Expert Opinion

Polymer conjugates as therapeutics: future trends, challenges and opportunities

María J Vicent†, Lucile Dieudonné, Rodrigo J Carbajo & Antonio Pineda-Lucena
†*Centro de Investigación Príncipe Felipe, Polymer Therapeutics Laboratory, Avenue Autopista del Saler 16, E-46012 Valencia, Spain*

Objective: Clinical proof of concept for polymer conjugates has already been achieved over the last 30 years, with a family of polymer–protein conjugates reaching the market and an exponentially growing list of polymer–drug conjugates currently in clinical trials. However, many challenges and opportunities still lie ahead, providing scope to develop this platform technology further. *Methods*: The delivery of new anticancer agents aimed at novel molecular targets and their combination, the development of both new polymeric materials with defined architectures and the treatment of diseases other than cancer are the most exciting and promising areas. The latest advances and future trends in the polymer conjugate field will be presented in this article, providing an insight into their potential in the clinics and offering a wide range of research approaches within the scientific community. *Results/conclusion*: Polymer therapeutics is a rapidly emerging field with exponentially growing opportunities to achieve medical treatments with highly enhanced therapeutic value.

Keywords: biophysical characterisation, combination therapy, polymer architecture, polymer conjugates, polymer therapeutics

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1. Introduction

Research carried out at the interface of polymer chemistry, cell biology and the biomedical sciences led to the first polymer-based nanomedicines used clinically to treat life-threatening and debilitating diseases [1-3]. These water soluble, multi-component constructs, polymer–drug [4] and polymer–protein [5,6] conjugates, are designed for parenteral administration and are considered 'new chemical entities' by the regulatory agencies [7] as the bioactive agent is covalently bound to the polymer carrier, rather than being entrapped within the complex [8]. Due to an existing chemical linkage, a better defined macromolecule tailoring strategy can be achieved taking always into account the location of the selected molecular target and the nature of the bioactive moiety to be delivered.

On one hand, novel proteins, peptides and antibody-based drugs are rapidly emerging as bioactive agents due to '-omics' research programmes, including metabolomics, genomics or proteomics. However, the development of protein therapeutics has been challenging due to short blood circulation time and non-specific toxicity [9]. Their conjugation to polymers can be used to overcome these drawbacks: it reduces immunogenicity, prolongs plasma half-life and enhances protein stability. On the other hand, high-throughput screening (HTS) provides with numerous lead compounds, mainly hydrophobic low molecular weight (Mw) drugs that require a rationally designed delivery system to achieve therapeutic value. Conjugation to a polymer boosts drug solubility, increases the Mw of the drug, which reduces drug renal clearance and prolongs circulation lifetime and consequently

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Table 1. Polymer–protein conjugates in the market or that have undergone/are in clinical evaluation [1,3].

Polymer–protein conjugates	Name	Status	Indication
PEG-adenosine deaminase	Adagen	1990	SCID syndrome
SMANCS	Zinostatin Stimalmer®	1993 (Japan)	Hepatocellular carcinoma
PEG-L-asparaginase	Oncaspar®	1994	Acute lymphoblastic Leukaemia
PEG-G-CSF	Neulasta™	2002	Prevention of neutropenia Associated with cancer Chemotherapy
PEG-interferon α 2a	PEG-Asys®	2002 Phase I/II	Hepatitis B and C Melanoma and renal cell carcinoma
PEG-interferon α 2b	PEG-Intron™	2000 Phase I/II	Hepatitis C Melanoma, chronic myelogenous leukaemia and renal cell carcinoma
PEG-arginine deiminase	ADI-PEG20	Phase I	Hepatocellular carcinoma
PEG-human growth hormone (HGR)	Pegvisomant	2002 (EU)	Acromegaly
PEG-glutaminase combined with a glutamine antimetabolite 6-diazo-5-oxo-L-norleucine (DON)	PEG–PGA and DON	Phase I/II	Various cancers
PEG-recombinant mammalian urate oxidase	Puricase® or peglotinase	Phase III	Resistant gout and hyperuricemia
PEG-anti-TNF-α Fab	Cimzia™, CD870, Certolizumab pegol	2008 (US) Phase III finished (filed to FDA and EMEA) Phase II – III	Crohn's disease Rheumatoid arthritis Psoriasis

G-CSF: Granulocyte colony-stimulating factor; PEG: Poly(ethylene glycol); SCID: Severe combined immunodeficiency disease; SMANCS: Styrene maleic anhydride-neocarzinostatin.

promotes tumour targeting by the enhanced permeability and retention (EPR) effect [10,11]. Furthermore, and depending on the polymer's chemical nature, intracellular endo- or lyso-somotropic drug delivery is allowed after endocytic capture at the cellular level [8,12].

