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(54) Title: ENCAPSULATION OF BIOLOGICALLY ACTIVE AGENTS

(57) Abstract: The present invention provides methods of encapsulating biologically active agents such as proteins in particulate carriers such as nanoparticles using Hip agents. Also provided are compositions comprising particulate carriers obtainable by such methods and uses of such compositions in treatment.

Encapsulation of Biologically Active Agents

Background

A number of drugs have activity at targets in the brain or in the eye, in order to get these to their target they must pass through a biological barrier such as the blood brain barrier. While some molecules are able to cross biological barriers, there are others which do not pass these barriers efficiently or in fact at all. Many drugs are also only efficient when given directly into the target tissue and if this target tissue cannot be reached the drug simply cannot work. Therefore many potentially potent drugs are not useful clinically due to their inability to pass such biological barriers.

A number of approaches have been described in the art to increase drug penetration through these biological barriers.

One approach has been to alter the function of the barrier itself. For instance, osmotic agents or cholinomimetic arecolines result in the opening, or a change in the permeability, of the blood brain barrier *(Saija A et al, J Pharm. Pha. 42:135-138 (1990))*.

Another approach resides in the modification of the drug molecules themselves. For instance modifications of proteins to attempt passage across the blood brain barrier include glycating such proteins, or alternatively by forming a prodrug. *(WO/2006/029845).*

Still another approach is the implantation of controlled release polymers which release the active ingredient from a matrix system directly into the nervous tissue. However, this approach is invasive and requires surgical intervention if implanted directly into the brain or spinal cord (sable et al. US patent no. 4,833,666) this presents problems with patient compliance and often only allows for localised delivery within the brain with the administered drug usually draining away very quickly. *(WO/2006/029845).*

To overcome these limitations drug carrier systems have been used however, a major problem in targeted drug delivery is the rapid opsonisation and uptake of injected carriers by the reticuloendothelial system (RES) especially by the macrophages in the liver and spleen.

There remains therefore a need for an efficient and effective means of delivering macromolecules such as proteins to the brain and to the eye. In particular, it would be desirable to find a method of delivery of macromolecules across the blood brain barrier, which would retain activity on entry into the brain, and which may also provide desirable release kinetics, maintain protein stability and activity, and have the ability to evade clearance mechanisms.

Brief Description of the Figures.

Figure 1 Sizing data obtained by DLS that indicate the presence of nanoparticles in suspension.

Fig 1(a) - Correlogram obtained following analysis of a nanoparticle suspension by dynamic light scattering.

Fig 1(b) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes.

Fig 1(c) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes.

Fig 1(d) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes.

Figure 2. Comparison of the amounts of encapsulated Dalargin achieved with the HIP method to those achieved by the common method of adsorption onto the particle surface.

Figure 3 - Dalargin levels in the brain following delivery with HIP-PBCA nanoparticles. The peptide was detectable in the brain only when encapsulated within the particles using the HIP process.

Figure 4. - Encapsulation of dalargin into PBCA nanoparticles using the HIP process. Determination of the effect of pH of the aqueous phase on the encapsulation efficiency.

Figure 5. - Encapsulation of anti-hen egg lysozyme domain antibody into PBCA nanoparticles using the HIP process. The nanoparticles were analysed by Edman sequencing.

Figure 6 – Confirmation of encapsidation of dAbs into HIP-PBCA nanoparticles by SDS-PAGE analysis. The nanoparticles were centrifuged to remove any free dAb and the pellets analysed by SDS-PAGE to visualise encapsidated dAb.

Figure 7 – Determination of the loading of VEGF dAb (DOM15-26-593) into HIP-PBCA nanoparticles by SDS-PAGE analysis. Nanoparticle formulations were compared to dAb standards in order to quantify the amount of dAb present in the nanoparticles. A total of 3.31 mg of dAb had been encapsidated in the nanoparticles

out of the starting input of 12 mg. Therefore, the loading efficiency was 27.6 %. The dAb loading was 3.31 % w/w.

Figure 8 – Results from the i*n vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies for their ability to deliver their protein load to the brain in the mouse via the intravenous route. At 10 minutes post administration, the dAb in nanoparticles resulted in detectable brain uptake which amounted to 8.0 ng/ml. The free dAb was also detectable in the brain at the slightly lower concentration of 3.3 ng/ml (preliminary data). Therefore, the nanoparticles appeared to marginally increase the brain uptake of the protein (preliminary data). At 60 minutes the opposite was observed as the free dAb appeared to accumulate into the brain resulting in a further increase in its brain levels to 13.5 ng/ml. Brain levels were corrected.

Figure 9 – Brain to blood ratios of dAb derived from the i*n vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies via the intravenous route. The results show that higher proportions of dAb were present in the brain compared to the blood when given with nanoparticles compared to when the dAb was given free in solution.

Figure 10 – Results from the i*n vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies for their ability to deliver their protein load to the brain in the mouse via the intracarotid route. At 10 minutes post administration, the dAb in nanoparticles group exhibited high levels of dAb in the brain, at an average of 627.60 ng/ml.

Figure 11 – Brain to blood ratios of dAb derived from the i*n vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies via the intracarotid route. The dAb in nanoparticles group exhibited brain to blood ratios that were greater than 1 at both time points (1.569 and 1.845 at10 and 60 minutes respectively) suggesting that the majority of formulated dAb had successfully reached the brain.

Figure 12 – Confirmation of generation of microspheres by light microscopy. Formulations of microspheres were all generated by the HIP process using polycaprolactone.
(a) Vit E TPGS 2% surfactant 4000rpm 2 min 20x mag
(b) Vit E TPGS 2% surfactant 7500rpm 2min 20x mag
(c) Vit E TPGS 2% surfactant 7500rpm 2min + dAb1 20x mag

(d) Vit E TPGS 2% surfactant 7500rpm 2min + dAb2 20x mag

Figure 13 – Confirmation of generation of microspheres by laser diffraction. Formulations of microspheres were all generated by the HIP process using polycaprolactone.

(a) Vit E TPGS 2% surfactant 4000rpm 2 min 20x mag

(b) Vit E TPGS 2% surfactant 7500rpm 2min 20x mag

(c) Vit E TPGS 2% surfactant 7500rpm 2min + dAb1 20x mag

(d) Vit E TPGS 2% surfactant 7500rpm 2min + dAb2 20x mag

Figure 14 - Confirmation of encapsidation of dAbs into HIP-PC microspheres by SDS-PAGE analysis. The microspheres were filtered, (F) centrifuged, (3k or 13K rpm) to remove any free dAb and the supernatant, (S), and the pellets, (P), analysed by SDS-PAGE to visualise encapsidated dAb.

Figure 15 - Confirmation of release of encapsidated dAb from HIP-PC microspheres by SDS-PAGE analysis. The microspheres were washed and then heat treated at 56^0C for 0, 20, 40 or 60 mins to release dAb, the debris pelleted, (5 mins @ 5k) and the supernatant, (S), analysed by SDS-PAGE to visualise encapsidated dAb. Molecular markers – SeeBlue Plus 2 pre-stained standard, (invitrogen), molecular weight (kd), The gel confirmed that release of the dAbs had taken place. The gel also confirmed that the dAbs were intact and that they had not fragmented due to the release process.

Summary of Invention

In one aspect of the present invention there is provided a method of encapsulating biologically active agents in particulate carriers such as methods of encapsulating proteins and or peptides in, or in and on, or with nanoparticles and a method of delivery of proteins and or peptides across the blood brain barrier by encapsulation in, or in and on, or with nanoparticles and a method of delivery of proteins and or peptides to the eye by encapsulation in, or in and on, or with particulate carriers.

In another embodiment of the present invention there are provided particulate carriers comprising a particle forming substance and a biologically active agent such as a protein and or peptide, for delivery of a protein and or peptide from the blood to the brain across the blood brain barrier or for delivery to the eye. In another embodiment of the invention are compositions of nanoparticles and their use in treating disorders or diseases of the central nervous system and or eye.

Detailed Description of invention

The present invention provides particulate carriers comprising a particle forming substance and a biologically active agent, and methods of making said particulate carriers.

In one embodiment of the present invention there is provided a method of encapsulating a biologically active agent in a particulate carrier comprising the steps of:

a) solubilising a biologically active agent in the presence of a hydrophobic ion pairing (HIP) agent and in an organic solvent;

b) dissolving a monomer and or oligomer of a polymer forming substance in the organic phase formed in (a);

c) forming an emulsion of the organic phase formed in (b) in a continuous aqueous phase to allow polymerisation of the monomer; and

d) obtaining particulate carriers formed from the emulsion.

In a further embodiment of the present invention there is provided a method of encapsulating biologically active agents in a particulate carrier comprising the steps of:

a) mixing a biologically active agent in an aqueous phase with a hydrophobic ion pairing (HIP) agent in an organic solvent phase to form a biologically active agent-HIP complex;

b) separation of the complex from the aqueous phase.

c) removal of the aqueous phase and homogenisation of the complex with the organic phase;

d) (i) dissolving a polymer in the organic phase formed in (c) and then forming an emulsion of the organic phase in a continuous aqueous phase; or

(ii) dissolving a monomer or oligomer of a polymer forming substance, in the organic phase formed in (c) and then forming an emulsion of the organic phase in a continuous aqueous phase; to allow polymerisation of the monomer or oligomer to form a polymer; and

e) obtaining a particulate carrier formed from the emulsion of step (d).

This method using hydrophobic ion pairing agents allows encapsulation of biologically active agents for example proteins such as for example hydrophilic proteins within the core of the hydrophobic polymer particles. Hydrophobic ion pairing

allows extraction of protein into an organic medium and therefore the method enables preparation of a particulate carrier with a single emulsion.

In a further embodiment the particulate carriers of the present invention comprise biologically active agents such as proteins or peptides. Such proteins may be antigen binding molecules which as used herein refers to antibodies, antibody fragments and other protein constructs which are capable of binding to a target.
Antigen binding molecules may comprise a domain. A “domain” is a folded protein structure which has tertiary structure independent of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain. A “single antibody variable domain” is a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains and modified variable domains, for example, in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least the binding activity and specificity of the full-length domain.

Antigen binding molecules may comprise at least one immunoglobulin variable domain, for example such molecules may comprise an antibody, a domain antibody, Fab, Fab', F(ab')2, Fv, ScFv, diabody, heteroconjugate antibody. Such antigen binding molecules may be capable of binding to a single target, or may be multispecific, i.e. bind to a number of targets, for example they may be bispecific or trispecfic. In one embodiment the antigen binding molecule is an antibody. In another embodiment the antigen binding molecule is a domain antibody (dAb). In yet a further embodiment the antigen binding molecule may be a combination of antibodies and antigen binding fragments such as for example, one or more dAbs and or one or more ScFvs attached to a monoclonal antibody. In yet a further embodiment the antigen binding molecule may be a combination of antibodies and peptides. Antigen binding molecules may comprise at least one non-Ig binding domain such as a domain that specifically binds an antigen or epitope independently of a different V region or domain, this may be a dAb, for example a human, camelid or shark immunoglobulin single variable domain or it may be a domain which is a derivative of a scaffold selected from the group consisting of CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain

(Avimer/Maxibody); Heat shock proteins such as GroEl and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human -crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin); which has been subjected to protein engineering in order to obtain binding to a ligand other than the natural ligand.

CTLA-4 (Cytotoxic T Lymphocyte-associated Antigen 4) is a CD28-family receptor expressed on mainly CD4+ T-cells. Its extracellular domain has a variable domain-like Ig fold. Loops corresponding to CDRs of antibodies can be substituted with heterologous sequence to confer different binding properties. CTLA-4 molecules engineered to have different binding specificities are also known as Evibodies. For further details see Journal of Immunological Methods **248 (1-2)**, 31-45 (2001)

Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid -sheet secondary structure with a numer of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are derived from lipocalins. For further details see Biochim Biophys Acta **1482**: 337-350 (2000), US7250297B1 and US20070224633

An affibody is a scaffold derived from Protein A of *Staphylococcus aureus* which can be engineered to bind to antigen. The domain consists of a three-helical bundle of approximately 58 amino acids. Libraries have been generated by randomisation of surface residues. For further details see Protein Eng. Des. Sel. **17**, 455-462 (2004) and EP1641818A1

Avimers are multidomain proteins derived from the A-domain scaffold family. The native domains of approximately 35 amino acids adopt a defined disulphide bonded structure. Diversity is generated by shuffling of the natural variation exhibited by the family of A-domains. For further details see Nature Biotechnology **23(12)**, 1556 - 1561 (2005) and Expert Opinion on Investigational Drugs **16(6)**, 909-917 (June 2007)

A transferrin is a monomeric serum transport glycoprotein. Transferrins can be engineered to bind different target antigens by insertion of peptide sequences in a permissive surface loop. Examples of engineered transferrin scaffolds include the Trans-body. For further details see J. Biol. Chem **274**, 24066-24073 (1999).

Designed Ankyrin Repeat Proteins (DARPins) are derived from Ankyrin which is a family of proteins that mediate attachment of integral membrane proteins to the cytoskeleton. A single ankyrin repeat is a 33 residue motif consisting of two -helices and a -turn. They can be engineered to bind different target antigens by randomising residues in the first -helix and a -turn of each repeat. Their binding interface can be increased by increasing the number of modules (a method of affinity maturation). For further details see J. Mol. Biol. **332**, 489-503 (2003), PNAS **100(4)**, 1700-1705 (2003) and J. Mol. Biol. 369, 1015-1028 (2007) and US20040132028A1.

Fibronectin is a scaffold which can be engineered to bind to antigen. Adnectins consists of a backbone of the natural amino acid sequence of the 10th domain of the 15 repeating units of human fibronectin type III (FN3). Three loops at one end of the -sandwich can be engineered to enable an Adnectin to specifically recognize a therapeutic target of interest. For further details see Protein Eng. Des. Sel. **18**, 435-444 (2005), US20080139791, WO2005056764 and US6818418B1.

Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein, typically thioredoxin (TrxA) which contains a constrained variable peptide loop inserted at the active site. For further details see Expert Opin. Biol. Ther. 5, 783-797 (2005).

Microbodies are derived from naturally occurring microproteins of 25-50 amino acids in length which contain 3-4 cysteine bridges – examples of microproteins include KalataB1 and conotoxin and knottins. The microproteins have a loop which can be engineered to include upto 25 amino acids without affecting the overall fold of the microprotein. For further details of engineered knottin domains, see WO2008098796.

Other non Ig binding domains include proteins which have been used as a scaffold to engineer different target antigen binding properties include human -crystallin and human ubiquitin (affilins), kunitz type domains of human protease inhibitors, PDZ-domains of the Ras-binding protein AF-6, scorpion toxins (charybdotoxin), C-type lectin domain (tetranectins) are reviewed in Chapter 7 – Non-Antibody Scaffolds from Handbook of Therapeutic Antibodies (2007, edited by Stefan Dubel) and Protein Science **15**:14-27 (2006). Non Ig binding domains of the present invention could be derived from any of these alternative protein domains.

In one embodiment of the invention the antigen binding molecule binds to a target found in the central nervous system such as for example in the brain or spinal cord, or for example in neuronal tissue.

In yet a further embodiment of the invention described herein the antigen binding molecule specifically binds to a target known to be linked to neurological diseases or disorders such as for example MAG (myelin associated glycoprotein), NOGO (neurite outgrowth inhibitory protein) or β-amyloid.

Such antigen binding molecules include antigen binding molecules capable of binding to NOGO for example anti-NOGO antibodies. One example of an anti-NOGO antibody for use in the present invention is the antibody defined by the heavy chain of SEQ ID NO 1 and the light chain of SEQ ID NO 2 or an anti-NOGO antibody or antigen binding fragment thereof which comprises the CDRs of the antibody set out in SEQ ID NO 1 and 2.. Further details of this antibody (H28 L16) can be found in PCT application WO2007068750 which is herein incorporated by reference.

Such antigen binding molecules include antigen binding molecules capable of binding to MAG for example anti-MAG antibodies. One example of the anti-MAG antibody for use in the present invention is the antibody defined by the heavy chain variable region of SEQ ID NO 11 and the light chain variable region of SEQ ID NO 12 or an anti-MAG antibody or antigen binding fragment thereof which comprises the CDRs of the antibody set out in SEQ ID NO 1 and 2.. Further details of this antibody (BvH1 CvL1) can be found in PCT application WO2004014953 which is herein incorporated by reference.

Such antigen binding molecules include antigen binding molecules capable of binding to β-amyloid for example anti- β-amyloid antibodies. One example of the anti- β-amyloid antibody for use in the present invention is the antibody defined by the heavy chain of SEQ ID NO 5 and or the light chain of SEQ ID NO 6 or an anti- β-amyloid antibody or antigen binding fragment thereof which comprises the CDRs of the antibody set out in SEQ ID NO 5 and 6. Further details of this antibody (H2L1) can be found in PCT application WO2007113172 which is herein incorporated by reference. An alternative anti- β-amyloid antibody Which is of use in the present invention is the anticody defined by the heavy chain of SEQ ID NO 7 and or the light chain of SEQ ID NO 8 or an anti- β-amyloid antibody or antigen binding fragment thereof which comprises the CDRs of the antibody set out in SEQ ID NO 7 and 8.

The CDR sequences of such antibodies can be determined by the Kabat numbering system (Kabat *et al; Sequences of proteins of Immunological Interest* NIH, 1987), the Chothia numbering system (Al-Lazikani *et al.*, (1997) JMB **273**,927-948), the contact definition method (MacCallum R.M., and Martin A.C.R. and Thornton J.M, (1996), Journal of Molecular Biology, 262 (5), 732-745) or any other established method for numbering the residues in an antibody and determining CDRs known to the skilled man in the art.

In one embodiment of the invention the antigen binding protein binds to a target found in the eye such as for example TNF, TNFr-1, TNFr-2, TGFbeta receptor-2, VEGF, NOGO, MAG, IL-1, IL-2, IL-6, IL-8, IL-17, CD20, Beta amyloid, FGF-2, IGF-1, PEDF, PDGF or a complement factor for example C3, C5, C5aR , CFD, CFH, CFB, CFI, sCR1 or C3.

In a further embodiment of the invention the antigen binding protein binds to VEGF. In an alternative embodiment of the invention the antigen binding protein binds to β-amyloid.

In one embodiment of the present invention the particulate carriers may be microspheres or nanoparticles. In one such embodiment the particulate carrier is a nanoparticle and the biologically active agent is a protein. In another embodiment the particulate carrier is a nanoparticle and the biologically active agent is a peptide. In a further embodiment the particulate carrier is a nanoparticle and the biologically active agent comprises an antigen binding molecule for example a domain antibody or antibody. In yet a further embodiment the particulate carrier is a nanoparticle and the biologically active agent comprises a domain. In another embodiment the particulate carrier is a microsphere and the biologically active agent is a protein. In a further embodiment the particulate carrier is a microsphere and the biologically active agent is a peptide. In yet a further embodiment the particulate carrier is a microsphere and the biologically active agent comprises an antigen binding molecule for example a domain antibody or antibody. In yet a further embodiment the particulate carrier is a microsphere and the biologically active agent comprises a domain.

In one embodiment of the present invention there is provided a composition comprising nanoparticles according to the method of the present invention. In a further embodiment at least about 90% of the nanoparticles by number are within the range of about 1nm to about 1000nm when measured using dynamic light scattering

techniques. In a further embodiment at least about 90% of the nanoparticles by number are within the range of about 1nm to about 400nm, or about 1nm to about 250nm or about 1nm to about 150nm, or about 40nm to about 250nm, or about 40nm to about 150nm, or about 40nm to about 100nm when measured using dynamic light scattering techniques.

