asp-Val-Gly-His-Phe-Ser-Ser-Phe85 Gly-Pro-His-Leu-Leu-Val-Glu-Ala29	5.49
MMP Families	MMP-11	Stromelysin-3, Furin motif	51	46	Laminin; fibronectin; aggrecan;; IGFBP-1	Ala-Ala-Gly-Ala-Met-Phe-Leu-Glu354 Arg-Val-Gly-Phe-Tyr-Glu-Ser-Asp688 Lys-Ala-Leu-His-Val-Thr-Asn-Ile144 Dns-Pro-Leu-Ala-Cys(OmeBzl)-Trp-Ala-Arg-NH ₂	6.25

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Proteases	Enzyme Name(s)		MW (kDa)		Substrate	Substrate specificity	pI
	MMP	Other names	latent	active			
Other MMPs	MMP-12	Macrophage metalloelastase, metalloelastase	52	20	Collagen IV; gelatin; elastin; casein; fibronectin; vitronectin; laminin; entactin; MBP; fibrinogen/fibrin; plasminogen	Leu-Val-Glu-Ala~Leu-Tyr~Leu-Val-CysO ₃ H-Gly ₂₀ Dnp-Arg-Pro-Leu-Ala~Leu-Trp-Arg-Ser-NH ₂ Arg-Pro-Phe-Glu~Val-Lys-Asp-Thr ₂₀₃ Gly-Ala-Met-Phe~Leu-Glu-Ala-Ile ₃₅₆	8.75
	-	-	28.57806		Elastin	MeO-Suc-Ala-Ala-Pro-Val-pNA	9.31
	-	Factor II, coagulation			Collagens	Benzoyl-Phe-Val-Arg-pNA, HCl	
Serine Proteases	-	-	25		Elastin		8.0 - 8.5