Clinical proof of concept has been already gained with an increasing number of polymer–protein conjugates reaching the market since the 1990s (e.g., PEGylated enzymes and cytokines) (**Table 1**) [5] and the promising results arising from clinical trials with polymer-bound chemotherapy (e.g., doxorubicin [Dox], paclitaxel [PTX], camptothecins [CPT]) [1,4]. Poly(-L-glutamic acid) (PGA)–paclitaxel conjugate (Xyotax™, CT-2103 or PPX from Cell Therapeutics Inc.) [13,14] is expected to be the first polymer–drug conjugate to be commercialised (**Figure 1** and **Table 2**).

Lessons have been learnt over the last 30 years, which have laid a firm foundation for the development of more sophisticated second generation polymer conjugates. However, there are still opportunities to take and challenges to address in order to move this technology forward. An exhaustive historical overview is beyond the scope of this review, but detailed information can be found in a vast number of publications (see [1-6,8,15,16]).

Here, we aim to analyse instead the latest advances and future trends in this exciting field of polymer conjugates, mainly focusing on four key aspects: i) the development of new polymeric carriers and the importance of polymer architecture; ii) the delivery of newly emerging target-directed bioactive agents (e.g., modulators of the cell cycle, signal transduction inhibitors, apoptosis modulators and antiangiogenic drugs) with application in cancer but also in a much broader range of diseases (e.g., antiviral diseases, ischaemia, eye-related diseases, arthritis etc); iii) polymer-based combination therapy to better tackle the complex molecular basis of disease; and iv) the importance of an exhaustive physicochemical characterisation of these complex hybrid constructs.

2. Where should we focus to achieve second generation polymer conjugates?

2.1 Development of new polymeric carriers and the importance of polymer architecture

The rationale for the use of polymers as carriers of conjugated therapeutics was well described by Helmut Ringsdorf [17], tuning the pioneering work of Jatzkewitz, who used non-degradable or enzymatically degradable (glycyl-L-leucine) side chains to conjugate the psychedelic alkaloid drug mescaline to *N*-vinylpyrrolidine-based polymers [18]. Since then, it has become clear that the molecular mass and physicochemical