In yet a further embodiment of the present invention at least about 90% of the nanoparticles by number are within the range of about 40nm to about 250 nm when measured using dynamic light scattering techniques.

In yet a further embodiment of the present invention at least about 90% of the nanoparticles by number are within the range of about 40nm to about 150 nm when measured using dynamic light scattering techniques.

In yet a further embodiment there is provided a composition comprising the nanoparticles of the present invention wherein the median size of the nanoparticles in the composition is less than about 1000nm in diameter, for example is less than about 400nm in diameter for example is less than about 250nm in diameter, for example is less than about 150nm in diameter when measured by light scattering techniques.

In yet a further embodiment the median size of the nanoparticles in the composition is about 40nm to about 250nm.

In yet a further embodiment the median size of the nanoparticles in the composition is about 40nm to about 150nm.

In one embodiment of the present invention there is provided a composition comprising microspheres according to any method of the invention as presented herein. In a further embodiment at least about 90% of the microspheres by number have a diameter within the range of about 1µm to about 100µm when measured using Low angle laser light scattering techniques. In a further embodiment at least about 90% of the particles by number are within the range of about 1µm to about 80µm, or about 1µm to about 60µm or about 1µm to about 40µm, or about 1µm to about 30µm or about 1µm to about 10µm when measured using Low angle laser light scattering techniques.

In yet a further embodiment of the present invention at least about 90% of the microspheres by number are within the range of about 1 µm to about 60 µm when measured using Low angle laser light scattering techniques.

In yet a further embodiment of the present invention at least about 90% of the microspheres by number are within the range of about 1µm to about 30µm when

measured using Low angle laser light scattering techniques.

In yet a further embodiment there is provided a composition comprising the microspheres of the present invention wherein the median size of the microspheres in the composition is less than about 100μm in diameter, for example is less than about 80μm in diameter for example is less than about 60μm in diameter, for example is less than about 40μm in diameter when measured by Low angle laser light scattering techniques.
In yet a further embodiment the median size of the microspheres in the composition is about 1μm to about 6μm, or 1μm to about 30μm.

In another embodiment of the invention the particulate carriers continue to release therapeutic amounts of active biological molecules over a period of at least 3 months or longer, or of up to 6 months or longer or of up to 12 months or longer.

In one embodiment the biologically active agent is insoluble in the organic phase without the presence of hydrophobic ion pairing agents.

In one embodiment of the invention as herein described the hydrophobic ion pairing agent is a cationic HIP agent when the protein is anionic. In another embodiment the hydrophobic ion pairing agent is an anionic HIP agent when the protein is cationic. In a further embodiment the anionic HIP agent is selected from the group consisting of Alkyl quaternary ammonium cations, preferably alkyl ammonium bromides, more preferably tetrabutyl ammonium bromide, tetrahexyl ammonium bromide, tetraoctyl ammonium bromide, Sodium dodecyl sulphate (SDS), sodium oleate or docusate sodium (aka Aerosol OT™) and the HIP agent is present in stoichiometric amounts equal to or greater than the number of net positive charges on the protein. In another embodiment, the cationic HIP agent is selected from the group consisting of: dimethyldioctadecyl-ammonium bromide (DDAB18); 1,2-dioleoxy-3-(trimethylammonium propane (DOTAP); or cetrimonium bromide (CTAB) and the HIP agent is present in stoichiometric amounts equal to or greater than the number of net negative charges on the protein.

In a further embodiment any hydrophobic cation or anion could potentially be used as a HIP agent to solubilise the protein. Hydrophobic ion pairing (HIP) involves stoichiometric replacement of polar counter ions with a species of similar charge but less easily solvated. As disclosed herein, the invention provides a method that uses

HIP to change the solubility properties of proteins, allowing extraction of the protein into an organic solvent, such as methylene chloride. Docusate sodium (Bis(2-ethylhexyl) sodium sulfosuccinate) is one example of a suitable ion-pairing agent. In one embodiment, methylene chloride containing docusate sodium is mixed with an aqueous protein solution. This results in ion pairing of the docusate ion with the protein and subsequent partitioning of the protein into the oil phase. Dissolution of the protein in methylene chloride allows the protein to be encapsulated in nanoparticles or microspheres prepared via a single oil-in-water emulsion method.

In one embodiment of the invention herein described the continuous aqueous phase has a pH of about 7.0 or higher when the protein is anionic and the HIP agent is cationic, for example the pH may be at least about 8.0 or at least about 10.0 or is at least about 12.0.

In an alternative embodiment of the invention herein described the continuous aqueous phase has a pH of about 7.0 or lower when the protein is cationic and the HIP agent is anionic, for example the pH may be less than about 6.0 or less than about 4.0 or less than about 2.0.

In one such embodiment the weight/weight (w/w) ratio of protein to polymer may be 0.5% to 90% for example is at least about 0.5% or is at least about 1% or is at least about 2% or is at least about 2.5% or is at least about 5% or is at least about 9% or is at least about 10% or is at least about 15% or is at least about 20% or is at least about 40%, or is at least about 50%, or is at least about 60%, or is at least about 70%, or is at least about 80% or is at least about 90%. For example when the protein is a peptide the peptide to polymer ratio may be at least about 9%, when the protein is an antibody the antibody to polymer ratio may be at least about 2%, or when the protein is a domain antibody the domain antibody to polymer ratio may be at least about 2.5%.

In one embodiment of the present invention the w/w ratio of protein to total formulation (polymer +HIP and optionally surfactants) may be 0.5% to 50% for example is at least about 5% or at least about 9% or at least about 15% or at least about 16% or at least about 20% or at least about 25%. For example when the protein is a peptide the peptide to total formulation ratio may be at least about 16% or when the protein is an antibody the antibody to polymer ratio may be at least about 1%, or when the protein is a domain antibody the domain antibody to total formulation ratio may be at least about 9%.

In one embodiment of the present invention the encapsulation efficiency of the particles is at least about 1% or is at least about 2% or is at least about 10% or is at least about 20% or is at least about 40% or is at least about 50% or is at least about 60% or is at least about 70% or is at least about 80% or is at least about 90% or is alt least about 95% or is least about 97% or is at least about 99%. For example when the protein is a peptide the encapsulation efficiency may be at least about 90%, when the protein is an antibody the encapsulation efficiency may be at least about 1%, or when the protein is a domain antibody the encapsulation efficiency may be at least about 70%.

In one embodiment of the present invention the monomer or oligomer is selected from the group consisting of: methylmethacrylates, alkylcyanoacrylates, hydroxyethylmethacrylates, methacrylic acid, ethylene glycol dimethacrylate, acrylamide, N, N'-bismethylene acrylamide and 2-dimethylaminoethyl methacrylate. In a further embodiment the monomer is an alkylcyanoacrylate for example is butylcyanoacrylate (BCA).

In a further embodiment the polymer used in any of the methods as described herein is selected from but not limited to: poly – L –lactide (PLA), poly(lacto-co-glycolide) (PLG), poly(lactide), poly(caprolactone), poly(hydroxybutyrate) and/or copolymers thereof. Suitable particle-forming materials include, but are not limited to, poly(dienes) such as poly(butadiene) and the like; poly(alkenes) such as polyethylene, polypropylene, and the like; poly(acrylics) such as poly(acrylic acid) and the like; poly(methacrylics) such as poly(methyl methacrylate), poly(hydroxyethyl methacrylate), and the like; poly(vinyl ethers); poly(vinyl alcohols); poly(vinyl ketones); poly(vinylhalides) such as poly(vinyl chloride) and the like; poly(vinyl nitriles), poly(vinyl esters) such as poly(vinyl acetate) and the like; poly(vinyl pyridines) such as poly(2-vinyl pyridine), poly(5-methyl-2-vinyl pyridine) and the like; poly(styrenes); poly(carbonates); poly(esters); poly(orthoesters); poly(esterarnides); poly(anhydrides); poly(urethanes); poly(amides); cellulose ethers such as methyl cellulose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, and the like; cellulose esters such as cellulose acetate, cellulose acetate phthalate, cellulose acetate butyrate, and the like; poly(saccharides), proteins, gelatin, starch, gums, resins, and the like. These materials may be used alone, as physical mixtures (blends), or as copolymers.Also polyacrylates, polymethacrylates, polybutylcyanoacrylates, polyalkylcyanoacrylates, polyarylamides, polyanhydrates,

polyorthoesters, N,N-L-lysinediylterephthalate, polyanhydrates, desolvated biologically active agents or carbohydrates, polysaccharides, polyacrolein, polyglutaraldehydes and derivatives, copolymers and polymer blends.

Examples of organic solvents suitable for use with the methods of the invention include but are not limited to water-immiscible esters such as ethyl acetate, isopropyl acetate, n-propyl acetate, isobutyl acetate, n-butyl acetate, isobutyl isobutyrate, 2-ethylhexyl acetate, ethylene glycol diacetate; water-immiscible ketones such as methyl ethyl ketone, methyl isobutyl ketone, methyl isoamyl ketone, methyl n-amyl ketone, diisobutyl ketone; water-immiscible aldehydes such as acetaldehyde, n-butyraldehyde, crotonaldehyde, 2-ethylhexaldehyde, isobutylaldehyde and propionaldehyde; water-immiscible ether esters such as ethyl 3-ethoxypropionate; water-immiscible aromatic hydrocarbons such as toluene xylene and benzene; water-immiscible halohydrocarbons such as 1,1,1 trichloroethane; water-immiscible glycol ether esters such as propylene glycol monomethyl ether acetate, ethylene glycol monoethyl ether acetate, ethylene glycol monobutyl ether acetate, diethylene glycol monobutyl ether acetate; water-immiscible phthalate plasticisers such as dibutyl phthalate, diethyl phthalate, dimethyl phthalate, dioctyl phthalate, dioctyl terephthalate, butyl octyl phthalate, butyl benzyl phthalate, alkyl benzyl phthalate; water-immiscible plasticisers such as dioctyl adipate, triethylene glycol di-2-ethylhexanoate, trioctyl trimellitate, glyceryl triacetate, glyceryl/tripropionin, 2,2,4-trimethyl-1,3-pentanediol diisobutyrate, methylene chloride, ethylacetate or dimethylsulfoxide, carbon tetrachloride, chloroform, cyclohexane, 1,2-dichloroethane, dichloromethane, diethyl ether, dimethyl formamide, heptane, hexane and other hydrocarbons, methyl-tert-butyl ether, pentane, toluene, 2,2,4-trimethylpentane, 1-octanol and its isomers or benzyl alcohol.

In one embodiment of the invention the solvent used in the methods of the invention will be selected from methylene chloride, ethylacetate or dimethylsulfoxide, carbon tetrachloride, chloroform, cyclohexane, 1,2-dichloroethane, dichloromethane, diethyl ether, dimethyl formamide, heptane, hexane and other hydrocarbons, methyl-tert-butyl ether, pentane, toluene, 2,2,4-trimethylpentane, 1-octanol and its isomers, benzyl alcohol.

The particulate carriers, compositions comprising them or methods of making them in all aspects of the present invention as herein described may further comprise the addition of a surfactant such as but not limited to: sodium cholate, poloxamer 188

(pluronic F68™, or F127), polyvinyl alcohol, polyvinyl pyrrolidone, polysorbate 80, dextrans. poloxamers, poloxamines, carboxylic acid esters of multifunctional alcohols, alkoxylated ethers, alkoxylated esters, alkoxylated mono-, di and triglycerides, alkoxylated phenols and diphenols, ethoxylated ethers, ethoxylated esters, ethoxylated triglycerides, substances of the GenapolR™ and BaukiR™ series, metal salts of fatty acids, metal salts of carboxylic acids, metal salts of alcohol sulfates, and metal salts of fatty alcohol sulfates and metal salts of sulfosuccinates and mixtures of two or more of said substances.

In a further embodiment the surfactant is selected from sodium cholate, poloxamer 188 (pluronic F68™), polyvinyl alcohol, polyvinyl pyrrolidone, polysorbate 80 and dextrans.

In one embodiment of the present invention there is provided particulate carriers comprising biologically active agents, obtainable by any of the methods of the invention herein described.

The biologically active agent encapsulated in particulate carriers and or compositions of the present invention retains at least some biological activity on its release from the particulate carrier, for example, a proportion of the molecules in the composition may retain at least some ability to bind to their target when the agent is a binding agent and elicit a biological response on the release of the biologically active agent from the particles. Such binding can be measured in a suitable biological binding assay, examples of suitable assays include but are not limited to ELISA or Biacore™.

In a further embodiment the composition retains at least 50% of its affinity for the target, or at least 70% or at least 90% of its affinity (Kd) for the target when measured by a biological binding assay on release from the particles for example in one embodiment as determined by ELISA, Biacore. In one embodiment the composition will be capable of eliciting a therapeutic effect in the subject to which it is administered. The biological activity of the compositions of the invention can be measured by any suitable assay which measures activity of the encapsulated biologically active molecule, for example where the biologically active molecule is a VEGF dAb, the assay described in Example 18 can be used.

In another embodiment there is provided a pharmaceutical composition comprising a biologically active agent encapsulated in a particulate carrier of the present invention as herein described.

In a further embodiment there is provided a pharmaceutical composition comprising a

protein encapsulated in the nanoparticles of the present invention as herein described.

In a further embodiment a composition of the present invention may be used to treat and or prevent disorders or diseases which involve the particulate carriers crossing the blood brain barrier.

In a further embodiment a composition of the invention as herein described may be used to treat and or prevent disorders or diseases of the Central nervous system, for example it may be used to treat and or prevent Alzheimer's disease, Huntington's disease, bovine spongiform encephalopathy, West Nile virus encephalitis, Neuro-AIDS, brain injury, spinal cord injury, metastatic cancer of the brain, or multiple sclerosis, stroke.

In a further embodiment the composition may comprise an anti-MAG antibody for the treatment and or prevention of stroke or neuronal injury.
In another embodiment the composition may comprise an anti-NOGO antibody for the treatment and or prevention of stroke or neuronal injury or for example for the treatment or prophylaxis of neurodegenerative diseases such as Alzheimer's disease.
In another embodiment the composition may comprise an anti-ßamyloid antibody for the treatment and or prevention of stroke or neuronal injury or for example for the treatment or prophylaxis of neurodegenerative diseases such as Alzheimer's disease.

In one embodiment of the invention as herein described the particulate carriers may be administered to the patient by parenteral injection or infusion, intravenous, or intraarterial administration.

In a further embodiment the compositions of the invention as herein described may be used to treat and or prevent disorders or diseases of the eye. In a further embodiment a composition of the invention as herein described may be used to treat and or prevent disorders such as but not limited to age related macular degeneration (neovascular/ wet), diabetic retinopathy, retinal venous occlusive disease, uveitis, corneal neovascularisation or glaucoma.

In yet a further embodiment the composition is used to treat and or prevent AMD

(age related macular degeneration), for example wet AMD, or dry AMD.

In another embodiment of the present invention there is provided biologically active agents encapsulated in nanoparticles and or microspheres as described herein for use in medicine.

In one embodiment of the present invention there is provided the use of compositions of the invention as described herein in the manufacture of a medicament for the treatment and or prevention of a disease of the central nervous system. In yet another embodiment there is provided the use of a composition of the invention as described herein in the manufacture of a medicament for the treatment and or prevention of Alzheimer's disease. In yet a further embodiment there is provided the use of a composition of the invention as described herein in the manufacture of a medicament for the treatment and or prevention of stroke or neuronal injury.

In another embodiment of the invention there is provided the use of a composition of the invention as described herein in the manufacture of a medicament for the treatment or prevention of ocular diseases such as for example in the manufacture of a medicament for the treatment and or prevention of AMD.

The invention provides methods of treating and or preventing a disease of the central nervous system using a composition of the present invention. In a further embodiment there is provided a method of treating Alzheimer's disease using a composition of the present invention. In yet another embodiment of the present invention there is provided a method of treating and or preventing stroke or neuronal injury using a composition of the present invention.

The invention also provides methods of treating and or preventing ocular disease using a composition of the present invention. In a further embodiment there is provided a method of treating and or preventing AMD using a composition of the present invention.

Definitions:

As used herein the term "particle forming substance" is used to describe any monomer and or oligomer capable of polymerising, or a polymer which can form an insoluble particle in an aqueous environment for example PBCA, PLGA. The particle

forming substance will be soluble in an organic solvent when not polymerised.

The term "particulate carrier" as used throughout this specification is used to cover both nanoparticles and microspheres. "Microspheres" are particles composed of various natural and synthetic materials with diameters larger than 1µm whereas "nanoparticles" as used herein are submicron sized particles such as for example 1-1000nm.
In one embodiment the terms particulate carrier, nanoparticles and microspheres as used herein denotes a carrier structure which is biocompatible and sufficiently resistant to chemical and/or physical destruction by the environment of use such that a sufficient amount of the particles remain substantially intact after entry in to the human or animal body following administration and for sufficient time so as to be able to reach the desired target organ or tissue e.g. the brain or the eye.

The term "Biologically active agent" as used herein is a term used to indicate that the molecule must be capable of at least some biological activity when reaching their desired target. For the avoidance of doubt the term "Biologically active agent" and the "biologically active molecule" as used throughout the specification are intended as to have the same meaning and able to be used interchangeably.

The term "solubilisation" is defined as either formation of a solution, in the form of individual molecules in the solvent, or formation of a solid in liquid suspension, in the form of fine solid aggregates of molecules suspended in the liquid. The solubilisation process may also result in a mixture of fully dissolved molecules and suspended solid aggregates.

The term "protein" as used throughout this specification for encapsulation in particulate carriers includes proteins having a molecular weight of at least 11kDa, or at least 12kDa, or at least 50kDa, or at least 100kDa, or at least 150kDa or at least 200kDa. Proteins for encapsulation may also be of considerable length such as at least 70 amino acids in length or at least 100 amino acids in length or at least 150 amino acids in length or at least 200 amino acids in length.

The term "peptide" as used throughout this specification for encapsulation in particulate carriers includes shorter sequences of amino acids having a molecular weight of no more than about 10 kDa, or no more than about 8 kDa, or no more than about 5 kDa, or no more than about 2 kDa or no more than about 1 kDa or is less

than 1Kda. Peptides for encapsulation are no more than 70 amino acids in length or are no more than 50 amino acids in length, or are no more than are no more than 40 amino acids in length, or are no more than 20 amino acids in length or are less than 10 amino acids in length.

The term “Peri-ocular” refers to local administration to positions surrounding the outside of the eye and includes but is not limited to:
“Sub-conjuctival” - underneath the conjuctiva - a clear mucus membrane that covers the eyeball over the sclera; “Sub-tenon” - underneath the Tenon's membrane that envelopes the eye but outside of the sclera; “peribulbar” - the space underneath the globe of the eye where it sits in the eye socket; “retrobulbar” - the space at the very back of the globe of the eye, close to the optic nerve; “supra-choroidal” - underneath the sclera but outside of the choroid into the supra-choroidal space; “trans-scleral” - this term can also be used to mean delivery across, i.e. from outside of the sclera.

The phrase “immunoglobulin single variable domain” refers to an antibody variable domain (V_H, V_{HH}, V_L) that specifically binds an antigen or epitope independently of a different V region or domain. An immunoglobulin single variable domain can be present in a format (e.g., homo- or hetero-multimer) with other, different variable regions or variable domains where the other regions or domains are not required for antigen binding by the single immunoglobulin variable domain (i.e., where the immunoglobulin single variable domain binds antigen independently of the additional variable domains). A “domain antibody” or “dAb” is the same as an “immunoglobulin single variable domain” which is capable of binding to an antigen as the term is used herein. An immunoglobulin single variable domain may be a human antibody variable domain, but also includes single antibody variable domains from other species such as rodent (for example, as disclosed in WO 00/29004, nurse shark and Camelid V_{HH} dAbs. Camelid V_{HH} are immunoglobulin single variable domain polypeptides that are derived from species including camel, llama, alpaca, dromedary, and guanaco, which produce heavy chain antibodies naturally devoid of light chains. Such V_{HH} domains may be humanised according to standard techniques available in the art, and such domains are still considered to be “domain antibodies” according to the invention. As used herein “V_H includes camelid V_{HH} domains.