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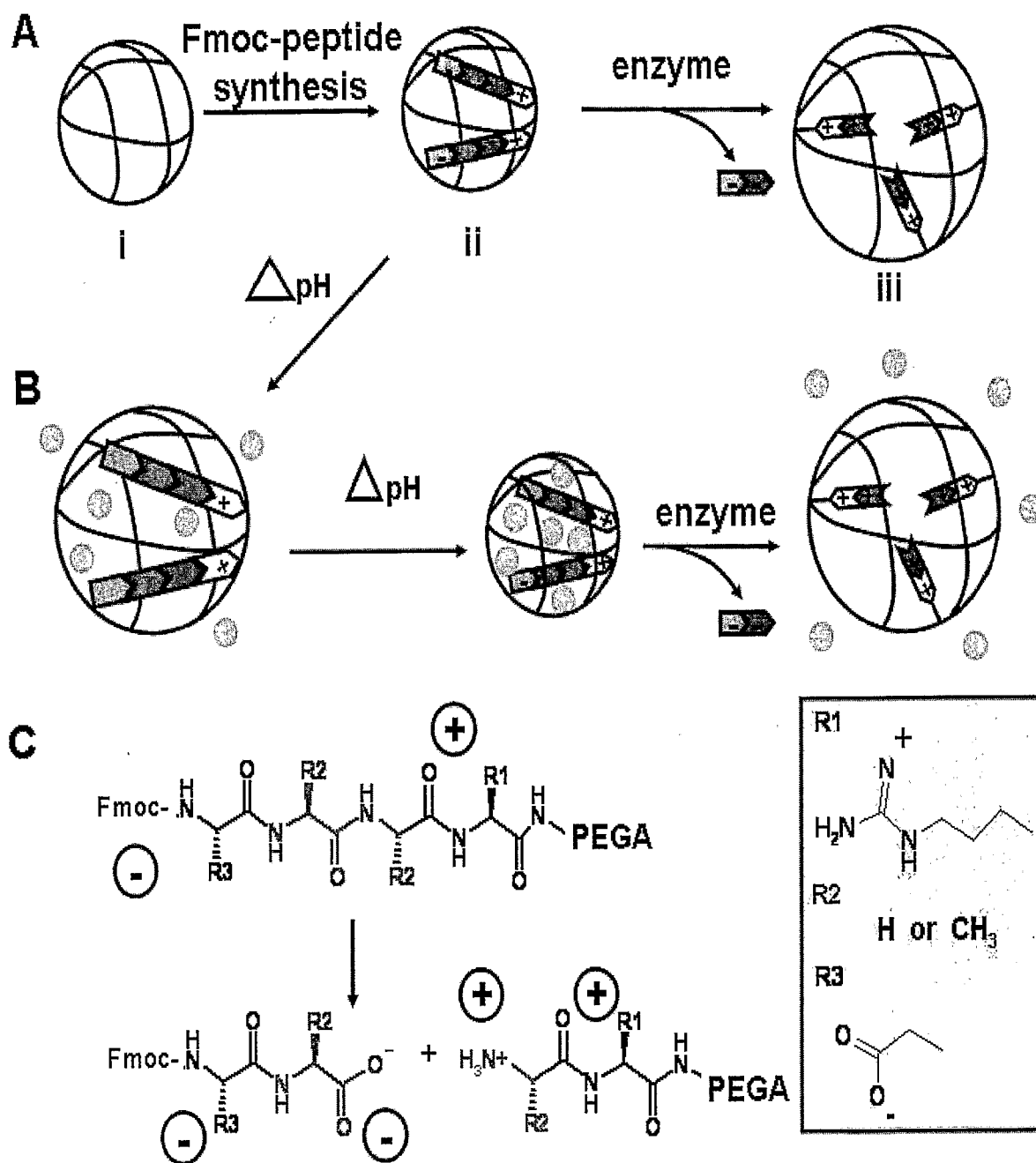
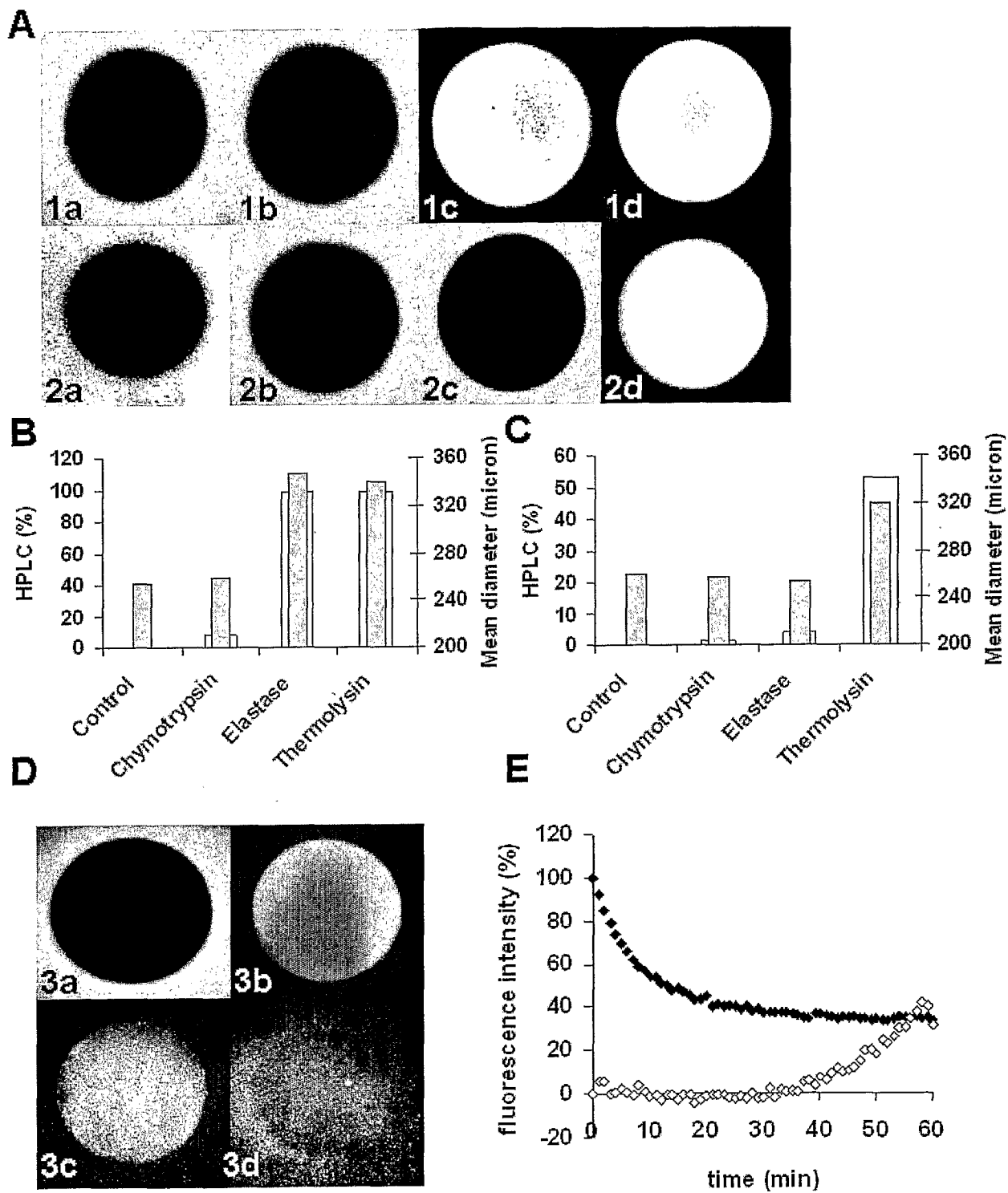
Fig. 11