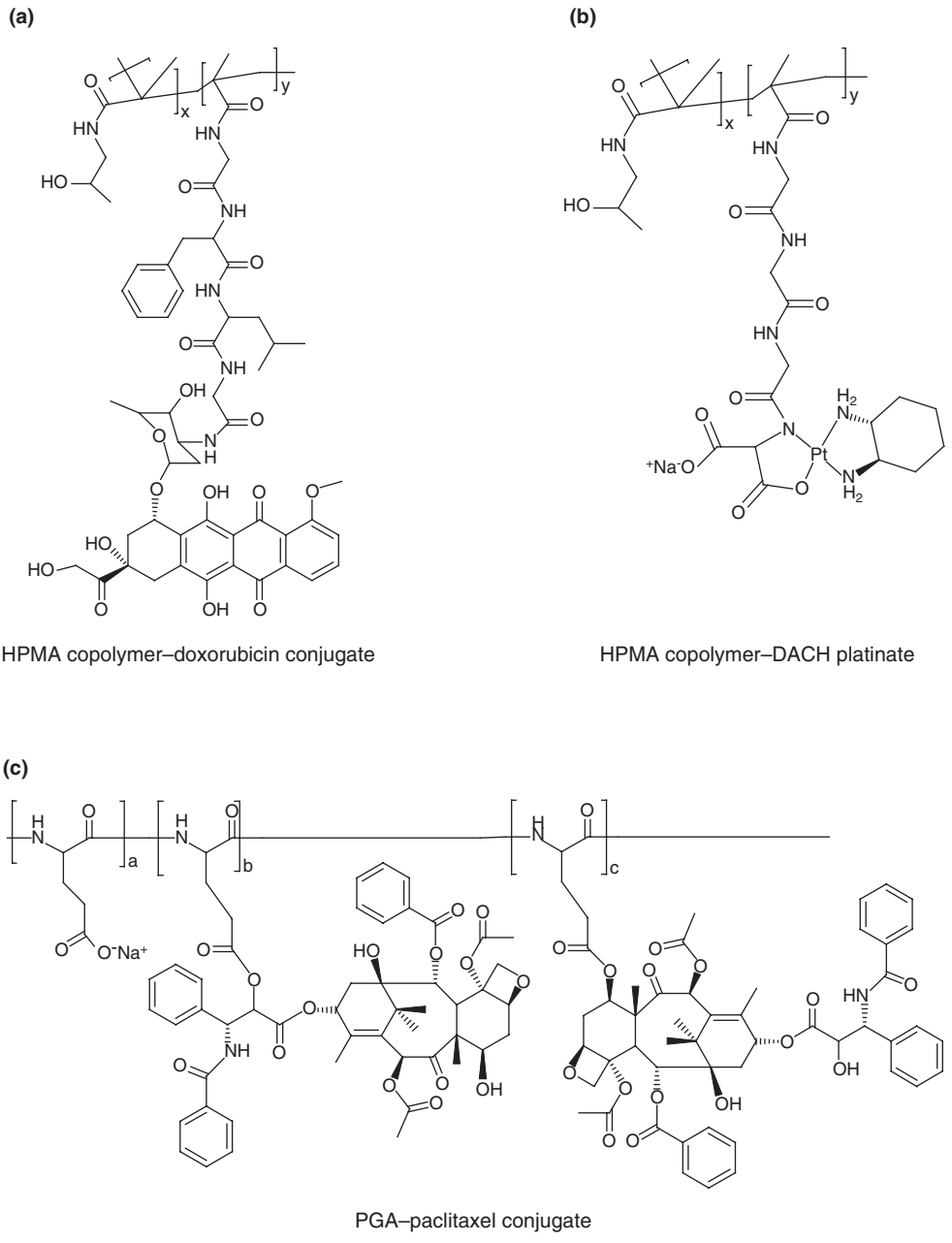

Figure 1. Polymer–anticancer drug conjugates. Examples of first generation polymer–drug conjugates containing: **(a)** doxorubicin (PK1, FCE28068), **(b)** platinum (ProLindac™, AP5346) and **(c)** paclitaxel (Xyotax™, CT2103) that have progressed to clinical trial.
DACH: Diaminocyclohexane; HPMA: *N*-2-hydroxypropyl methacrylamide; PGA: poly(-L-glutamic acid).

properties of the polymer are frequently the most important drivers governing biodistribution, elimination and metabolism of the conjugate as a whole. The choice of the polymeric carrier is therefore critical, and thus the development of improved polymeric carriers is an ongoing challenge.

From a wide range of natural and synthetic polymers currently available, only a few linear random coils have been fully exploited for conjugation to drugs and proteins in the clinical setting, namely, poly(ethylene glycol) (PEG) [5], poly(*N*-hydroxypropyl methacrylamide)s (PHPMA) [19,20], PGA [14] and dextran (**Tables 1** and **2**) [21].

There is a need to develop biodegradable polymeric carriers with a higher Mw that can maximise EPR-mediated tumour targeting, which is ultimately driven by the circulating plasma concentration of the polymer conjugate [22]. Following this trend, very recently three novel biodegradable polymers have been successfully transferred to the clinic: i) a 70 kDa hydrophilic polyacetal, poly(1-hydroxymethylene

Table 2. Polymer–drug conjugates that have undergone/are in clinical evaluation [1,3,4].