The term “antigen binding molecule” as used herein refers to antibodies, antibody fragments and other protein constructs which are capable of binding to a target.

A "domain" is a folded protein structure which has tertiary structure independent of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain. A "single antibody variable domain" is a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes complete antibody variable domains and modified variable domains, for example, in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains which retain at least the binding activity and specificity of the full-length domain.

The term "Light scattering techniques" as used herein is a means used to determine the size distribution profile of small particles in solution – one example of light scattering technique is dynamic light scattering which may be used to measure nanoparticles and another example of light scattering is static light scattering or low angle light scattering which may be used to measure microspheres.

The term "Dynamic light scattering" (DLS) as used herein is a method which utilises the light scattered by particle dispersions to derive information on the size of the particles. Dynamic light scattering relies on the fact that when in liquid suspension, the Brownian motion of particles is dependent on particle size and that the Brownian motion of the particles produces fluctuations in the intensity of light scattered from a particle sample. The particle diameter is derived by analysing these fluctuations by means of a correlation function. The Stokes-Einstein equation is then applied to yield the mean hydrodynamic diameter of the particles.

A multi-exponential analysis can produce a size distribution, providing insight into the presence of different species inside a sample. DLS is generally accepted for the analysis of nanoparticles.

"Static light scattering" or "Low angle laser light scattering" which are used interchangeably throughout the present specification is sometimes referred to as Laser diffraction. Laser diffraction relies on the fact that the diffraction angle is inversely proportional to particle size. The method utilises the full Mie theory which completely solves the equations for the interaction of light with matter. Laser diffraction can be used for the analysis of nanoparticles and microparticles (0.02 to 2000 micrometers in diameter).

The term “Blood brain barrier” (BBB) as used herein is a membranic structure that acts primarily to protect the brain from chemicals in the blood, while still allowing essential metabolic function. It is composed of cerebral microvascular endothelial cells, which are packed very tightly in brain capillaries. This higher density restricts passage of substances from the bloodstream much more than endothelial cells in capillaries elsewhere in the body.

Throughout this specification the percentage drug loading is defined as the percentage of weight of drug per weight of material used in the particle formulation (polymer weight) w/w.

% drug loading = (weight of drug / weight of material used in the particle formulation) x 100%.

Within this specification the invention has been described, with reference to embodiments, in a way which enables a clear and concise specification to be written. It is intended and should be appreciated that embodiments may be variously combined or separated without parting from the invention.

Examples

Example 1 - Preparation of PBCA nanoparticles by the HIP process

The nanoparticles were prepared by adding 100 µl BCA monomer to an organic phase containing solubilised HIP ion (docusate sodium, 3.058-6.116 % w/v in 1 ml dichloromethane). The resulting solution was pipetted into an aqueous phase (1% w/v dextran, 0.2 % w/v pluronic F68, 10 ml, pH 7.0) with homogenisation at 7,000 using a Silverson L4RT homogeniser. Exposure to the neutral pH of the aqueous phase resulted in rapid polymerisation of the BCA monomer to form PBCA polymer. The emulsion that was formed was homogenised for 45 seconds and then incubated in a fume hood for 3 hours to allow the organic solvent to evaporate and nanoparticles to form. The resulting nanoparticle suspension was stored at 4°C.

Example 2 - Confirmation of nanoparticle formation by dynamic light scattering

The formation of PBCA nanoparticles by the HIP process was confirmed by sizing using dynamic light scattering (DLS). The particles were analysed using a Brookhaven Instruments corporation particle size analyser (BIC 90 plus). Figure 1 shows the sizing data obtained by DLS that indicate the presence of nanoparticles in suspension. Sizing by DLS showed that nanoparticles of a mean hydrodynamic diameter of 291.4 nm had formed (figure 1a). The particle population was also found to be relatively monodisperse, with the polydispersity index, which is a measure of how broad the range of particle sizes in the sample is, at 0.242 (figure 1a). This is below the maximum acceptable value of 0.300 for a particle formulation. In general, the correlogram confirmed that the particle preparation process had successfully generated a good quality suspension of PBCA nanoparticles.

The derived data suggest that the majority of the particles are small (Figures 1b-d). The results suggest that approximately 96.3 % of the particle population had a diameter of 201.37 nm or lower (figure 1b). The suspension also appeared to be generally free of large aggregates and did not contain any particles that exceeded 732.05 nm in diameter, with the majority of the particle population being significantly smaller (figure 1c). The formulation also did not seem to contain any particles that were smaller than 143.38 nm (figure 1d). Therefore, the majority of the particles were of a diameter between 143.38 and 201.37 nm, a size that is safe for intravenous administration but not too small so that it reduces drug loading efficiency.

Fig 1(a) - Correlogram obtained following analysis of a nanoparticle suspension by dynamic light scattering. According to the data obtained, the mean hydrodynamic diameter of the particles was 291.4 nm and the polydispersity index 0.242.

Fig 1(b) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes. The data suggest that 96.3% of the particle population appeared to have a diameter of 201.37 nm or lower.

Fig 1(c) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes. The data suggest that 96.3% of the particle population appeared to have a diameter of 201.37nm or lower and that 100% of the particle sample possessed a diameter of 732.05 nm or lower. Therefore, the suspension was found to be free of large aggregates and was therefore considered to be safe for intravenous administration.

Fig 1(d) - Multimodal size distribution (derived data) of the nanoparticles plotted to depict the distribution of the particle population (number) over a range of sizes. The data suggest that 6.2% of the particle sample possesses a diameter of 143.38 nm or lower.

The process was found to yield similar nanoparticle sizes when different nanoparticle formulations were prepared. Table 1 summarises the sizing data obtained from a series of six different formulations of varying compositions:

Table 1

Formulation	Mean hydrodynamic diameter (nm)	Polydispersity index
1	367.7	0.280
2	291.4	0.242
3	236.3	0.259
4	244.7	0.256
5	255.4	0.213
6	269.8	0.221
Average	294.22	0.245

In general, the HIP process was found to generate nanoparticle suspensions that

were of the desired diameter and polydispersity.

Example 3 - Solubilisation of peptides into the organic phase using the HIP process and encapsulation into PBCA nanoparticles

A solution of the hexapeptide dalargin was prepared by dissolving 30-60 mg of the peptide into 3 ml of CaCl2 (18.3 mM) and lowering the pH to 3.05 by addition of concentrated HCl (2M). The resulting solution (500 µl, 10-20 mg/ml, and total amount of peptide 5-10 mg) was added to a solution of the HIP agent docusate sodium in dichloromethane (1ml, 3.058-6.116 % w/v) in a 2 ml eppendorf tube. The volume of HIP solution used was twice that of the peptide solution (1 ml HIP solution for 500 µl peptide solution). The molar ratio of HIP: peptide was 10:1 with 5 mg peptide and 5:1 with 10 mg peptide. The organic and aqueous phases were mixed by vortexing at maximum speed for 1 minute. The resulting suspension was then centrifuged to separate the two phases at 20,817 rcf for 50 minutes. The organic layer (containing solubilised peptide) was collected and used to prepare nanoparticles.
In order to confirm that the process had been successful at solubilising the peptide into the organic phase, the amount of peptide remaining in the aqueous phase was determined. Analysis by LC-MS and Edman sequencing showed that at least 99% of the peptide had been successfully extracted into the organic phase.

Example 4 - Encapsulation of peptide within the PBCA nanoparticles

The nanoparticles were prepared by adding 100 µl BCA monomer to the organic phase containing solubilised peptide and HIP (1 ml). The resulting solution was pipetted into an aqueous phase (1% w/v dextran, 0.2 % w/v pluronic F68, 10 ml, pH 7.0) with homogenisation at 7,500 using a Silverson L4RT homogeniser (fine emulsor screen, ¾ inch probe). Exposure to the neutral pH of the aqueous phase resulted in rapid polymerisation of the BCA monomer to form PBCA polymer. The emulsion that was formed was homogenised for 45 seconds and then incubated in a fume hood with stirring (IKA magnetic stirrer, speed setting 4) for 1 hour in order to evaporate the organic phase. The speed setting was then lowered to 3 and the formulations incubated for a further 2 hours to ensure evaporation of the organic phase and nanoparticle formation. The nanoparticle suspensions were collected and stored at 4 degrees C.

The resulting nanoparticles were centrifuged to remove any free peptide and re-suspended in water or PBS.

The encapsulation efficiency was determined by analysing the particles by LC-MS. It was found that approximately 90% of the peptide dose was encapsulated, even when high peptide amounts were used (10mg). Comparison of the amounts of encapsulated peptide achieved with the HIP to those achieved by the common method of adsorption onto the particle surface clearly demonstrated the superiority of the HIP-PBCA process (figure 2). When the adsorption method was used, a mere 1.5% of the peptide dose was loaded onto the particles. The analysis of the nanoparticles loaded with dalargin by the adsorption method was performed at a different time. The Kreuter adsorbed particles were made and analysed prior to the development of the current HIP method as a means aimed at evaluating the prior art. The LC/MS method and HPLC method that were used are equally as sensitive.

Example 5 - Evaluation of the HIP-PBCA nanoparticle delivery system in vivo (mouse model)

The ability of the HIP-PBCA nanoparticles to deliver their peptide load to the brain was determined in vivo in the mouse model. HIP-PBCA nanoparticles containing encapsulated dalargin using the HIP process were compared to HIP-PBCA nanoparticles that had had the peptide adsorbed onto the particle surface as reported by Kreuter et al. The nanoparticles were prepared for brain delivery via the intravenous route by coating their surface with polysorbate 80 surfactant. Briefly, the nanoparticles were incubated in PBS containing 1% w/v surfactant for 30 minutes prior to injection. The surfactant has been reported in the literature to indirectly target the nanoparticles to the brain by promoting adsorption of serum apolipoproteins onto the nanoparticle surface. This allows the particles to bind to the apolipoprotein receptor on the blood brain barrier and transcytose to reach the brain. The following formulations were compared:

1. HIP-PBCA nanoparticles alone (5:1 HIP content)
2. HIP-PBCA nanoparticles alone (10:1 HIP content)
3. Dalargin in solution (2.0 mg/kg)
4. HIP-PBCA nanoparticles with dalargin adsorbed onto the surface (2.0 mg/kg total dose used in formulation)
5. HIP-PBCA nanoparticles (5:1 HIP:dalargin molar ratio) with dalargin encapsulated (2.0 mg/kg total dose used in formulation)

6. HIP-PBCA nanoparticles (5:1 HIP:dalargin molar ratio) with dalargin encapsulated (2.0 mg/kg total dose used in formulation) – the same formulation as above, but injected at 1/10 of the dose.
7. HIP-PBCA nanoparticles (10:1 HIP:dalargin molar ratio) with dalargin encapsulated (2.0 mg/kg total dose used in formulation)

The mice were sacrificed at 20 minutes following injection, and the brains and blood samples collected and analysed for the presence of peptide by LC-MS-MS. The brain data were corrected for blood contamination assuming a blood contamination of 15 µl per gram of brain. The results obtained are shown in figure 3:

The results of the in vivo study suggest that encapsulation of the peptide within the core of HIP-PBCA nanoparticles using the HIP process, is superior to adsorption of the peptide on the particle surface.

Example 6 – Effect of pH on the encapsulation efficiency of dalargin in HIP-PBCA nanoparticles

In the prior art, the PBCA nanoparticles are formed by slow polymerisation of the BCA monomer in an acidic water in oil emulsion, where the pH of the aqueous phase is around 2.0 (0.01 N HCl). The polymerisation reaction under acidic conditions requires a period of at least 3 hours to reach completion. This method however, employs a neutral pH to allow rapid polymerisation. The aqueous phase that is used is phosphate buffered saline (PBS, pH 7.2). At neutral pH, the BCA monomer is known to polymerise rapidly (within seconds). As a result, the production of HIP-PBCA nanoparticles requires the very quick formation of an emulsion. This is achieved in this method by means of homogenisation at high speed (7,500 rpm or higher) using a Silverson L4RT homogeniser. It was hypothesized that a faster polymerisation reaction at neutral pH would improve the encapsulation efficiency, by quickly entrapping the peptide in the particles. In contrast, prolonged polymerisation could lead to gradual loss of peptide from the emulsion into the aqueous phase.
To test the hypothesis, nanoparticles were prepared, using the HIP-PBCA process, by emulsifying the BCA monomer with extracted peptide in either PBCS or the original medium of the prior art, 0.01 N HCl. Both the acidic and neutral aqueous phases contained the required stabilisers (0.2% pluronic F68, 1% dextran). The nanoparticles were prepared following the procedure described in example 3. The amount of peptide used per formulation was 5 mg. The following formulations were

prepared (one preparation each):

1. HIP-PBCA nanoparticles alone (35:1 HIP content), pH2
2. HIP-PBCA nanoparticles alone (35:1 HIP content). pH7
3. HIP-PBCA nanoparticles (35:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH2
4. HIP-PBCA nanoparticles (35:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH7
5. HIP-PBCA nanoparticles (10:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH2
6. HIP-PBCA nanoparticles (10:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH7
7. HIP-PBCA nanoparticles (5:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH2
8. HIP-PBCA nanoparticles (5:1 HIP:dalargin molar ratio) with dalargin encapsulated (5.0 mg input), pH7

The nanoparticle formulations were centrifuged to remove any free peptide and re-suspended in water or PBS. The encapsulation efficiency was determined by breaking up the particles in 10 mM NaOH (overnight incubation at room temperature) and then analysing by LC-MS. The results obtained are shown in figure 4.

The results support the hypothesis that rapid formation of the PBCA polymer at neutral pH would result in higher peptide encapsulation efficiency than slow formation of the polymer at acidic pH as is the case in the prior art. Despite loss of some of the peptide due to degradation by the treatment with NaOH, the results obtained clearly show the benefits of forming the particles at neutral pH. At a HIP:dalargin ratio of 10:1, 63.23% of the input peptide was entrapped into the nanoparticles when the particles were formed at pH 7. At pH 2, the encapsulation efficiency was significantly lower at 2.36%. Overall, the encapsulation efficiency was higher when the particles when the particles were prepared at pH 7 than when they were prepared at pH 2.

Example 7 - Encapsulation of domain antibody in PBCA nanoparticles using the HIP process

A domain antibody (anti-hen egg lysozyme dAb) was formulated in PBCA nanoparticles following the procedure described in example 3. The amount of protein

used in the formulation was 10 mg. A total of two formulations were prepared. In order to determine the amount of encapsulated dAb, the particles were centrifuged to remove any free protein and then analysed by Edman sequencing. In addition to sequence information, Edman sequencing can also be used to provide quantitative information. The process involves harsh chemical treatment which destroys the particle and allows detection of the encapsulated material. The results obtained are shown in figure 10. The results suggest that it is possible to encapsulate a larger molecule using the HIP-PBCA process, but at a lower efficiency. However, it may be possible to increase the efficiency of encapsulation by optimising the protocol for use with the domain antibody. With the current protocol, which has been optimised for dalargin, it was possible to encapsulate approximately 2.56 mg of the 10 mg used. This amounts to an encapsulation efficiency of 25.6%, which is high for a single emulsion process where a protein is entrapped within a hydrophobic particles matrix.

Example 8 – Optimisation of the HIP process for improved encapsulation of domain antibody in PBCA nanoparticles

In order to improve the loading of domain antibodies, the dalargin protocol was optimised further. The protocol for dalargin that was used as a starting point for optimisation is described in examples 3 and 4.

The aim of the protocol modification was to achieve full solubilisation of the antibody in the organic phase and efficient incorporation into the nanoparticles.

This was accomplished by including an additional homogenisation step to form a suspension of the HIP dAb complex in the organic phase. In general, the following changes were made to the dalargin protocol:

The dAb that was used was the VEGF-myc dAb. The dAb is named DOM15-26-593 and is disclosed in PCT WO2008/149147

The dAb was formulated at an input amount of 12 mg (0.843 μmol) per 100 mg PBCA polymer (12% w/w dAb / PBCA, 12 mg dAb per 100 mg PBCA polymer.

The dAb was complexed with the HIP (docusate sodium) at a molar ratio of 82:1. The HIP solution concentration was 30.581 mg/ml (0.06879 mmol in 1 ml).

The acidification of the dAb solution was carried out gradually and with constant mixing to prevent exposure of the molecule to too low pH values and degradation.

The pH of the dAb soltuion was lowered to a pH of 3.6 with HCl.

The $CaCl_2$ was not used as it could interfere with binding of the HIP to the dAb.

The acidified dAb was extracted from the aqueous phase by vortex mixing 500 µl acidified dAb solution (24 mg/ml, 12 mg protein) with1,000 µl docusate sodium in DCM (30.581 mg/ml, 3.058 %w/w) followed by centrifugation to separate the two phases. Unlike dalargin, the dAb was found not to fully solubilise into the organic phase. Instead, it formed a white precipitate at the interface. The precipitate clearly consisted of the dAb:HIP complex as its volume seemed to be proportional to the amounts of HIP and dAb used in the extraction. Attempts to fully extract the dAb using a reduced volume of aqueous phase from 500 µl to 367.76 µl (no addition of water) were less successful than using the 500 µl volume. A series of experiments suggested that a high isoelectric point was favourable, but it was possible to successfully precipitate dAbs with low pI's (VEGF dAb-myc, pI=6.6 in this case) as well. Following centrifugation, the aqueous layer was collected and stored at 4 degrees C. The organic layer, including the HIP-dAb solid pellet was used to prepare the nanoparticles.

In order to prepare the nanoparticles, it was necessary to solubilise the HIP-dAb pellet into the organic phase. This was achieved by introducing the following, additional homogenisation step to the process:

The aqueous phase was removed and the organic phase and dAb precipitate were homogenised in the 2 ml eppendorf using an Ultra-Turrax homogeniser (T25 basic, speed setting 1). The formulation was homogenised for 15 seconds to form a white suspension.

It was important to ensure that the HIP-dAb pellet came into contact with the homogeniser probe and started mixing immediately.

Homogenisation for a longer period of 1 minute gave a better suspension, but the dAb lost activity.

Following homogenisation, the organic phase was left into the 2 ml eppendorf and 100 µl BCA monomer was added. The liquid monomer was found to easily mix with the organic phase. The organic phase was then used to prepare the nanoparticles as described in example 1.

The modified procedure was also applied to the preparation of HIP-PBCA nanoparticles containing encapsidated mAb. A full length monoclonal antibody (anti-CD23 mAb as disclosed in PCT WO99/58679 , 150,000 Da, 12 mg per 100 mg PBCA polymer, 860:1 HIP:mAb molar ratio) was formulated by following the protocol developed for dAbs. The following observations were made:

The mAb (anti-CD23 as disclosed in WO99/58679) was found to require higher amounts of HCl than the VEGF dAb. When extracted using the HIP agent, mAbs

were found to behave similarly to the dAbs: they did not fully solubilise into the organic phase and formed a white precipitate at the interface.

The high concentration of the mAb stock solution allowed to experiment with the use of a 250 μl volume of aqueous phase instead of 500 μl in order to achieve full extraction of the mAb into the organic phase. This ratio of 4:1 organic phase to aqueous phase worked less well, it appeared to compromise the activity of the mAb as it was exposed to more solvent. A 1:1 ratio of organic to aqueous phase was clearly preferable with both mAbs and dAbs.