Fig. 12

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Fig. 13

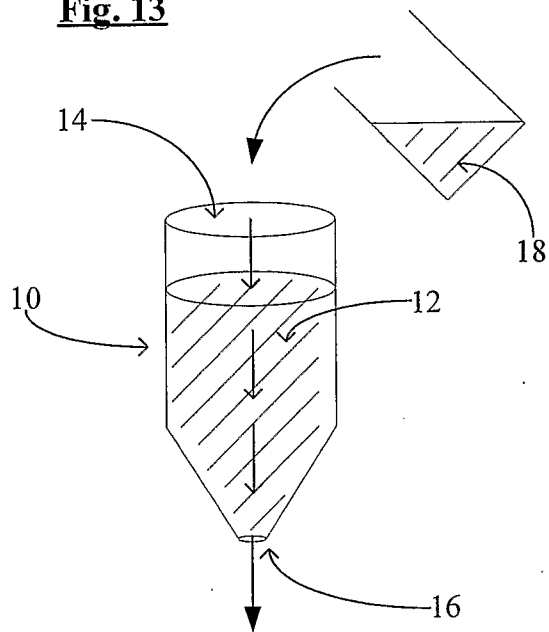
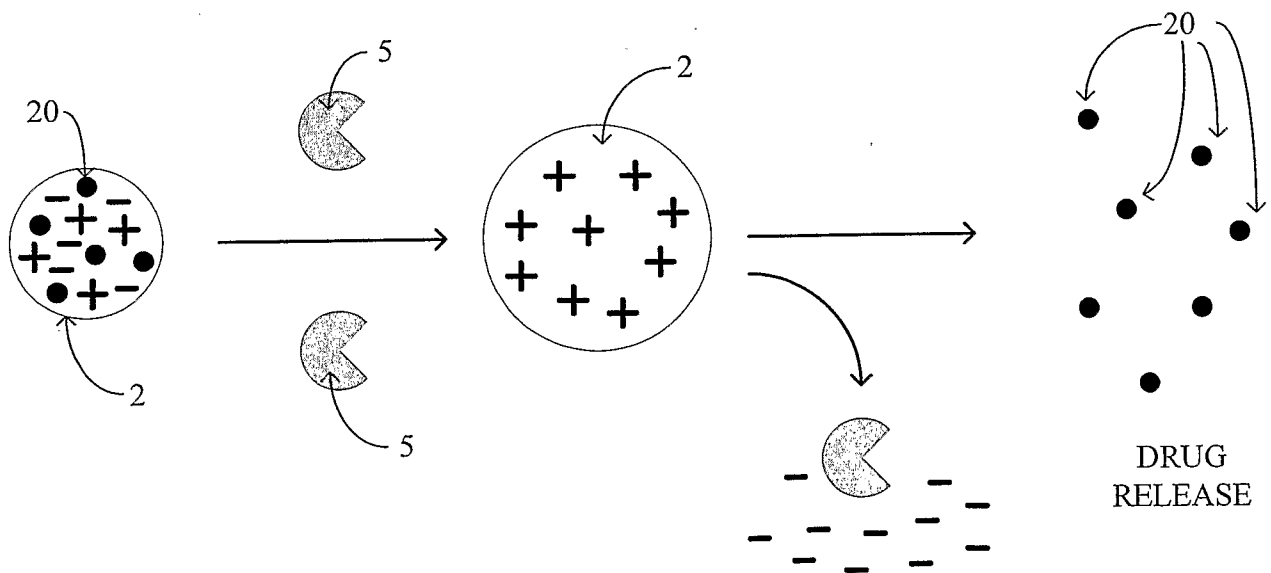