In order to prepare the nanoparticles the HIP-mAb pellet was solubilised into the organic phase by homogenisation by means of the same homogenisation step that was employed with dAbs. The homogenisation was found to be successful, but the suspension was less smooth, probably due to the larger size of the HIP-mAb complexes. Homogenisation for a longer period of 1 minute gave a better suspension, but the mAb appeared to denature. Therefore, the homogenisation step was kept brief at 15 seconds.

The nanoparticles were then prepared following the dAb protocol as described earlier in this example.

In general, the encapsidation of biopharms larger than peptides was found to require substantial modifications to the dalargin protocol.

Both dAbs and mAbs required higher HIP:biopharm molar ratios for solubilisation (82:1 and 860 respectively).

Both dAbs and mAbs were found not to fully solubilise into the organic phase. Instead, the formed a precipitate at the interface. In order to solubilise into the organic phase, the precipitate was homogenised into the organic phase to form a solid in oil suspension. This resulted in successful particle formation.

Example 9 –Encapsulation of domain antibodies in PBCA nanoparticles using the modified HIP process.

The modified HIP protocol for dAbs as described in example 8 was used to encapsidate a series of dAb molecules. The dAbs were selected on the basis of their isolelectric point. The aim was to cover the range of isolelectric points (pI) that would be likely to be used in the process in order to confirm that the process was versatile and suitable for a range of dAbs. The following dAbs were selected for the experiment (table 2):

Table 2

dAb	DOM 15-10-11 Vk, NT tagless	DOM 15-10-11, Vk, myc	DOM 15-10-11, Vk, HA
Concentration (mg/ml)	39.734	22.585	12.523
Isoelectric point (pI)	9.0	6.8	8.2

The DOM number refers to the domain antibody as disclosed in WO2008/149146 . The myc refers to the myc-tag on the domain antibody or HA refers to an HA tag on the domain antibody.

Each dAb was individually formulated into PBCA nanoparticles using docusate sodium as the HIP agent at molar ratio of 70:1.

The reagents used in the HIP extractions are listed in the table below (table 3):

Table 3

Formulation	DOM15-10-11 NT	DOM15-10-11 myc	DOM15-10-11 HA
dAb amount (mg)	6.0	6.0	6.0
dAb amount (μmol)	0.490	0.439	0.451
dAb solution concentration (mg/ml)	39.734	22.585	12.523
Volume of dAb solution (μl)	151.00	265.66	479.12
Amount of docusate sodium (mg) Mr = 444.55 Da	15.248	13.661	14.034
Amount of docusate sodium (μmol) Mr = 444.55 Da	34.30	30.73	31.57
Docusate sodium solution concentration (mg/ml)	15.248	13.661	14.034
Volume of docusate sodium solution (μl)	1000	1000	1000

Acidification of dAb solution for extraction.

The dAb solutions were diluted prior to acidification by addition of water as follows (table 4):

Table 4

Formulation	DOM15-10-11 NT	DOM15-10-11 myc	DOM15-10-11 HA
Volume of dAb solution needed (μl)	151.00	265.66	479.12
Volume of water needed (μl)	328.12	213.46	0
Total volume of aqueous phase	479.12	479.12	479.12
Volume of HCL (2 M) added (μl)	2.00	5.00	3.00
Final pH	3.4	2.5	3.0

The solutions were acidified by addition of HCl (2 M). All dAb solutions were acidified to a pH of around 3.0 as measured by indicator strips. The final volume of each acidified solution was brought up to 500 μl with water.

The dAbs were then extracted into the organic phase as described in example 16. All dAbs were found not to fully solubilise in the organic phase and to form a precipitate at the interface. The untagged dAb (NT) was found to yield a precipitate that was much thinner than those of the other dAbs. The high isolelectric point and strong positive charge of the dAb had apparently permitted the formation of a stronger, more hydrophobic complex with the HIP and resulted in a greater degree of solubilisation and transfer into the organic layer.

Following removal of the aqueous phase (top layer) the organic phase and dAb precipitate were solubilised by homogenisation and the nanoparticles prepared as described in example 8.

Analysis of the nanoparticles by SDS-PAGE to assess loading of the dAbs.

In order to analyse the nanoparticle suspensions by SDS-PAGE, samples were spun

for 10minutes at 13000rpm in a micro-centrifuge. The supernatant was aspirated and the pellet resuspended in 100µl of PBS. Supernatant and pellet fractions were disrupted with 1x NuPAGE LD and reducing agent, heated to 80°C for 4 minutes, and examined by SDS PAGE using commercially obtained NuPAGE gels. A sample of supernatant from hollow formulations containing dAb was also examined and used as a positive control.

Lanes 1 and 6: molecular weight markers. Lane 2: VEGF dAb DOM15-10-11, untagged encapsidated in HIP-PBCA nanoparticles. Lane 3: VEGF dAb DOM15-10-11, myc tagged, encapsidated in HIP-PBCA nanoparticles. Lane 4: VEGF dAb DOM15-10-11, HA tagged, encapsidated in HIP-PBCA nanoparticles. Lane 5: VEGF dAb DOM15-10-11, untagged, encapsulated in hollow nanoparticles (positive control). The gel confirmed that encapsidation of the dAbs had taken place. The gel also confirmed that the dAbs were intact and that they had not fragmented due to the particle preparation process.

The gel clearly showed that the dAb had been encapsidated within the particles, as they co-localised with the nanoparticle pellets following removal of any free dAb (figure 6).

The dAb had clearly been encapsidated within the nanoparticles, as analysis of the pellet under non-denaturing conditions (native gel) did not yield any bands on the gel as the dAb remained in the particles (results not shown). It was necessary to analyse the particles by SDS-PAGE, as the denaturing conditions (heat treatment in the presence of SDS) were required for the dAb to be released from the particles and run on the gel. The encapsidation was found to be successful with all dAbs tested. This suggests that the additional homogenisation step successfully solubilised the HIP-dAb complex into the organic phase to allow entrapment of the dAb into the particles. As a result, the fact that the HIP-dAb complexes precipitated at the interface following extraction, as was the case especially with low pI dAbs, did not compromise the encapsidation of the dAbs in the particles. Therefore, the modified protocol for the encapsidation of dAbs into HIP-PBCA particles was found to be independent of pI for the ranges tested and suitable for a range of dAbs.

Example 10 – Encapsulation of domain antibodies in PBCA nanoparticles using the modified HIP process; determination of loading efficiency and measurement of activity of formulated dAb.

In order to determine the loading efficiency that the modified HIP protocol could achieve, a HIP PBCA nanoparticle formulation was prepared with a tool dAb (VEGF-

myc dAb, DOM15-26-593 as disclosed in WO2008/149147. The dAb was formulated at an input amount of 12 mg (0.843 μmol) per 100 mg PBCA polymer (12% w/w dAb / PBCA, 12 mg dAb per 100 mg PBCA polymer. The formulations were prepared using the modified HIP protocol for dAbs as described in example 9.

Following preparation, the nanoparticles were characterised by SDS-PAGE in order to confirm that the dAb had remained intact and that it had been successfully entrapped within the particles. Analysis by SDS-PAGE was performed as described in example 9. A set of dAb standards of known amounts were also analysed on the gel alongside the formulation and were used to determine the amount of encapsidated dAb (figure 7). This was achieved by photographing the gel and measuring the signal intensity from the bands of the standards using labworks V4.6. The gel was set up as follow:

Lanes 1 and 7: molecular weight markers. Lanes 2-4: dAb nanoparticle formulations. Lane 5: empty nanoparticles (negative control). Lanes 7-10: dAb standards (500, 125, 31.25 and 7.8 μg/ml). Lanes 11-14: dAb standards (7.8, 31.25, 125 and 500μg/ml).The gel confirmed that encapsidation of the dAbs had taken place and that the dAb was intact. Comparison of the sample band intensities to those of the standards suggested that the concentration of the dAb in the nanoparticle sample was 413.7 μg/ml.

The band intensities were used to construct a standard curve. The curve was then used to calculate the amount of dAb in the nanoparticle formulation from the intensity of the band of the nanoparticle sample.

The gel confirmed that the dAb had been successfully entrapped within the particles and that it had remained intact following encapsidation. From comparison to the standards, the concentration of dAb in the nanoparticle formulation was found to be 413.7 μg/ml. This translated to a total of 3.31 mg of encapsidated dAb in the nanoparticles out of the 12 mg input. Therefore, the loading efficiency was 27.6 %. The dAb loading was 3.31 % w/w.

In order to release the encapsidated dAb and assess its activity, nanoparticle samples were also subjected to heat treatment. The dAb was released from the nanoparticles by incubation at temperatures ranging from 4 to 65°C for 1 hour in the presence of 1% Tween 20. The process is known to achieve release of at least part of the encapsidated dAb from the particles, however it can also cause some loss of dAb activity. To minimise loss of dAb activity as a result of the heat treatment, samples were also incubated at 65°C for 5 minutes, followed by a milder treatment at the lower temperature of 37°C for 55 minutes.

Following the incubation, the samples were centrifuged at 10,000 rcf for 10 minutes to separate any released dAb from the particles. The supernatants, which contained released dAb, were collected and analysed by ELISA for activity.

The released dAb was analysed by ELISA as follows:

Nunc maxisorb 96 well plates were coated with 0.5 μg/ml rVEGF overnight at 4°C. The plates were then washed with wash buffer (PBS + 0.1% Tween) 4 times and then blocked with blocking buffer (PBS + 1% BSA) for 1 hour at room temperature whilst being rocked. Plates were washed as above, then 50 μl triplicate supernatant samples were added to the wells and plates were incubated as above. The plates were washed, then 50 μl per well anti-myc Ab (mouse) solution was added and the plates were again incubated as described above. Following washing of the plates, 50 μl per well anti mouse HRP was added and the plates were incubated as above. The plates were finally washed as above and then 50 μl/well TMB reagent was added. The colour was allowed to develop and the reaction was stopped by addition of 50 μl per well HCl (1M). The absorbance was measured at 450 nm using a Versamax plate reader and Softmax Pro V5.3 software.

The results obtained from the ELISA assay are shown in table 5:

Table 5

Temp	4°C	56°C	65°C	65/37°C
Conc. (μg/ml)	0.44	4.0	>50	61

The released dAb was found to be active, with the high temperatures releasing a greater amount of protein from the particles. The samples treated at the two temperatures of 65 and 37 degrees C were found to exhibit the highest amount of released active dAb. The original level of activity in the formulation was hard to estimate as the release method is known to compromise activity, but, considering the PAGE result, the 65/37 method produced material with approximately 50% of the specific activity of the standards.

The released dAb was analysed by ELISA, which gave a reading of active dAb, as well as by SDS-PAGE, which detected total dAb. The dAb was analysed on the gel alongside a series of standards. The amount of dAb was then determined by means of a standard curve that was constructed by measuring the band intensities of the standards. It was found that the concentration of active dAb (61 ug/ml, as measured by ELISA) was 44% of that of total dAb (137.89 ug/ml as measured by SDS-PAGE).

Therefore, at least 50% of the formulated dAb in the nanoparticles was found to be active. This was considered to be a very good level of activity, considering that the formulation process involved solubilisation in an organic phase followed by exposure to mixing by homogenisation. Therefore, the particle preparation process appears to be suitable for the formulation of domain antibodies.

Example 11 – *In vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies for their ability to deliver their protein load to the brain in the mouse via the intravenous route.

The nanoparticle formulation described in example 10 was evaluated for its ability to deliver its dAb load to the brain in the mouse model.
Nanoparticles containing encapsidated VEGF dAb were compared to free dAb in order to determine whether the particles could increased the brain uptake of the dAb compared to that of free dAb molecules. A batch of empty nanoparticles was also prepared and evaluated as a negative control.

Design of the *in vivo* study.

The study involved two different time points at which dAb brain levels were to be assessed: 10 minutes and 60 minutes post administration. The earlier time point was chosen in case the dAb concentration in the brain peaked within minutes after injection, as was found with the dalargin peptide. The later time point was selected in order to allow for some clearance of the dAb from the blood circulation to occur. Any dAb that was present in the blood could contaminate the brain samples and distort the data obtained. The short half life of the dAb in the blood circulation (20 minutes) could perhaps limit the blood contamination at the later time point, thus permitting a clearer reading of brain penetration.

Correction of blood contamination in brain samples:
In order to account for the amount of dAb in the brain samples that was not due to brain uptake but was merely present into the brain blood vessels and not in the brain tissue itself (blood contamination), a start-chase study was performed. All mice received a dose of a chase molecule that is known to remain in the blood and not to penetrate the brain. The chase molecule that was chosen was the anti-CD23 full length antibody as disclosed in WO99/58679 that exhibits negligible brain uptake. Therefore, any amount of anti-CD23 mAb detected in the brain samples would be

solely due to its presence in the blood contaminating the brain tissue. The chase was given to the animals 5 minutes before sacrificing them to ensure that the antibody remained in the blood and no tissue uptake took place in other areas of the body.

Groups of animals:

A: Control particles, given at t=0, followed by chase at t=5 minutes.
B: dAb in nanoparticles, given at t=0, followed by chase at t=5 minutes.
C: dAb free in solution (control), given at t=0, followed by chase at t=5 minutes.

The above groups were sacrificed at t=10 minutes, at 5 minutes post administration of the chase.

D: Control particles, given at t=0, followed by chase at t=55 minutes.
E: dAb in nanoparticles, given at t=0, followed by chase at t=55 minutes.
F: dAb free in solution (unformulated control), given at t=0, followed by chase at t=55 minutes.

The above groups were sacrificed at t=60 minutes, at 5 minutes post administration of the chase.

Preparation of the doses.

Chase: anti-CD23 mAb as disclosed in PCT WO99/58679, 2.0 mg/kg.
The doses were prepared by diluting the 68 mg/ml mAb stock solution to 500 µg/ml. This amounted to a 50 µg dose in a 100 µl volume for a 25 g mouse.

Nanoparticles with dAb: 1.584 mg/kg, 50 mg/kg PBCA polymer.
The nanoparticle suspension was prepared for injection by adding 160 µl polysorbate 80 solution (25 % w/w) to 3,600 µl nanoparticle suspension. This resulted in a final concentration of formulated dAb of 396.1 µg/ml. This amounted to a dAb dose of 39.6 µg in a 100 µl volume for a 25 g mouse.

Empty nanoparticles (negative control): 50 mg/kg PBCA polymer.
The nanoparticle suspension was prepared for injection by adding 160 µl polysorbate 80 solution (25 % w/w) to 3,600 µl nanoparticle suspension as above. This resulted in

a final concentration of PBCA polymer of 1.25 mg/ml. This amounted to a PBCA dose of 125 μg in a 100 μl volume for a 25 g mouse.

Free dAb in solution (unformulated control): 1.584 mg/kg.

The dAb solution was prepared for injection by diluting the 2.0 mg/ml stock solution to 396.1 μg/ml. This amounted to a dAb dose of 39.6 μg in a 100 μl volume for a 25 g mouse.

Injection of the mice: the CD1 mice were injected intravenously (tail vein injection). The injection volumes were calculated on the basis of the weight of the mice. Following the end of the *in vivo* procedure, brains and serum samples were collected from all the mice and frozen. The tissue samples were snap frozen in liquid nitrogen. All samples were stored at -80°C.

Homogenisation of brains for analysis.
The brains were thawed and weighed. A volume of PBS that was twice the weight of the brain volume was added to each brain. The brains were then homogenized using a Covaris acoustic tissue processor (Covaris E210).

Analysis of brains by Meso Scale Discovery (MSD)
The brain homogenates and serum samples were analysed by MSD. This was achieved by adapting the anti-VEGF ELISA assay described in example 18 to an MSD format. The serum samples were analysed at 1:1,000 in 1:10,000 dilutions. The brain samples were analysed in 1:5 dilutions.

Results
The data was processed and the results shown in figure 8 were generated. At 10 minutes post administration, the dAb in nanoparticles resulted in detectable brain uptake which amounted to 8.0 ng/ml. The free dAb was also detectable in the brain at the slightly lower concentration of 3.3 ng/ml (preliminary data). The preliminary data did not include the readings from two animals that could not be corrected for blood contamination as the readings from the serum were too high to be quanitified. In general, the nanoparticles appeared to marginally increase the brain uptake of the protein at the 10 minute time point.
However, at 60 minutes, the situation was reversed. The free dAb appeared to accumulate in the brain resulting in a further increase in its brain levels to 13.5 ng/ml. The observed result can be explained as follows:

1. The half life (t½) of the free dAb in the blood circulation was likely to be longer than that of the particles because of its hydrophilicity. This probably meant that more of the free dAb was available for brain uptake compared to dAb formulated in nanoparticles.
2. The loading of dAb into the particles was not high enough to offset the loss of formulation due to rapid elimination from the systemic circulation. The drug loading in the particles was 3.31% w/w. The dalargin formulations had previously required a loading of 5.0 % w/w in order to generate brain levels of 45 ng/ml. Higher peptide loadings of 8.9 % had given peptide concentrations in the brain that were up to 833 ng/ml. It seems that a loading of 3.31 % w/w, especially for the high molecular weight biopharm, is not sufficient for significant brain delivery therefore further optimisation of loading would be necessary.
3. The 10 and 60 minute time points were perhaps too late. Previous studies with dalargin and loperamide had all demonstrated that delivery is rapid, within 2-3 minutes of injection. The previous studies had also suggested that the highest drug levels in the brain were achieved within 5 minutes of administration or earlier. A time point of 10 minutes was chosen to ensure sufficient time to perform a start chase study and 60 minutes chosen to detect any long lasting effect of domain antibody.
4. The HIP PBCA system is only passively targeted to the brain. When given intravenously, such particulates that exhibit a hydrophobic surface are known to be passively targeted to a number of organs in addition to the brain. These organs include the liver and the spleen. When given intravenously, the nanoparticles will reach the liver and the spleen first, before they encounter the brain. As a result, the majority of the injected dose could be delivered to those tissues, leaving only a fraction available for delivery to the brain. This could have severely compromised the ability of the particles to reach the brain in this experiment.

To highlight the impact of the loss of dAb from the blood circulation, the brain to blood ratios were also calculated (figure 9). The results clearly show that much higher proportions of dAb were present in the brain compared to the blood when given with nanoparticles compared to when the dAb was given free in solution. In fact, the formulated dAb exhibited a brain to blood ratio of 0.04 (60 minutes), which is above the ratio at which a compound is considered to be brain penetrant. The free dAb did not exceed this brain penetration threshold at any of the time points that were

analysed. Therefore, despite significant loss of the injected dose, in terms of overall ability to penetrate the blood brain barrier the particles may ultimately be superior to free dAb.

In general, the intravenous route is known to be the most challenging route of administration for passively targeted particles such as the HIP-PBCA system. Therefore, intravenous administration was not the ideal method of assessing the ability of the HIP-PBCA system to deliver its drug load across the BBB from the blood. For this reason, an intracarotid study was also carried out. Administration via the intracarotid route by-passes tissues such as the liver and spleen and provides a more direct route to the brain. As a result, more of the injected nanoparticle dose is available for brain delivery. In a head to head comparison between free and formulated drug, the intracarotid route is more likely to provide a true measure of the ability of the nanoparticles to overcome the BBB.

Example 12 – *In vivo* evaluation of HIP PBCA nanoparticles containing domain antibodies for their ability to deliver their protein load to the brain in the mouse via the intracarotid route.

In vivo evaluation of the nanoparticle formulation – intracarotid administration.

The nanoparticle formulation was evaluated for its ability to deliver its dAb load to the brain in the mouse via the intracarotid route.

The route was selected because it provides a direct avenue to the brain. When a substance is given into the carotid artery, the first tissue that is reached is the brain. In contrast, when a drug is given intravenously, it will encounter tissues such as the liver prior to reaching the brain. This was found to limit the ability of the nanoparticles to deliver to the brain, as they are also known to be taken up by tissues such as the liver and spleen. In fact, Kreuter *et al* had observed that with their PBCA adsorbed particles, the majority of the injected empty nanoparticle dose (~60%) when given via the tail vein was taken up by the liver.

In general, the intravenous route is well known to be the least favourable and most challenging route of administration for passively targeted delivery systems such as the HIP-PBCA nanoparticles.

Therefore, the intracarotid route was thought more likely to provide an accurate indication of the ability of the nanoparticles to overcome the blood brain barrier.

Design of the *in vivo* study.

The study design was the same as for the intravenous study, with the only differences being the following:

1. The animals were kept under terminal anaesthesia throughout the experiment. This was necessary due to the complexity of the surgical procedures involved.
2. The nanoparticle formulations and free dAb were administered via the intracarotid route by means of a surgically prepared cannula.
3. The chase was given intravenously via the tail vein, but unlike the previous study the antibody was given by means of cannula.

Groups of animals:

A: Control particles, given at t=0, followed by chase at t=5 minutes.
E: dAb in nanoparticles, given at t=0, followed by chase at t=5 minutes.
C: dAb free in solution (control), given at t=0, followed by chase at t=5 minutes.

The above groups were sacrificed at t=10 minutes, at 5 minutes post administration of the chase.

B: Control particles, given at t=0, followed by chase at t=55 minutes.
F: dAb in nanoparticles, given at t=0, followed by chase at t=55 minutes.
D: dAb free in solution (unformulated control), given at t=0, followed by chase at t=55 minutes.

The above groups were sacrificed at t=60 minutes, at 5 minutes post administration of the chase.

Preparation of the doses.

Chase: anti-CD23 mAb as disclosed in PCT WO99/58679, 2.0 mg/kg.
The doses were prepared by diluting the 68 mg/ml mAb stock solution to 500 μg/ml. This amounted to a 50 μg dose in a 100 μl volume for a 25 g mouse.

Nanoparticles with dAb: 1.584 mg/kg, 50 mg/kg PBCA polymer.
The nanoparticle suspension was prepared for injection by adding 160 μl polysorbate 80 solution (25 % w/w) to 3,600 μl nanoparticle suspension. This resulted in a final

concentration of formulated dAb of 396.1 μg/ml. This amounted to a dAb dose of 39.6 μg in a 100 μl volume for a 25 g mouse.

Empty nanoparticles (negative control): 50 mg/kg PBCA polymer.

The nanoparticle suspension was prepared for injection by adding 160 μl polysorbate 80 solution (25 % w/w) to 3,600 μl nanoparticle suspension as above. This resulted in a final concentration of PBCA polymer of 1.25 mg/ml. This amounted to a PBCA dose of 125 μg in a 100 μl volume for a 25 g mouse.

Free dAb in solution (unformulated control): 1.584 mg/kg.

The dAb solution was prepared for injection by diluting the 2.0 mg/ml stock solution to 396.1 μg/ml. This amounted to a dAb dose of 39.6 μg in a 100 μl volume for a 25 g mouse.

Results

The data was processed and the results shown in figure 9 were generated. At 10 minutes post administration, the dAb in nanoparticles group exhibited high levels of dAb in the brain, at an average of 627.60 ng/ml. The actual concentration of dAb in the brain was probably higher, as the above figure did not include the readings from two animals. The two samples gave signals that were too high for quantification but unfortunately were not analysed in time for inclusion into this document. One of the animals was a clear outlier that gave a relatively low brain concentration of 45.45 ng/ml. This resulted in the large errors observed with the group. Nevertheless, the average concentration of formulated dAb in the brain was nearly 9-fold higher than that of free dAb, which was 71.67 ng/ml.

At 60 minutes post injection, the levels of dAb in the brain remained high at 146.51 ng/ml. The concentration of free dAb had instead fallen to an average of 3.17 ng/ml. Therefore, at 60 minutes post injection the brain concentration of dAb given in nanoparticles was 46-fold higher than that achieved with naked dAb.

In general, the nanoparticles were found to be very successful at delivering the dAb to the brain via the intracarotid route.

This was also evident from the determination of the brain to blood ratios for the groups (figure 11). The dAb in nanoparticles group exhibited brain to blood ratios that were greater than 1 at both time points (1.569 and 1.845 at10 and 60 minutes respectively) suggesting that the majority of formulated dAb had successfully reached the brain. In contrast, the free dAb groups were characterised by brain to blood ratios

that were significantly lower, at 0.012 and 0.286 for the 10 and 60 minute points respectively.

In conclusion, the nanoparticle delivery system was found to greatly improve the delivery of dAb to the brain when given via the intracarotid route. This was because the route reaches the brain prior to liver and the spleen, which are tissues to which the formulation is also passively targeted in addition to the brain.

The intravenous route was not as successful, providing a transient hint of an increase in brain uptake of dAb. This was probably due to the insufficient dAb loading into the particles, as well as due to the delivery system being taken up by other tissues resulting in only a fraction of the injected particles reaching the brain.

Therefore, in order to improve the system and achieve efficient delivery to the brain via the intravenous route, it is necessary to further improve the loading of dAb into the nanoparticles. This could be achieved by employing the hollow PBCA system, which has been shown to have a greater capacity for the loading of dAbs than the HIP PBCA system. Provided that it is stable enough in vivo, the hollow PBCA particles may be more successful than the HIP PBCA system at delivering the dAb to the brain. In order to ensure their stability, it may be necessary to employ blends of the PBCA polymer with other polymers of higher molecular weight such as PLGA, PLA or PCL. The delivery system may also benefit from the use of pegylated copolymers. Such polymers could improve the circulation time of the nanoparticles in the blood and thus improve brain delivery.

An additional means of improving the delivery system is to alter its mechanism of brain targeting. An actively targeted nanoparticle that exhibits a ligand that binds a target on the BBB is likely to improve brain uptake and concomitantly limit the loss of particles to other tissues. In order to achieve active targeting it will probably be necessary to extensively pegylate the nanoparticle surface in order to limit any non-specific targeting to other organs.

In general, the nanoparticle system described in this document has great potential to achieve the efficient delivery of domain antibodies to the brain, however in order to achieve this significant optimisation is still required.

Example 13 –Encapsulation of domain antibodies in PCL microspheres using a modified HIP process.

The polymer polycaprolactone, (PCL, Lactel), was used as a test case both for the generation of microspheres and the use of slow-release polymers with the HIP process for dAb encapsidation. Initial experiments used modifications of the HIP

encapsulation protocols described in Examples 7 and 8 to accommodate the use of PCL to generate both empty nanoparticles and microspheres.

Initial formulations used 100mg/ml stocks of PCL in dichlorormethane, (DCM), and 1% pluronic F68, (Sigma) as surfactant with homogenisation speeds of 4000-7500rpm for 45 seconds, but few microspheres or nanoparticles were made, (data not shown). The process was further optimised by trying different surfactants: also 1% sodium cholate, (Sigma), or 1% Lutrol F127 Poloxamer 407, (BASF Corp.), or 1% Vitamin E TPGS, (d-ALPHA tocopheryl polyethylene glycol 1000 succinate), (Peboc / Eastman) with little initial improvement, (data not shown), until the amount of input polymer was reduced to 10mg/ml, when small numbers of large fragile microspheres of >20um could be seen under the light microscope,(data not shown), but the majority of PCL came out of suspension as macroscopic particles once the organic solvent had evaporated, (data not shown). The process was further improved in terms of particle stability by using the two most promising surfactants from the experiments described above at 2% to help stabilise the suspensions more: Lutrol F127 Polaxomer 407, (BASF Corp.) or Vitamin E TPGS and homogenisation speeds of 7500-9000rpm for 45 seconds to 2mins. Using this process around 1µm sized particles were generated in all cases, (data not shown), but 2% Vitamin E TPGS was chosen as surfactant with a 2 min. homogenisation for a protocol to encapsidate dAbs shown below.

Preparation of HIP-PCL microspheres, (M/P), which encapsidate dAb.

HIP-PCL microspheres were prepared according to the methodology of example 13 above and any changes to this protocol detailed below.

Formulations used:

Four PCL (poly-e-caprolactone) formulations were prepared:

i) empty particles with HIP for PCL as M/P – 4000 rpm (2% Vitamin E [TPGS] as surfactant) – 2mins

ii) empty particles with HIP for PCL as M/P – 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2mins

iii) Particles with HIP for PCL as M/P + dAb for analysis, (dAb1) – 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2 mins

iv) Particles with HIP for PCL as M/P + dAb for sizing, (dAb2)– 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2 mins

Solutions needed:

(1) dAb to be extracted: anti-VEGF (DOM15-26-593 as disclosed in WO2008/149147.), batch TB090220 1.5 mg/ml (14,246 Da) – used 4 x 5ml (actual re-reading of the dAb concentration using a Nanodrop 1000 Spectrophotometer,Thermo Scientific, confirmed concentration as in fact 1.04mg/ml)

(2) 82:1 HIP solution (1 ml, 30.58 mg/ml)

(3) Acidified dAb solution (25 mg/ml) N/A

(4) Aqueous phase: 1% w/v dextran , 2% w/v surfactant* in PBS

(5) PCL polymer dissolve in DCM

*Stock 10% Vitamin E [TPGS]

Preparation of PCL solution in DCM.

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Aim was to provide 10mg of PCL dissolved in DCM per formulation – solubility was about ~100mg / ml in DCM, maximum, but more could be dissolved @ ~10mg/ml. Enough PCL was made for 5 formulations i.e. 50mg in 5ml DCM. Weighed out 50mg PCL, (opened thawed vacuum dessicator when warmed to room temperature from -20°C), weighed (fine balance) – stirred in 10 ml beaker with PCL + 4mls DCM – stirred with glass stirrer in fume hood under cover. Once dissolved, measured in glass measuring cylinder, and topped up to 5ml with DCM then re-mix, (stirred beaker), and used quickly.

Concentration of dAb solution.

Preparation was done intially O/N at RT 600rpm and diluted back to correct concentration. The solution was concentrated using Vivaspin concentrators (Vivaspin 6, Sartorius, VS0691, MWCO 3,000 PES) and following manufacturers instructions in a Sorvall legend RT Bench Top centrifuge The concentration was from the expected 1.5 mg/ml (20.0 ml as 5ml in 4 x Vivaspin)) to 25 mg/ml (~700 µl). The process took 2 hours at 1000-1500 rpm and a further ~1 hours at 3000 rpm. Initial dAb 400ul at 1 in 75 dilution gave 0.56 mg/ml by Nanodrop – this was then diluted to 760ul at 25mg/ml and acid treated to reduce the pH to ~3.7.The mock material was acid treated at pH ~2.5. dAb was at 25mg/ml ~pH 5.0/4.5 – aim is to get down to pH3.7 – pH was

checked with pH 2.5 to 4.5 paper.380ul of dAb was used, for example only 9.5mg per formulation, (and fitted an inital input concentration of 1mg/ml not 1.5mg/ml)

Table 6: Preparation of acidified solutions.

Formulation	Empty control	dAb solution
Volume of 25.0 mg/ml dAb solution needed (µl)	0	760, (2x 380)
Volume of water needed (µl)	500.0 x 10	12.0
Volume of 2M HCl solution added (µl)	5.0 x 10	6.0
Volume of 1M CaCl2 added (µl) optional, ADDED LAST	0	0
pH following addition	<<=2.5	~3.7

Table 7; Formulations and reagents for HIP extractions (standard volume protocol).

(A) Organic phase dAb

Formulation dAb	10-12.0mg dAb, 82:1 HIP, 10 mg PCL
dAb amount (mg) or w/o dAb	~9.5.
Concentration (mg/ml)	25.00
Volume needed (µl)	380

(B) Organic phase HIP

Formulation HIP AOT	12.0 mg dAb, 82:1 HIP, 10 mg PCL
Concentration of AOT needed (mg/ml)	30.58
Volume of 30.58 mg/ml AOT solution needed (µl)	1000

Aim was to make a 1: 2 mix of dAb (aq) : DCM / HIP – above is for a 1x mix, i.e. ~500ul : 1000ul organic phase.

Table 8: Preparation of empty controls (organic phase).

Formulation	Empty control, 82:1 HIP, 100 mg BCA
dAb amount (mg)	0 use just PBS 480µl + 20µl of HCl to acidify
Concentration of AOT needed (mg/ml)	30.58
Volume of 30.58 mg/ml AOT solution needed (µl)	1000

Extraction of dAb or mock (empty particles) into the organic phase using docusate sodium as a HIP agent.

The acidified dAb or 'mock' solution and organic phase was mixed in 2 ml eppendorf tubes and added to the aqueous phase. The mixtures were vortex-mixed maximum speed for 1 minute and then placed in Bench top mixer 5432 for 5 minutes. The resulting white mixtures were centrifuged at maximum speed (20,817 rcf, 14000rpm in microfuge) for 50 minutes. The dAb-HIP complex appeared to form a thick white precipitate at the interface. The aqueous phase was collected and stored at 4 degrees C. The top aqueous phase was removed and stored and the procedure continued with the bottom organic phase.

Homogenisation of HIP-dAb complex into organic phase.

The organic phase was homogenised in the 2 ml eppendorf tube using an IKA T25 homogeniser (polytron, speed setting 1) for 7-10 seconds. The aim was to achieve complete homogenisation of the white precipitate (dAb and HIP complex) into the organic solvent (DCM).The HIP-dAb complex was easily solubilised into the organic phase to form an emulsion that appeared homogeneous. The organic phase was homogenised for a total of 10 seconds. There was little precipitate left in the tube following homogenisation and removal of the organic phase.

Preparation of microspheres.

The 1ml organic phase of the homogenates were taken and 1ml of PCL dissolved in DCM (100mg) was mixed in by pipetting up and down.
The resulting white suspension (2ml) was pipetted into the aqueous phase (10 ml dextran in water and 2% surfactant solution in PBS in a 25 ml beaker) at the point of probe entry below the liquid surface. The aqueous phase was being homogenised at either 7,500 rpm, (M/P) or 4000 rpm (M/P) using a Silverson L4RT homogeniser. The emulsion was homogenised for 2 minutes. The formulation was then incubated in a fume hood with stirring (speed setting 4) for 3 hours in order to evaporate the organic phase. The setting was lowered to 3 at 1 h into the incubation to prevent over-mixing of the emulsion as that resulted in the deposit of aggregates on the surface of the beaker.

Example 14 - Sizing of the microspheres.

(a) Light microscopy.

All four formulations, (i) to (iv) above were sized on the Nikon Eclipse E400 Microscope using visible light. The data showing images of these particles is shown in Fig. 12. Visible microspheres of a similar size range could be seen from all four formulations both those with and without dAb. The data suggests that microspheres are similarly formed in the presence of dAb using this process.

(b) Multi-angle static light scattering.

All four samples were sized on the Micromeritics Saturn DigiSizer 5200, High Definition Particle Size Analyser.

Samples were sized by loading sufficient material from the micro-particle sizings above into the low-volume sample handling unit attached to the Saturn Digisizer 5200 to allow an obscuration of 5-30%, preferably above 15% in a matrix of de-gassed PBS, (which needs 50-100% of the formulation). Samples were then analysed using a polycaprolactone model of analysis using a real partitioning of refractive index of 1.476 and an imaginary partitioning of refractive index of 0.0001. The flow rate was at 6L / min, the stop beam angle was at 45 degrees, the media was PBS and counts were made in triplicate. Both volume and number distribution were reported, data obtained was as a combined report, a cumulative graph and a frequency graph, for details of methods see the Micromeritics Saturn Digisizer 5200 Operators Manual V1.12, (March 2007) and the Quick reference guide.

The data is displayed for the relevant formulations: (i) to (iv) in Figs 13 (a) to (d) as graphs plotting frequency of number of particles against particle size. See below the data corresponding to these graphs.

Fig 13 a

Summary Report

Sample

Sample Concentration: 0.01069 %
Obscuration: 29.1 %

Volume Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	8.355	2.825	Mode	10.00	4.017
Median	9.764	3.861	Std. Dev. Log.	0.382	0.047
Skewness	-0.257	0.105	Kurtosis	-0.435	0.180

Number Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	1.393	0.070	Mode	1.000	0.000
Median	1.033	0.009	Std. Dev. Log.	0.214	0.022
Skewness	1.504	0.388	Kurtosis	2.001	2.445

Fig 13

(b)

Summary Report

Sample

Sample Concentration: 0.00284 %
Obscuration: 16.7 %

Volume Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	5.366	3.943	Mode	2.239	2.744
Median	4.304	5.016	Std. Dev. Log.	0.521	0.033
Skewness	0.523	0.459	Kurtosis	-0.379	0.178

Number Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	1.231	0.016	Mode	1.000	0.000
Median	1.048	0.013	Std. Dev. Log.	0.141	0.012
Skewness	1.854	0.198	Kurtosis	4.737	1.339

Fig 13 (c)

Sample

Sample Concentration: 0.00771 %
Obscuration: 21.6 %

Volume Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	9.248	7.388	Mode	2.239	12.53
Median	10.48	7.174	Std. Dev. Log.	0.421	0.115
Skewness	-0.234	0.252	Kurtosis	-0.185	3.125

Number Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	1.255	1.046	Mode	1.000	0.751
Median	1.048	0.731	Std. Dev. Log.	0.195	0.070
Skewness	1.711	0.234	Kurtosis	3.195	1.059

Fig 13 (d)

Summary Report

Sample

Sample Concentration: 0.01063 %
Obscuration: 29.1 %

Volume Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	8.355	2.028	Mode	10.00	4.017
Median	9.754	3.951	Std. Dev. Log.	0.382	0.047
Skewness	-0.267	0.106	Kurtosis	-0.428	0.180

Number Distribution Geometric Statistics

		Std Dev of 8			Std Dev of 8
Mean	1.393	0.070	Mode	1.000	0.000
Median	1.033	0.098	Std. Dev. Log.	0.214	0.022
Skewness	1.504	0.386	Kurtosis	2.091	2.445

The clearest determination of mean particle size is from the geometric mean number distribution which gave the following mean particle sizes:

i) empty particles with HIP for PCL as M/P – 4000 rpm (2% Vitamin E [TPGS] as surfactant) – 2mins mean particle size 1.231

ii) empty particles with HIP for PCL as M/P – 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2mins mean particle size 1.181

iii) Particles with HIP for PCL as M/P + dAb 1 for analysis – 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2 mins mean particle size 1.355

iv) Particles with HIP for PCL as M/P + dAb 2 for sizing– 7500 rpm (2% Vitamin E [TPGS] as surfactant) – 2 mins mean particle size 1.393

The conclusion was that although a lower speed under these conditions generated slightly large microspheres the impact was not great – the conditions described for homogenisation at 7,500 rpm generated microspheres in the presence of dAb with a mean diameter of 1.4µm so slightly larger than empty particles in this process.

Example 15 - Analysis of HIP-PCL Microspheres containing dAb.

HIP PCL microspheres containing dAb were prepared as in example 13 above. 50µl of each formulation, (dAb1 and dAb2) was taken and either:-

i) Spun in a 1.5ml microfuge tube at 3K rpm for 5' to generate a supernatant (S) – 30µl removed to a fresh microfuge tube and a pellet (P) fraction resuspended in 50µl PBS;

ii) Spun in a 1.5ml microfuge tube at 13K rpm for 5' to generate a supernatant (S) – 30µl removed to a fresh microfuge tube and a pellet (P) fraction resuspended in 50µl PBS;

iii) Spun in a Vivaspin 500 (1,000,000 molecular weight cut off) at 5K rpm for 5' to remove any incorporated dAb as particles are retained in the column and the 50µl PBS as supernatant (F) passed through and was collected. The Vivaspin 500 (Sartorius stedim biotech) was used according to manufacturer's instructions.