Fig. 14



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2006/002844

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61P35/00 A61P17/02 C08J7/12 A61K47/48 A61K38/05
C07K1/14 A61K9/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61P C08J A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BASSO A., DE MARTIN L., GARDOSSI L., MARGETTS G., BRAZENDALE I., BOSMA A.Y., ULIJN R.V., FLITSCH S.L.: "Improved biotransformations on charged PEGA supports" CHEMICLA COMMUNICATIONS, no. 11, 2003, pages 1296-1297, XP002406534 scheme 1,2 page 1296, left-hand column -----	1-58
A	BOSMA A.Y., ULJIN R.V., MCCONNELL G., GIRKIN J., HALLING P.J., FLITSCH S.L.: "Using two photon microscopy to quantify enzymatic reaction rates on polymer beads" CHEMICAL COMMUNICATIONS, no. 22, 2003, pages 2790-2791, XP002406535 page 3790, left-hand column ----- -/-	1-58

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

27 November 2006

Date of mailing of the international search report

12/12/2006

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KRESS J., ZANALETTI R., AMOUR A., LADLOW M., FREY J.G., BRADLEY M.: CHEMISTRY AN EUROPEAN JOURNAL, vol. 8, no. 16, 2002, pages 3769-3772, XP002406536 scheme 1table 1 -----	1-58
A	DATABASE WPI Week 200501 Derwent Publications Ltd., London, GB; AN 2005-012801 XP002408309 & WO 2004/100988 A1 (MIYAZAKI M) 25 November 2004 (2004-11-25) abstract -----	1-58
A	US 2005/019884 A1 (SPECTOR MARK S [US] ET AL) 27 January 2005 (2005-01-27) page 1, paragraph 4 page 4, paragraph 29 - paragraph 35 -----	1-58
A	US 6 362 276 B1 (HARRIS J MILTON [US] ET AL) 26 March 2002 (2002-03-26) column 3, line 44 - line 63; claims; example 4 -----	1-58
A	US 5 952 232 A (ROTHMAN JAMES EDWARD [US]) 14 September 1999 (1999-09-14) column 5, line 61 - column 6, line 37 -----	1-58
A	WO 98/19787 A (UNIV PITTSBURGH [US]) 14 May 1998 (1998-05-14) page 2, line 31 - page 3, line 24 page 4, line 11 - line 13 page 5, line 4 - line 7 page 7, line 9 - line 12 page 12, line 3 - line 18 page 13, line 13 - line 15 example 6 -----	1-58
A	KIM S.W., BAE Y.H., OKANO T.: "Hydrogels: Swelling, Drug Loading and release" PHARMACEUTICAL RESEARCH, vol. 9, no. 3, 1992, pages 283-290, XP002406537 the whole document -----	1-58
A	WO 97/09068 A2 (UNIV WASHINGTON [US]) 13 March 1997 (1997-03-13) page 8, line 10 - line 18; figure 11 -----	1-58
A	US 2002/068019 A1 (FUJIWARA SHOICHIRO [JP] ET AL) 6 June 2002 (2002-06-06) page 2, paragraph 29 page 3, paragraphs 39-41, 44 examples -----	1-58
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2006/002844