Samples were prepared for loading by adding 21µl of sample to 8µl of 4x loading dye to 3µl of 10x reducing agent to generate a final volume of 32µl of which 10µl was loaded after heating to 80 degrees C in a 96 well PCR plate placed in a PCR block, (PTC-100, MJ research Inc) for 5 minutes.

Samples were then loaded on to a gel run according to manufacturer's instructions in MES SDS (2-N morpholino ethanesulphonic acid, sodium dodecyl sulphate), buffer for 35 minutes,(invitrogen) andstained using the microwave mediated version of the SimplyBlue SafeStain protocol, (invitrogen) Loading standards of unencapsidated dAb were run also alongside on the gel to help calculate concentrations, i.e. as 3.28µg, 0.82µg, 0.21µg and 0.05µg from diluted, as described above in sample preparation, 10µl loadings of 500ng/µl, 125ng/µl, 31.25ng/µl and 7.8ng/µl stocks. An image of the stained gel is shown in Fig. 14

The gel was set up as follows:

Lane 1: Whole dAb1, Lane 2: dAb1 3K S, Lane 3: dAb1 3K P, Lane 4: dAb1 13K S, Lane 5: dAb1 13K P, Lane 6: dAb1 F, Lane 7: Whole dAb2, Lane 8: dAb2 3K S, Lane 9: dAb2 3K P, Lane 10: dAb2 13K S, Lane 11: dAb2 13K P,Lane 12: dAb2 F, Lane 13: Molecular markers – SeeBlue Plus 2 pre-stained standard, (invitrogen), molecular weight (kd), Lane 14: 3.28µg dAb standard, Lane 15: 0.82µg dAb standard, Lane 16: 0.21µg dAb standard, Lane 17: 0.05µg dAb standard, The gel confirmed that encapsidation of the dAbs had taken place. The gel also confirmed that the dAbs were intact and that they had not fragmented due to the particle preparation process.

The amount of material in the whole, supernatant and pellet fractions of the PCL HIP particles were ascertained for dAb 1 using band capture and the 1D Gel quantitation

package of Labworks 4.6 software (UVP). Images for analysis were captured using a Vision works station fitted with an Olympus camera under white light. The data is shown in Table 9.

Table 9: Determination of dAb loading in PCL-HIP microspheres.

Lanes	dAb band intensity	dAb in µg
1	997.63	3.5
2	277.54	1.0
3	865.5	3.0
4	241.3	0.9
5	927.56	3.1
6	214.26	0.73
7	1225.4	4.5
8	317.93	nd
9	628.75	nd
10	126.42	nd
11	1056	nd
12	23.896	nd
14	925.94	3.28*
15	272.34	0.82*
16	40.492	0.21*
17	6.7923	0.05*

Lane 1: Whole dAb1, Lane 2: dAb1 3K S, Lane 3: dAb1 3K P, Lane 4: dAb1 13K S, Lane 5: dAb1 13K P, Lane 6: dAb1 F, Lane 7: Whole dAb2, Lane 8: dAb2 3K S, Lane9: dAb2 3K P, Lane 10: dAb2 13K S, Lane 11: dAb2 13K P, Lane 12: dAb2 F, Lane13: Molecular markers – SeeBlue Plus 2 pre-stained standard, (Invitrogen), molecular weight (kd), Lane 14: 3.28µg dAb standard, Lane 15: 0.82µg dAb standard, Lane 16: 0.21µg dAb standard, Lane17: 0.05µg dAb standard,

Gel readings for intensity of dAb were converted to dAb amounts in µg, (Table 9) using a plot of the dAb standards against band intensity, (data not shown).

The mean whole dAb formulation was read as, (lanes 1 and 7), 3.5µg + 4.5µg / 2 = 4µg – sample was diluted 21 in 32 in loading the 10µl so total dAb in 10µl = 32/21 x 4 = 6µg.

dAb 1 supernatant, (lanes 2, 4 and 6) = 1.0 + 0.9 + 0.73 / 3 = 0.9 µg corrected for dilution is 1.4µg

dAb1 pellet, (lanes 3 and 5) = 3.0 + 3.1 / 2 = 3.0µg corrected for dilution is 4.6µg

The fractions appear to tally as whole dAb at 6µg = dAb 1 supernatant (1.4µg) + dAb1 pellet (4.6µg) using these figures the percentage of encapsidated dAb is 77% of whole dAb, (4.6/6.0)

However percentage of input dAb – 10µl (9.5µg) that is encapsidated is 4.6/9.5 x 100 = 48%.

Example 16 - Release of dAb from HIP PCL microspheres.

In order to release dAb from HIP PCL particles for analysis of functional activity – samples of 50µl aliquots were taken from the dAb1 and dAb2 HIP PCL formulations and placed in a 1.5ml eppendorf. These were washed 2x with 1ml of PBS, with a 5 min 5000 rpm spin in an Eppendorf 5417C microfuge. The pellet was re-suspended in 50µl PBS and a time course of 0, 20, 40 and 60 mins incubation at 56°C in a Techne heating block. The sample was then spun down, 5 min 5000 rpm spin and 30µl of supernatant, (S) removed and placed on ice for analysis. The pellet, (P), was then dried and re-suspended in 50µl.

All the fractions were subject to gel analysis – released supernatants were analysed on one gel and the released pellets were analysed on another gel. The supernatant gel is shown in Fig. 15 loadings were set up as described for the initial analysis gel in Fig 14.

The amount of material in the released supernatant and pellet fractions of the PCL HIP particles were ascertained for dAb 1 and dAb 2 using band capture and the 1D Gel quantitation package of Labworks 4.6 software (UVP). Images for analysis were captured using a Vision works station fitted with an Olympus camera under white light.

Gel readings for intensity of dAb were converted to dAb amounts in ng, (Table 10, column 2), using a graph plot of the dAb standards against band intensity, (data not shown).

Note that the amount of material released by this process ranged from 120-189 ng (data not shown), from a pellet source of around 882-1000 ng in the particles –where 12-19% of the material was released.

Functional analysis of dAb released from PCL HIP particles by ELISA.

ELISA assay protocol describes a binding assay for measuring the ability of soluble domain antibodies (VEGF dAb) to bind to recombinant VEGF. The assay uses recombinant human VEGF (R&D Systems) coated onto the surface of ELISA plates (Nunc Immunosorb) to capture VEGF dAb. The plates are washed to remove any unbound dAb. Bound dAb is subsequently detected using an antibody to the Myc tag of the VEGF dab (9E10, Sigma). Excess antibody is removed by washing and the bound anti-myc antibody is detected using an anti-mouse IgG peroxidase conjugate (Sigma). The assay is developed using TMB solution and stopped using acid. The signal from the assay is proportional to the amount of dAb.

An ELISA plate was set up to analyse the samples listed below and also dAb1 and dAb 2 samples 'released' after 0 mins. From the removed released sample of 30µl – 21µl was used for SDS PAGE analysis and 9µl was left to make dilutions of 1 in 100, 1 in 1000 and 1 in 10000.

A comparison of calculated total released dAb and functionally active dAb measured by ELISA is shown in Table 10.

Table 10 – Quantification of functionally active and total released dAb

Sample	corrected dAb ng/ µl from SDS PAGE intensity conversion	corrected dAb ng/ µl from functional ELISA	Percentage active dAb
dAb1 – 20 mins	20.57143	33	~100
dAb1 - 40 mins	28.8	22	76
dAb1 - 60 mins	18.59048	30	~100
dAb 2 – 20 mins	18.28571	14	77
dAb2 - 40 mins	18.28571	11	61

dAb2 - 60 mins	21.33333	17	80

It can be seen that using this release procedure functionally active dAb can be detected from material released from microspheres at a range consistent with 60-100% of the encapsidated retaining activity as measured by ELISA.

Total versus active dAb will fluctuate according to dAb released, heat inactivation or any degradation of dAb however, the variance is not considered to be significantly different.

Sequence Listing.

SEQ ID NO.	Description
1	Heavy chain amino acid sequence humanised construct H28 anti-NOGO antibody
2	2A10 light chain amino acid sequence humanised construct L16 anti-NOGO antibody
3	Heavy chain humanised DNA construct H28 anti-NOGO antibody
4	2A10 light chain humanised DNA construct L16 anti-NOGO antibody
5	Mature H2 heavy chain amino acid sequence, (Fc mutated double mutation bold) beta-amyloid antibody
6	Mature Light chain amino acid sequence beta-amyloid antibody
7	Mature H11 heavy chain amino acid sequence
8	Mature L9 light chain amino acid sequence
9	Dalargin hexapeptide
10	DOM15-26-593 VEGF dAb amino acid sequence
11	CvL1 variable region amino acid sequence MAG antibody
12	BvH1 variable region amino acid sequence MAG antibody
13	H2 Full length DNA beta-amyloid antibody
14	Optimised L1 light chain DNA beta-amyloid antibody
15	L1 Full length DNA beta-amyloid antibody

SEQUENCES

SEQ ID NO. 1: Heavy chain humanised construct H28

MGWSCIILFLVATATGVHSQVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHW
VRQAPGQGLEWIGNINPSNGGTNYNEKFKSKATMTRDTSTSTAYMELSSLRSEDTA
VYYCELMQGYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPS
NTKVDKKVEPKSCDKTHTCPPCPAPELAGAPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL
HNHYTQKSLSLSPGK

SEQ ID NO. 2: 2A10 light chain humanised construct L16

MGWSCIILFLVATATGVHSDIVMTQSPLSNPVTLGQPVSISCRSSKSLLYKDGKTYLN
WFLQRPGQSPQLLIYLMSTRASGVPDRFSGGGSGTDFTLKISRVEAEDVGVYYCQ
QLVEYPLTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKV
QWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC

SEQ ID NO. 3: Heavy chain humanised construct H28

ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCAC
TCCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCT
CAGTGAAGGTTTCCTGCAAGGCATCTGGATACACCTTCACCAGCTACTGGATGC
ACTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATCGGAAATATTAAT
CCTAGCAATGGTGGTACTAACTACAATGAGAAGTTCAAGAGCAAGGCCACCATG
ACCAGGGACACGTCCACGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATC
TGAGGACACGGCCGTGTATTACTGTGAACTGATGCAGGGCTACTGGGGCCAGG
GAACACTAGTCACAGTCTCCTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCC
TGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCT
GGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACTCAGGCGCCC
TGACCAGCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACT
CCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTAC
ATCTGCAACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAG
CCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTC
GCGGGGGCACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCAT
GATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAG
ACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCC
AAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGT
CCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGG
TCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAG

GGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTG
ACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGA
CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCA
CGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACC
GTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCA
TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAA
ATGA

SEQ ID NO. 4: 2A10 light chain humanised construct L16

ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCAC
TCCGATATTGTGATGACCCAGTCTCCACTCTCCAACCCCGTCACCCTTGGACAG
CCGGTCTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTATATAAGGATGGGAAG
ACATACTTGAATTGGTTTCTCCAGAGGCCAGGCCAATCTCCACAGCTCCTAATTT
ATTTGATGTCCACCCGTGCATCTGGGGTCCCAGACAGATTCAGCGGCGGTGGG
TCAGGCACTGATTTCACACTGAAAATCAGCAGGGTGGAGGCTGAGGATGTTGG
GGTTTATTACTGCCAACAACTTGTAGAGTATCCGCTCACGTTTGGCCAGGGGAC
CAAGCTGGAGATCAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCC
ATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAA
CTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGACAACGCCCTCCAATC
GGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGACAGCACCTACA
GCCTCAGCAGCACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTC
TACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTT
CAACAGGGGAGAGTGTTAG

SEQ ID NO. 5: Mature H2 heavy chain amino acid sequence

EVQLVESGGGLVQPGGSLRLSCAVSGFTFSDNGMAWVRQAPGKGLEWVSFISNLA
YSIDYADTVTGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCVSGTWFAYWGQGTLV
TVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC
PPCPAPELAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
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PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO. 6: Mature Light chain amino acid sequence

DIVMTQSPLSLPVTPGEPASISCRVSQSLLHSNGYTYLHWYLQKPGQSPQLLIYKVS
NRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCSQTRHVPYTFGGGTKVEIKR
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT
EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 7: Mature H11 heavy chain amino acid sequence

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GETIYTPKFQDKATLTVDTSTDTAYMELSSLRSEDTAVYYCVSSGYWGQGTLVTVS
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VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELAGAPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
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QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO. 8: Mature L9 light chain amino acid sequence

DIVMTQSPLSNPVTPGEPASISCRSSKSLLHRNGITYLYWYLQKPGQSPQLLIYQMS
NLASGVPDRFSSSGSGTDFTLKISRVEAEDVGVYYCAQNLELWTFGQGTKVEIKRT
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO. 9: Dalargin

YAGFLR

SEQ ID NO. 10: DOM15-26-593 VEGF dAb sequence:

EVQLLVSGGGLVQPGGSLRLSCAASGFTFKAYPMMWVRQAPGKGLEWVSEISPSG
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VTVSSAAAEQKLISEEDLN

SEQ ID NO. 11: CvL1 variable region

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ASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCHQYLSSLTFGQGTKLEIKR
TV

SEQ ID NO. 12: BvH1 variable region

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MDYWGQGTLVTVSS.

SEQ ID NO. 13: H2 Full length DNA sequence

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GGTAAA

SEQ ID NO. 14: Optimised L1 light chain DNA
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TCCGGCACCGACTTCACCCTGAAGATCAGCAGAGTGGAGGCCGAGGATGTGGG
CGTGTACTACTGCTCCCAGACCAGACACGTGCCTTACACCTTTGGCGGCGGAA
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CTACAGCCTGAGCAGCACCCTGACCCTGAGCAAGGCCGACTACGAGAAGCACA
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AGCTTCAACCGGGGCGAGTGC

SEQ ID NO. 15: L1 Full length
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ATTTACATTGGTACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATA
AAGTTTCCAACCGATTTTCTGGGGTCCCTGACAGGTTCAGTGGCAGTGGATCAG
GCACAGATTTTACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTT
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GGGGAGAGTGT

Claims.

1. A method of encapsulating biologically active agents in a particulate carrier comprising the steps of:

a) solubilising a biologically active agent in the presence of a hydrophobic ion pairing (HIP) agent and in an organic solvent to form an organic phase;

b) dissolving a monomer or oligomer of a polymer forming substance in the organic phase formed in (a);

c) forming an emulsion of the organic phase formed in (b) in a continuous aqueous phase to allow polymerisation of the monomer; and

d) obtaining a particulate carrier formed from the emulsion.

2. A method of encapsulating biologically active agents in a particulate carrier comprising the steps of:

a) mixing a biologically active agent in an aqueous phase with a hydrophobic ion pairing (HIP) agent in an organic solvent phase to form a biologically active agent-HIP complex;

b) separation of the complex from the aqueous phase.

c) removal of the aqueous phase and homogenisation of the complex with the organic phase;

d) (i) dissolving a polymer in the organic phase formed in (c) and then forming an emulsion of the organic phase in a continuous aqueous phase; or

(ii) dissolving a monomer or oligomer of a polymer forming substance, in the organic phase formed in (c) and then forming an emulsion of the organic phase in a continuous aqueous phase; to allow polymerisation of the monomer or oligomer to form a polymer; and

e) obtaining a particulate carrier formed from the emulsion of step (d).

3. The method of claims 1 or 2 wherein the monomer comprises alkylcyanoacrylate (ACA).

4. The method of claim 3 wherein the monomer comprises butylcyanoacrylate (BCA)

5. The method as claimed in claim 1 or claim 2 wherein the polymer comprises poly-L-lactide (PLA), poly butylcyanoacrylate (PBCA) or poly(lactide-co-glycolide)(PLG), or poly(caprolactone), poly(hydroxybutyrate) and/or copolymers thereof.

6. The method of claim 5 wherein the polymer comprises poly(caprolactone).

7. The method of claim 5 wherein the polymer comprises poly(lactide-co-glycolide)(PLG).

8. The method of claim 5 wherein the polymer comprises poly-L-lactide (PLA).

9. The method of claims 3 or 4 wherein the continuous aqueous phase has a pH of about 6 or higher.

10. The method of any one of claims 1 to 9 wherein the particulate carrier is a nanoparticle.

11. The method of any one of claims 1 to 10 wherein the biologically active agent is a protein or peptide.

12. The method of claim 11 wherein the biologically active agent is an antigen binding molecule.

13. The method of claim 12 wherein the antigen binding molecule comprises a domain.

14. The method of claim 12 wherein the antigen binding molecule is an antibody.

15. The method of claim 12 wherein the antigen binding molecule is a domain antibody.

16. The method of any preceding claim wherein the biologically active agent is insoluble in the organic phase without the presence of hydrophobic ion pairing agents.

17. The method of any one of claims 1 to 16, wherein the HIP agent is an anionic HIP agent when the biologically active agent is cationic.

18. The method of claim 17 wherein the HIP agent is docusate sodium.

19. The method of any one of claims 1 to 16, wherein the HIP agent is a cationic HIP agent when the biologically active agent is anionic.

20. The method of claim 19 wherein the HIP agent is dimethyldioctadecyl-ammonium bromide (DDAB18); 1,2-dioleoyloxy – 3(trimethylammonium)propane (DOTAP); or cetrimonium bromide (CTAB).

21. A particulate carrier comprising an encapsulated biologically active agent obtainable by the method of any preceding claim.

22. A particulate carrier according to claim 21 wherein the protein to polymer ratio is at least about 1.0% w/w or is at least about 2.5% w/w, or is at least about 5% w/w.

23. A particulate carrier according to claim 21 wherein the peptide to polymer ratio is at least about 5% w/w, or is at least about 9% w/w.

24. A particulate carrier according to claim 21 wherein the antibody to polymer ratio is at least about 1% w/w, or is at least about 2.5% w/w.

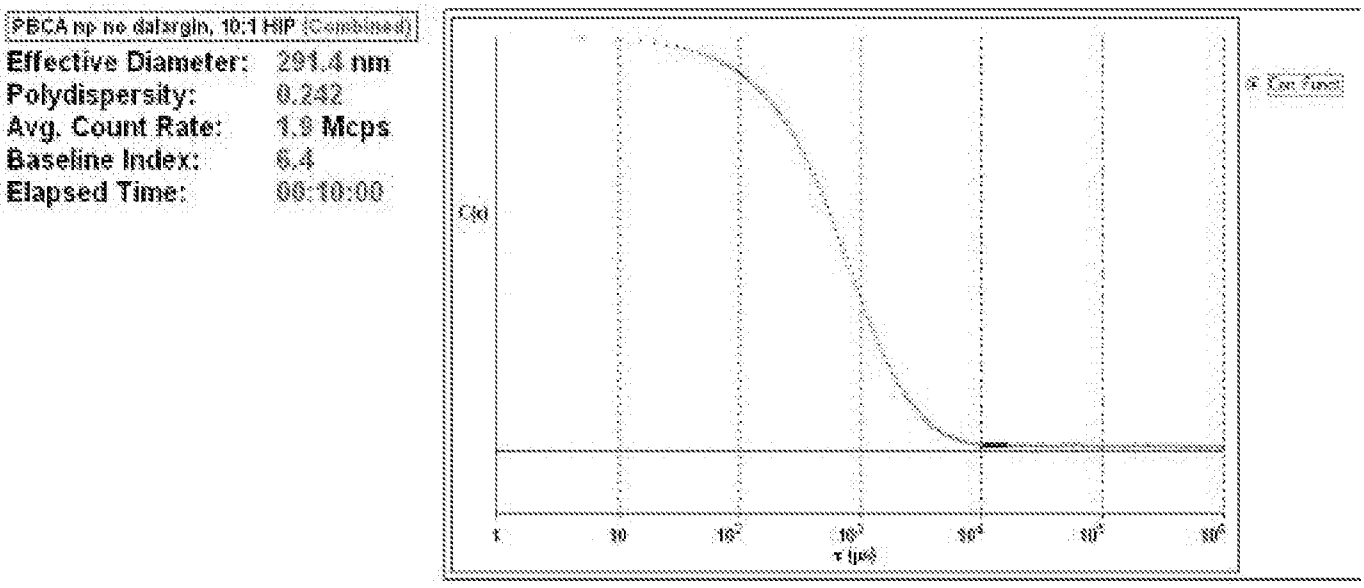
25. A particulate carrier according to claim 21 wherein the domain antibody to polymer ratio in the nanoparticle is at least about 5% w/w.

26. A pharmaceutical composition comprising the particulate carriers of any one of claims 21-25.

27. The pharmaceutical composition of claim 26 for the prophylaxis or treatment of disease.

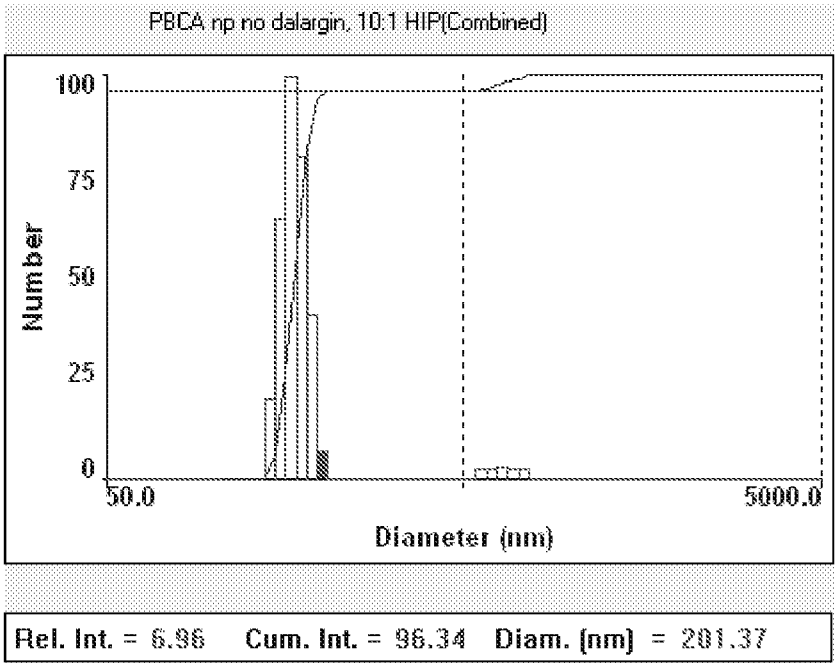
Figure 1

(1a)

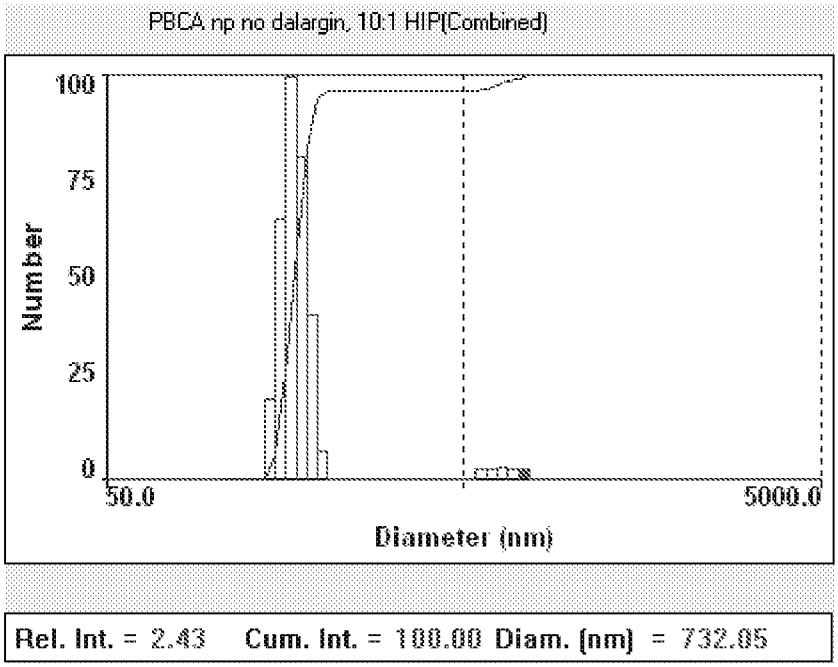


Run	Eff. Diam. [nm]	Half Width [nm]	Polydispersity	Baseline Index
1	302.8	135.1	0.200	8.8
2	290.2	151.0	0.270	10.0
3	291.0	131.7	0.205	7.8
4	293.1	148.2	0.253	9.2
5	298.8	149.4	0.258	9.4
6	289.2	145.8	0.241	8.6
7	320.1	138.3	0.188	9.7
8	281.3	133.7	0.226	7.9
9	293.6	143.1	0.238	9.7
10	282.7	147.5	0.272	7.4
Mean	294.3	142.6	0.237	9.5
Std. Error	3.5	2.3	0.010	0.3
Combined	291.4	143.2	0.242	6.4

(1b)


PBCA np no dalargin, 10:1 HIP(Combined)
100
75
50
25
0
Number
50.0
5000.0
Diameter (nm)
Rel. Int. = 6.96 Cum. Int. = 98.34 Diam. (nm) = 201.37

1c


PBCA np no dalargin, 10:1 HIP(Combined)
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75
50
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0
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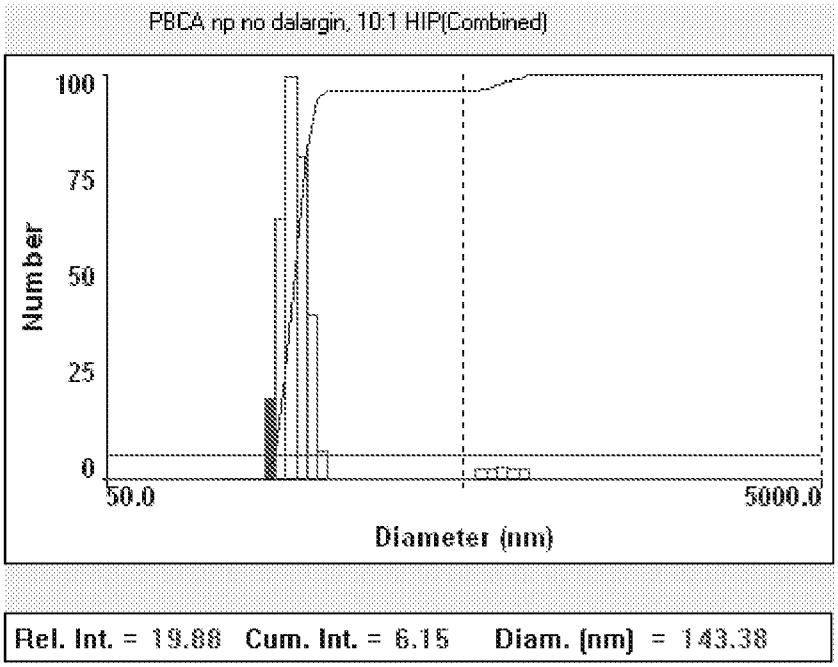
50.0
5000.0
Diameter (nm)
Rel. Int. = 2.43 Cum. Int. = 100.00 Diam. (nm) = 732.05

1d


PBCA np no dalargin, 10:1 HIP(Combined)
100
75
50
25
0
Number
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5000.0
Diameter (nm)
Rel. Int. = 19.88 Cum. Int. = 6.15 Diam. (nm) = 143.38

Figure 2.

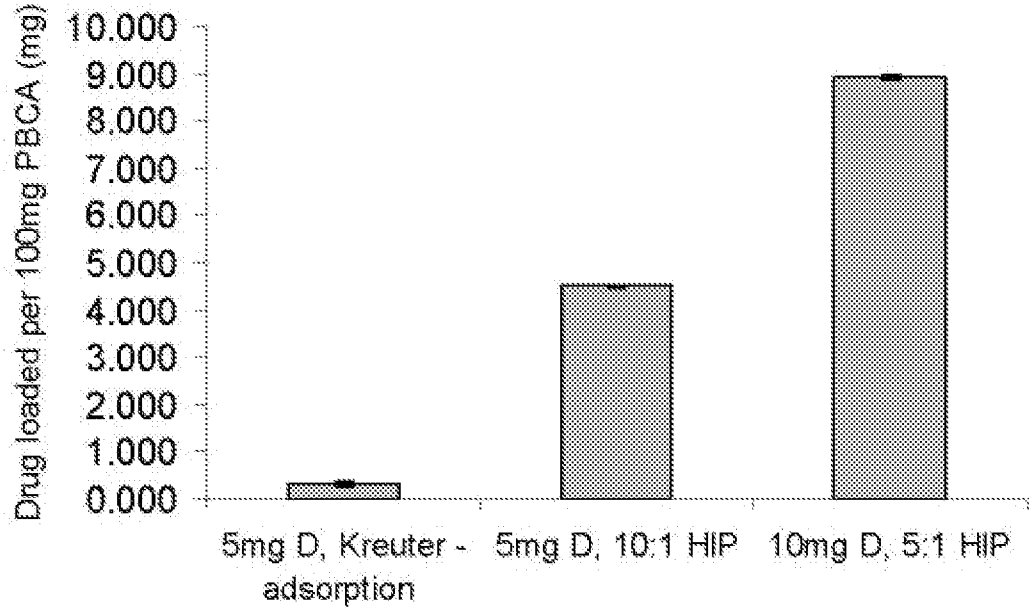


Figure 3

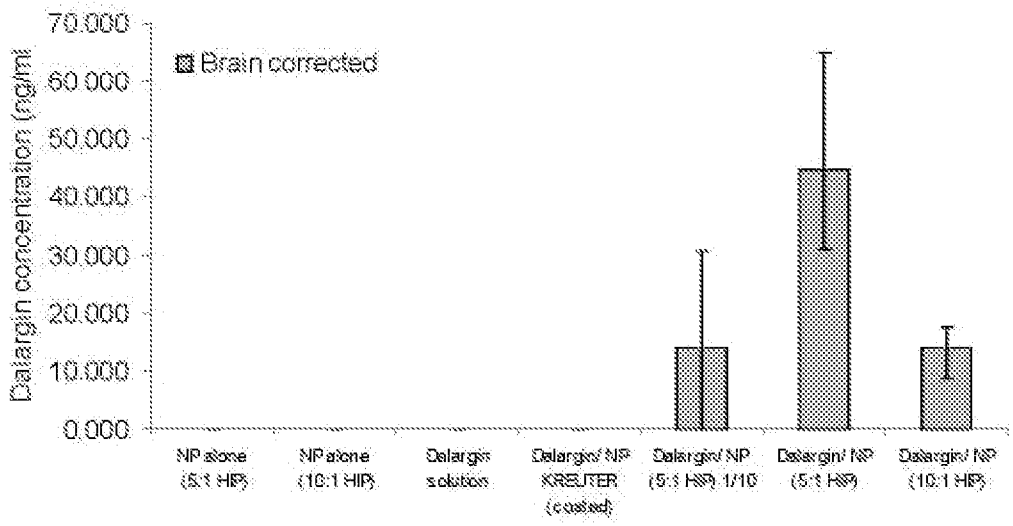


Figure 4

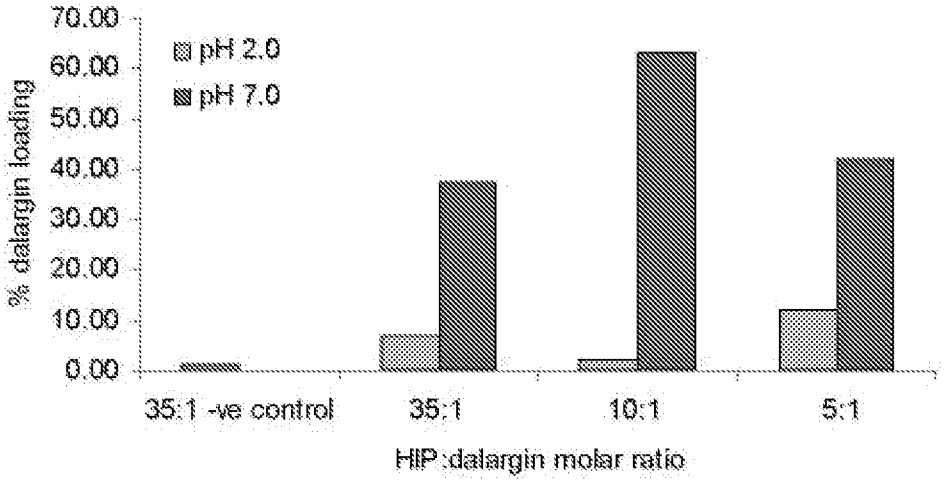


Figure 5

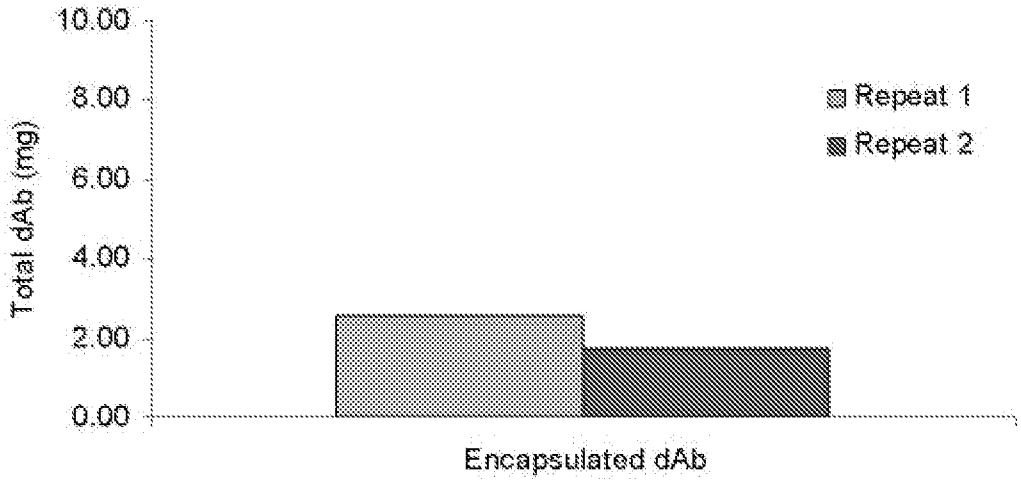


Figure 6.

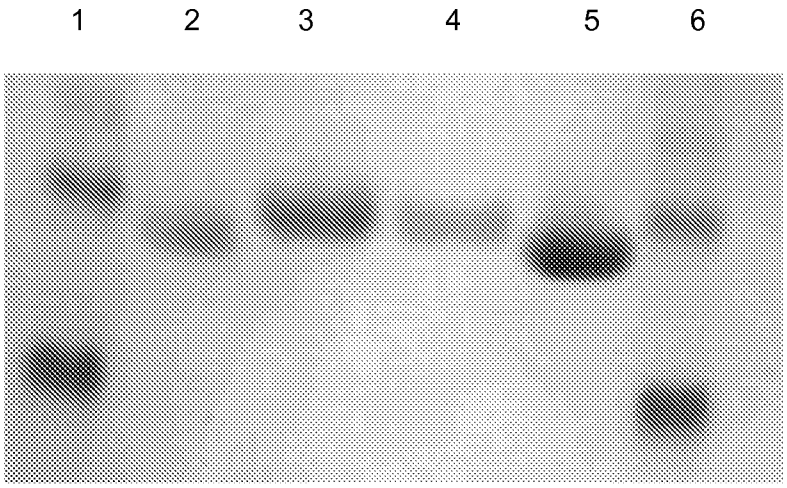


Figure 7

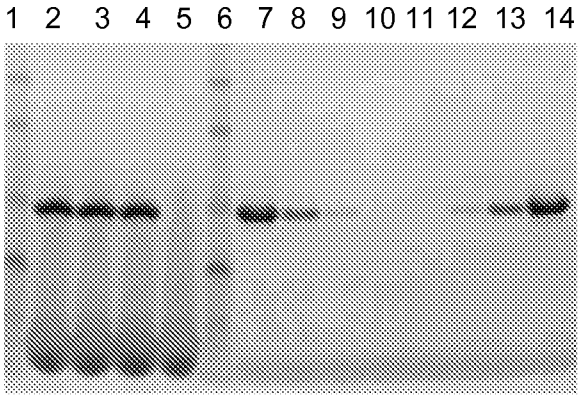


Figure 8

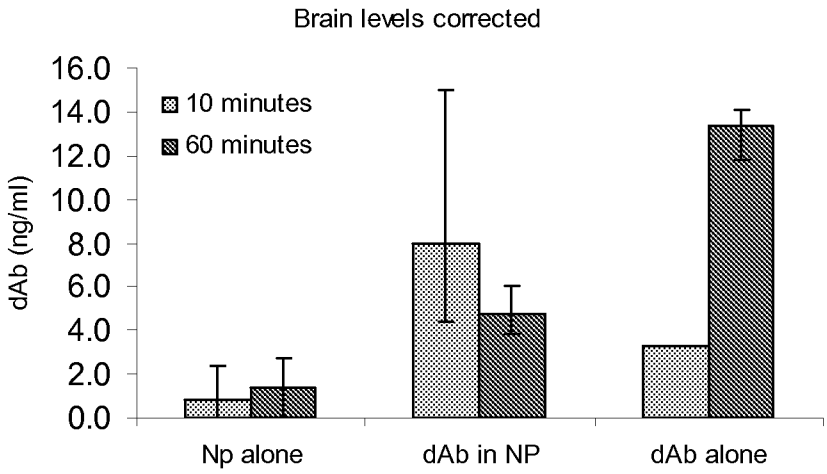

Brain levels corrected
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
dAb (ng/ml)
10 minutes
60 minutes
Np alone
dAb in NP
dAb alone

Figure 9

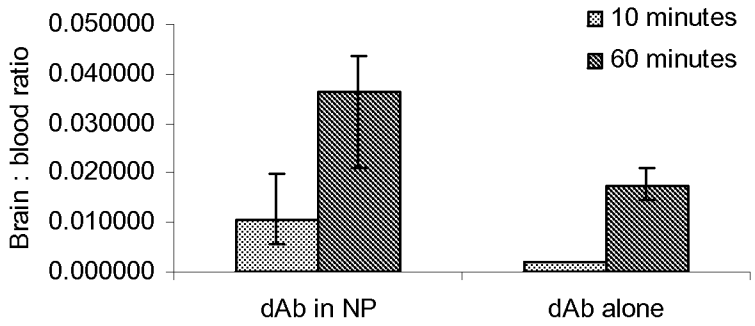


Figure 10

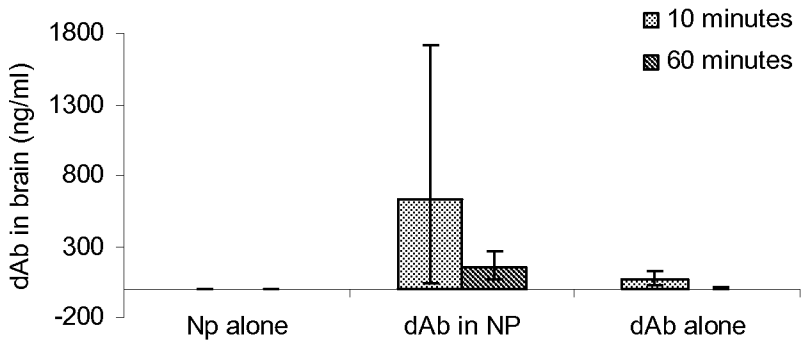


Figure 11

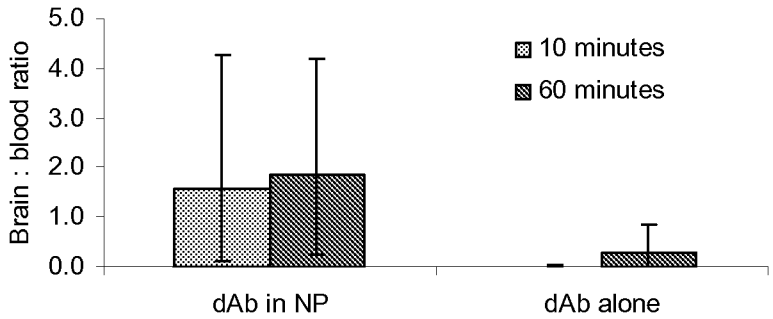


Figure 12.