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MASUDA T., NAKAYAMA Y.: "Development of a water-soluble Matrix Metalloproteinase Inhibitor as an Intra-arterial Infusion Drug for Prevention of Restenosis after Angioplasty" JOURNAL OF MEDICINAL CHEMISTRY, vol. 46, 2003, pages 3497-3501, XP002406538 page 3497, right-hand column scheme 1 -----	57
A	WO 2004/103351 A (UNIV MANCHESTER [GB]; TIRELLI NICOLA [GB]; CELLESI FRANCESCO [GB]) 2 December 2004 (2004-12-02) page 2, line 14 - line 26; claims -----	1-58
A	US 2004/072961 A1 (PATHAK CHANDRASHEKHAR P [US] ET AL) 15 April 2004 (2004-04-15) page 3, paragraph 39 -----	1-58
A	WO 99/03454 A1 (INFIMED INC [US]) 28 January 1999 (1999-01-28) page 3, line 11 - line 23 -----	1-58

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2006/002844

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2004100988	A1	25-11-2004	EP 1640020 A1 US 2006234916 A1	29-03-2006 19-10-2006
US 2005019884	A1	27-01-2005	CA 2533924 A1 EP 1649037 A2 US 2006246499 A1 WO 2005017180 A2	24-02-2005 26-04-2006 02-11-2006 24-02-2005
US 6362276	B1	26-03-2002	US 2001016624 A1	23-08-2001
US 5952232	A	14-09-1999	AU 771874 B2 AU 6044299 A CA 2344441 A1 EP 1113820 A1 HU 0103841 A2 JP 2002524536 T WO 0015263 A1	01-04-2004 03-04-2000 23-03-2000 11-07-2001 28-01-2002 06-08-2002 23-03-2000
WO 9819787	A	14-05-1998	AU 717930 B2 AU 5093698 A CA 2270346 A1 EP 0951348 A1 JP 2001505236 T US 5898004 A US 5854078 A	06-04-2000 29-05-1998 14-05-1998 27-10-1999 17-04-2001 27-04-1999 29-12-1998
WO 9709068	A2	13-03-1997	AT 216594 T AU 716154 B2 AU 7011096 A CA 2230557 A1 DE 69620898 D1 DE 69620898 T2 EP 0851768 A2 JP 2000500733 T	15-05-2002 17-02-2000 27-03-1997 13-03-1997 29-05-2002 07-11-2002 08-07-1998 25-01-2000
US 2002068019	A1	06-06-2002	JP 2002174597 A	21-06-2002
WO 2004103351	A	02-12-2004	CA 2525756 A1 CN 1802150 A EP 1624865 A1	02-12-2004 12-07-2006 15-02-2006
US 2004072961	A1	15-04-2004	NONE	
WO 9903454	A1	28-01-1999	AU 748756 B2 AU 8490498 A BR 9811793 A CA 2296459 A1 CN 1271277 A EP 1019031 A1 JP 2001510151 T NZ 502358 A TR 200001021 T2	13-06-2002 10-02-1999 26-09-2000 28-01-1999 25-10-2000 19-07-2000 31-07-2001 26-11-2002 21-09-2000