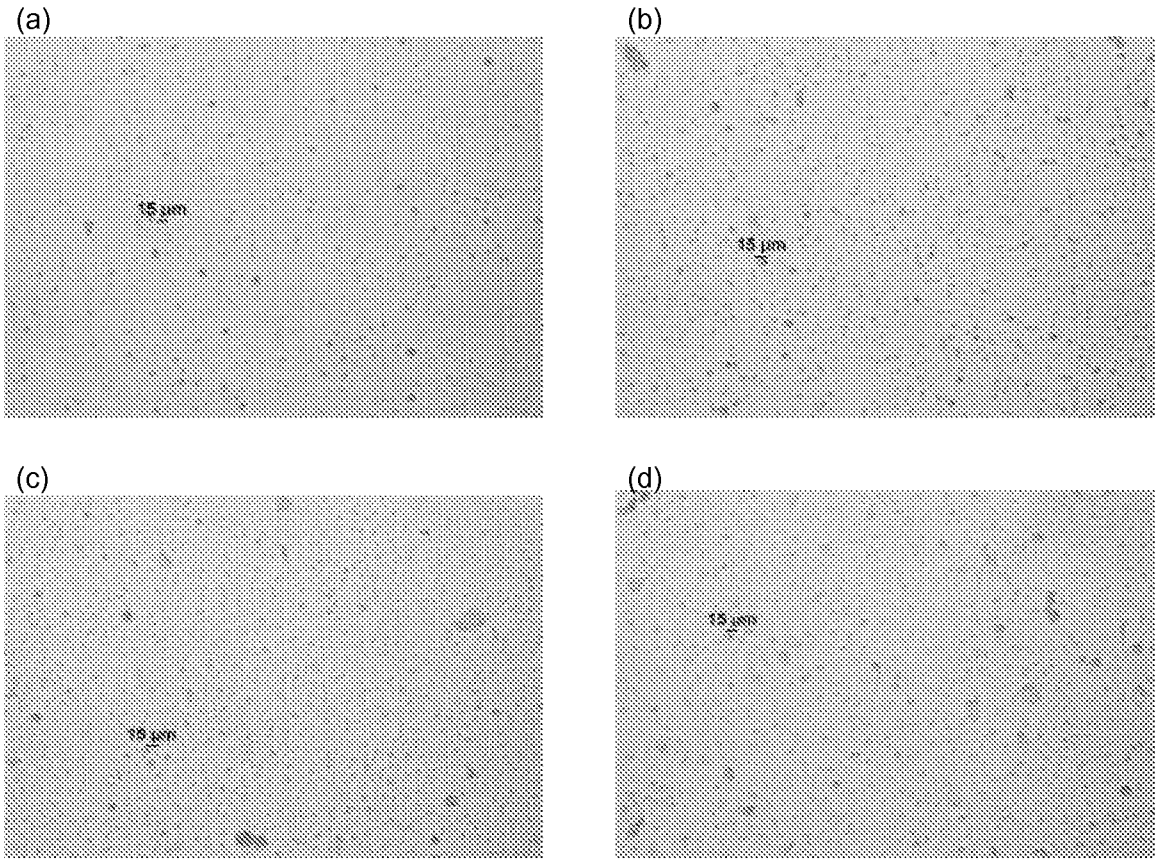
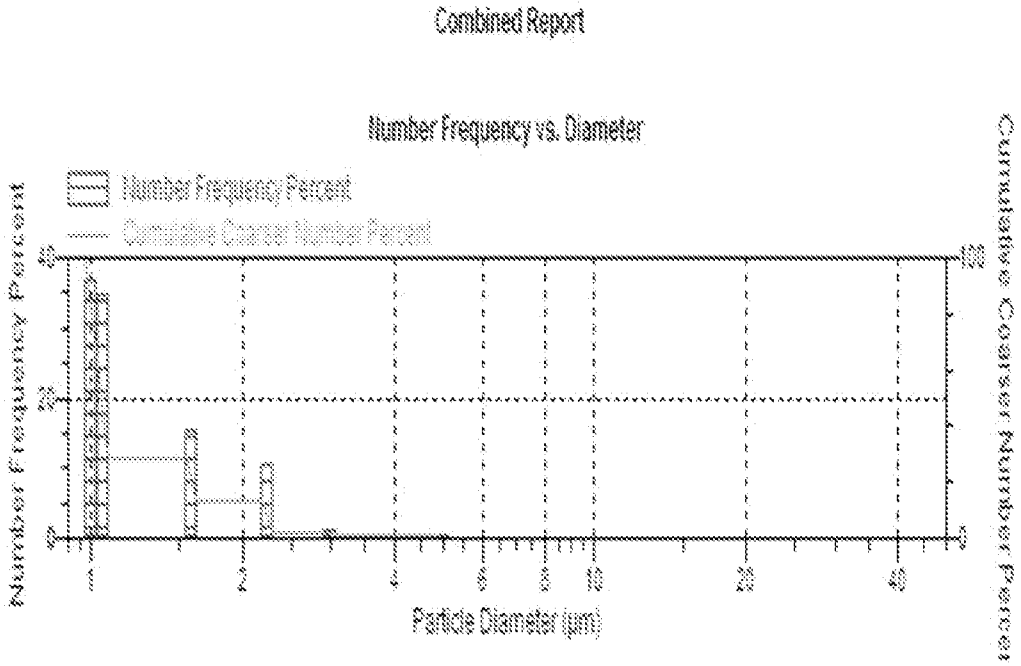


Figure 13

(a)


Combined Report
Number Frequency vs. Diameter
Number Frequency Percent
Cumulative Coarser Number Percent
Number Frequency Percent
Cumulative Coarser Number Percent
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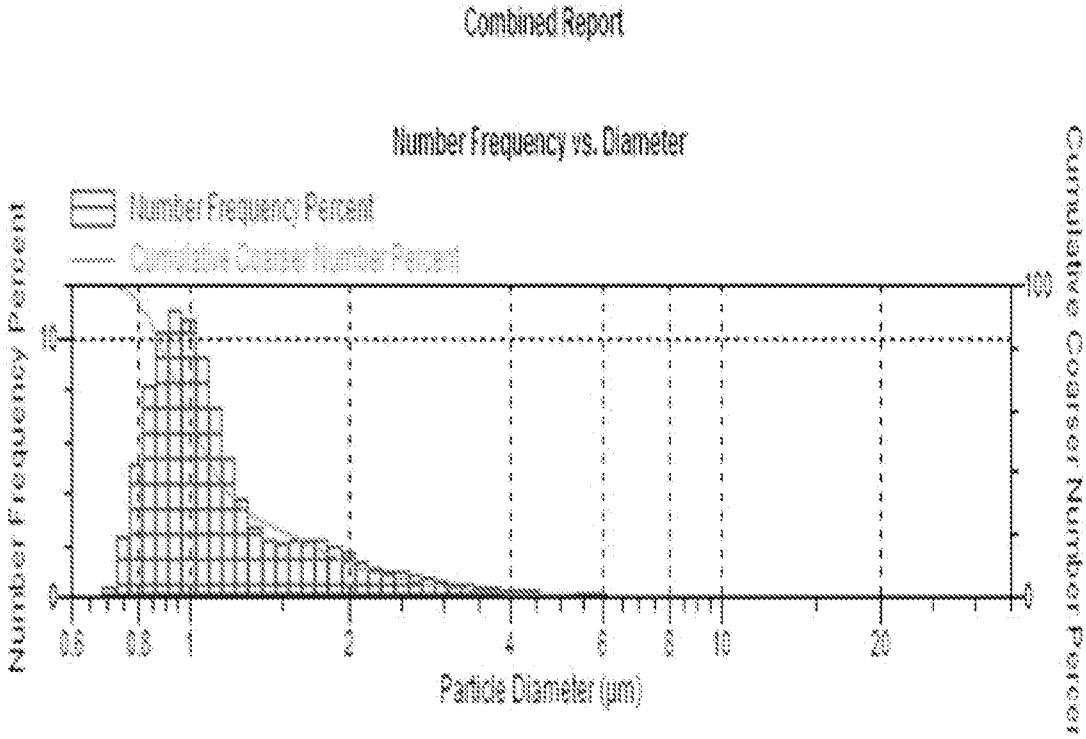
6
8
10
20
40
Particle Diameter (µm)

(b)


Combined Report
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Number Frequency Percent
Cumulative Coarser Number Percent
Number Frequency Percent
Cumulative Coarser Number Percent
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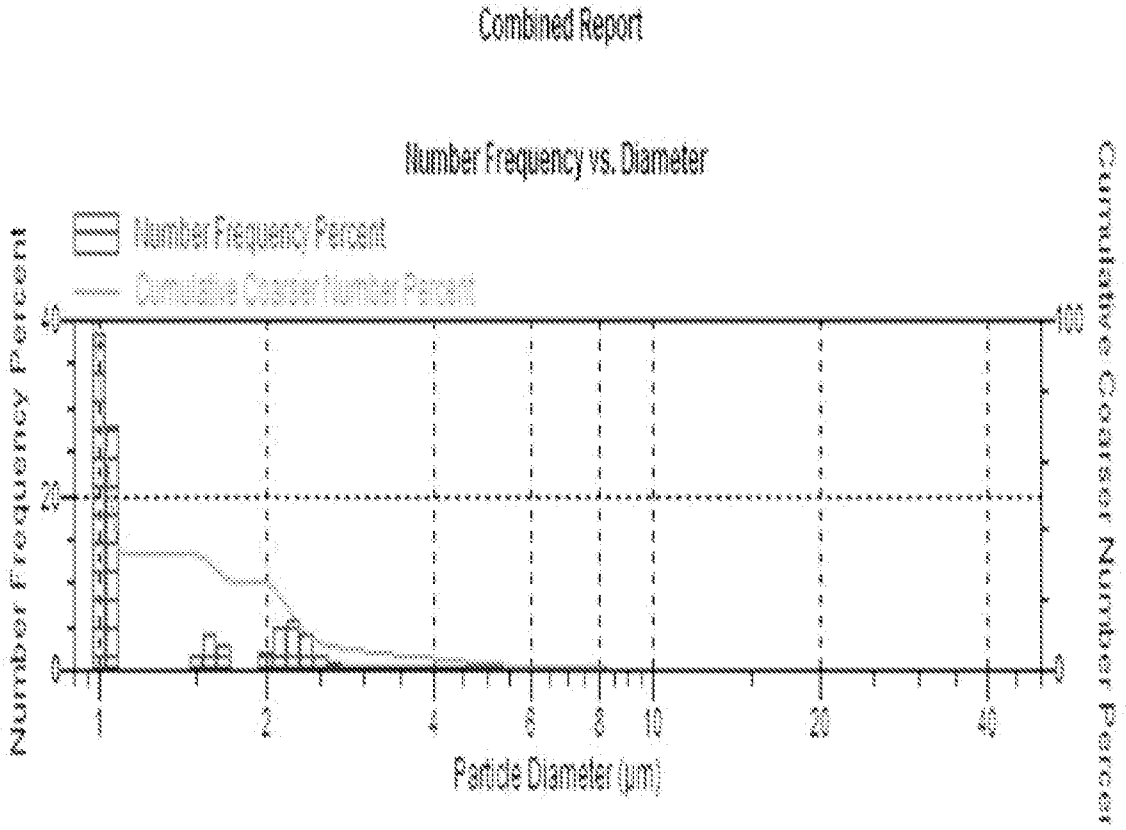
2
4
6
8
10
20
Particle Diameter (µm)

(c)


Combined Report
Number Frequency vs. Diameter
Number Frequency Percent
Cumulative Coarser Number Percent
Number Frequency Percent
Cumulative Coarser Number Percent
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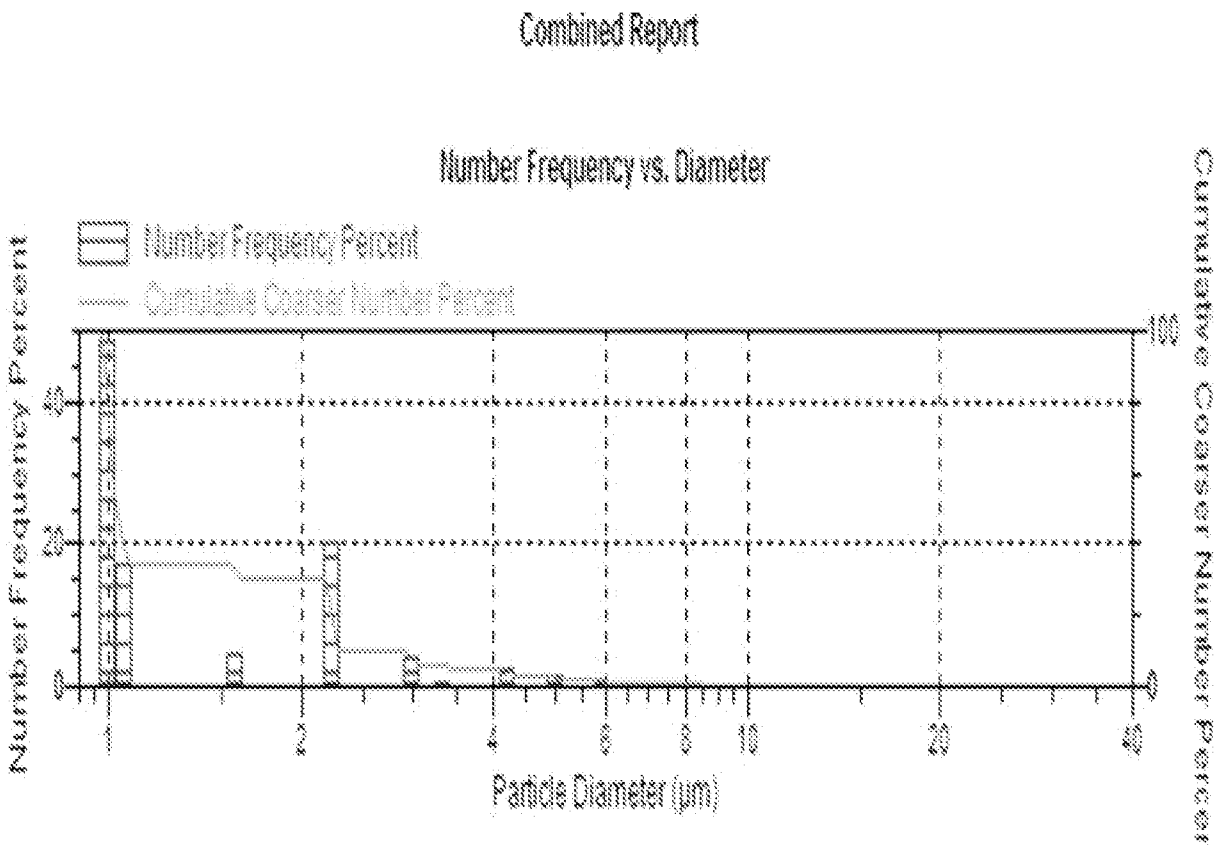
0
1
2
4
6
8
10
20
40
Particle Diameter (µm)

(d)


Combined Report
Number Frequency vs. Diameter
Number Frequency Percent
Cumulative Coarse Number Percent
Number Frequency Percent
Cumulative Coarse Number Percent
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1
2
4
6
8
10
20
40
Particle Diameter (µm)

Figure 14.

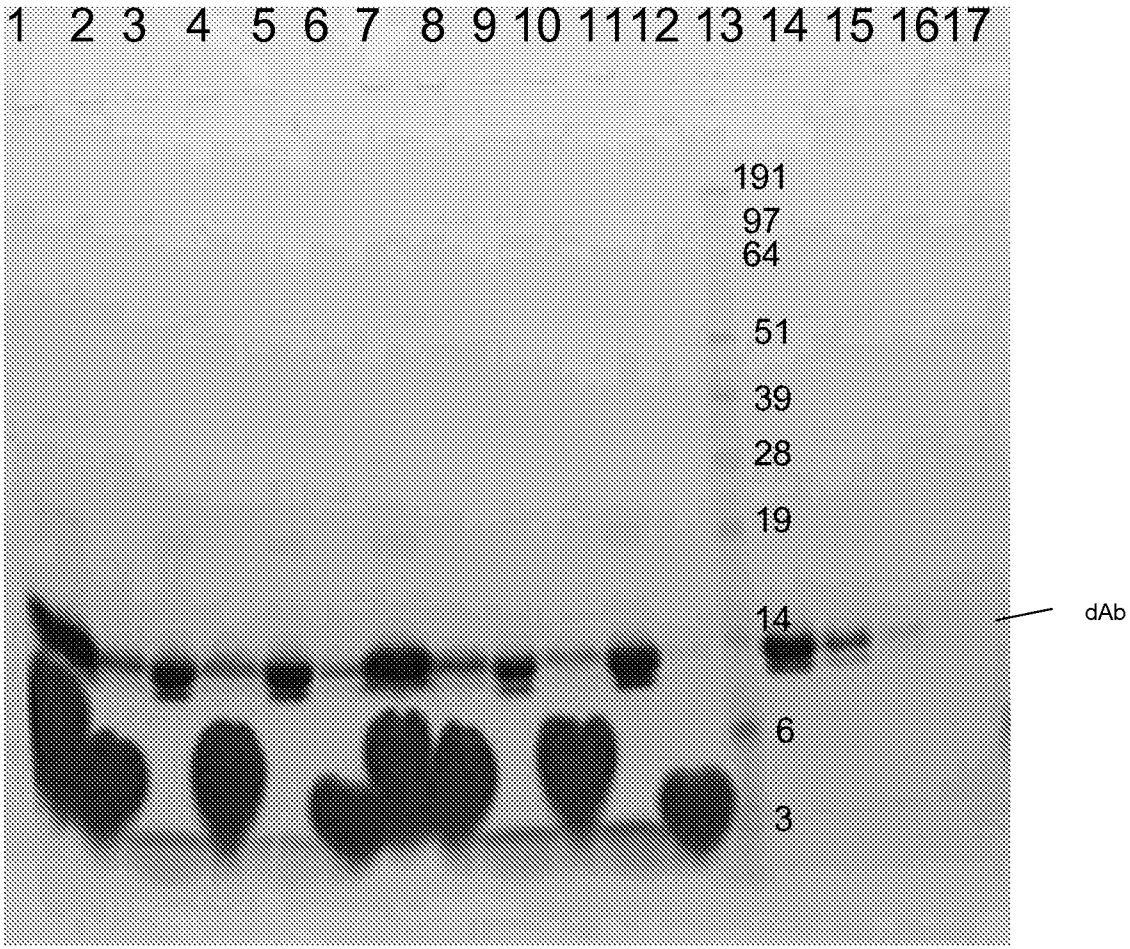

Figure 15.