Polymer–drug conjugates	Name	Status	Indication
Poly(glutamate) (PGA)–paclitaxel	CT-2103, Xyotax™ Paclitaxel Poliglumex (PPX)	Phase III	Various cancers, particularly non-small cell lung cancer and ovarian cancer
HPMA copolymer–doxorubicin	PK1; FCE28068	Phase II	Various, particularly lung and breast cancer
HPMA copolymer–doxorubicin–galactosamine	PK2; FCE28069	Phase I/II	Particularly hepatocellular carcinoma
Hyaluronic acid–doxorubicin	ONCOFID-D	Phase I/II	Superficial bladder cancer
Oxidised dextran–doxorubicin	AD-70, DOX-OXD	Phase I (discontinued)	Various cancers
HPMA copolymer–paclitaxel	PNU166945	Phase I (discontinued)	Various cancers
HPMA copolymer–malonato–platinate	AP5280	Phase I/II	Various cancers
HPMA copolymer–DACH–platinate	AP5346, ProLindac™	Phase II	Various cancers, particularly ovarian and colorectal
PEG–camptothecin	Pegamotecan, Prothecan™	Phase II (discontinued)	Various cancers
HPMA copolymer–camptothecin	MAG-CPT	Phase I (discontinued)	Various cancers
Poly(glutamate)–camptothecin	CT-2106	Phase I/II	Various cancers
Carboxymethyldextran–exatecan	DE-310	Phase I	Various cancers
β-Cyclodextrin–camptothecin	IT-101	Phase I	Various cancers
PHF–camptothecin	XMT-1001	Phase I	Various cancers
PEG–naloxol (oral administration)	NKTR-118	Phase I	Opioid-induced bowel dysfunction (OBD)

DACH: Diaminocyclohexane; HPMA: *N*-2-hydroxypropyl methacrylamide; PEG: Poly(ethylene glycol); PGA: Poly(-L-glutamic acid); PHF: Poly(1-hydroxymethylene hydroxylmethyl formal).

hydroxylmethyl formal) (PHF) carrying CPT (XMT-1001 from Mersana Therapeutics) [23,24]; ii) cyclodextrins, cyclic oligomers of alpha-1,4-linked glucopyranose units bioresponsive in presence of amylase [25], with IT-101 from Insert Therapeutics [26], a cyclodextrin-containing polymer conjugate of CPT currently into Phase I – II clinical trials [27]; and iii) hyaluronic acid (HA)-based conjugates, susceptible to degradation by hyaluronidases, like the ONCOFID™ platform [28] developed by Fidia Farmaceutici SPA [29], where cytotoxic drugs are covalently conjugated to the hyaluronic backbone. In particular, HA-Dox (ONCOFID™–D) conjugate is in Phase I – II trials for superficial bladder cancer.

Novel biodegradable PEGs such as PEG diacrylates [30] or PEG-polyacetals that show pH-dependent degradation [31] are another promising option. We described a novel system where the drug can be chemically incorporated within the polymer main chain and specifically designed for pH-triggered activation in the endosomes and lysosomes. The non-steroidal oestrogen diethylstilbestrol (DES) was used to achieve proof of concept (**Figure 2**) [32].

Typically, for optimised synthesis of polymer–protein conjugates there are specific requirements, such as the use of a semitelechelic polymer to avoid protein crosslinking during conjugation, site-specific protein modification and appropriate linking chemistry that maintains the biological activity of the protein without generation of toxic or immunogenic by-products. For this reason, PEGylation [5] has been the technique of choice in the last three decades. Moreover, it has been fully demonstrated that PEG associated with a protein or other biological molecules does not represent a significant additional unquantified risk to humans due to its low exposure, low toxicity profile and the similarity of the metabolites that are formed in all species [33].

Current efforts are devoted to developing more sophisticated PEG bioconjugation approaches [34,35]. However, new concepts are also arising, always trying to mimic nature. It has been shown recently that biodegradable polymers such as dextrin and hyaluronic acid can be used to generate bioresponsive protein conjugates in the context of polymer-masking–unmasking protein therapy (PUMPT) [36]. This approach uses a biodegradable polymer to transiently mask a protein during transportation (therefore stabilising/inactivating the protein), while subsequently allowing triggered polymer degradation, protein unmasking and thus restoration of bioactivity. The so-called 'responsive polymers' [37] or 'smart polymers' are also found along this line. These are polymers that exhibit discontinuous, sometimes large changes in their physical state as a result of small changes in their surrounding

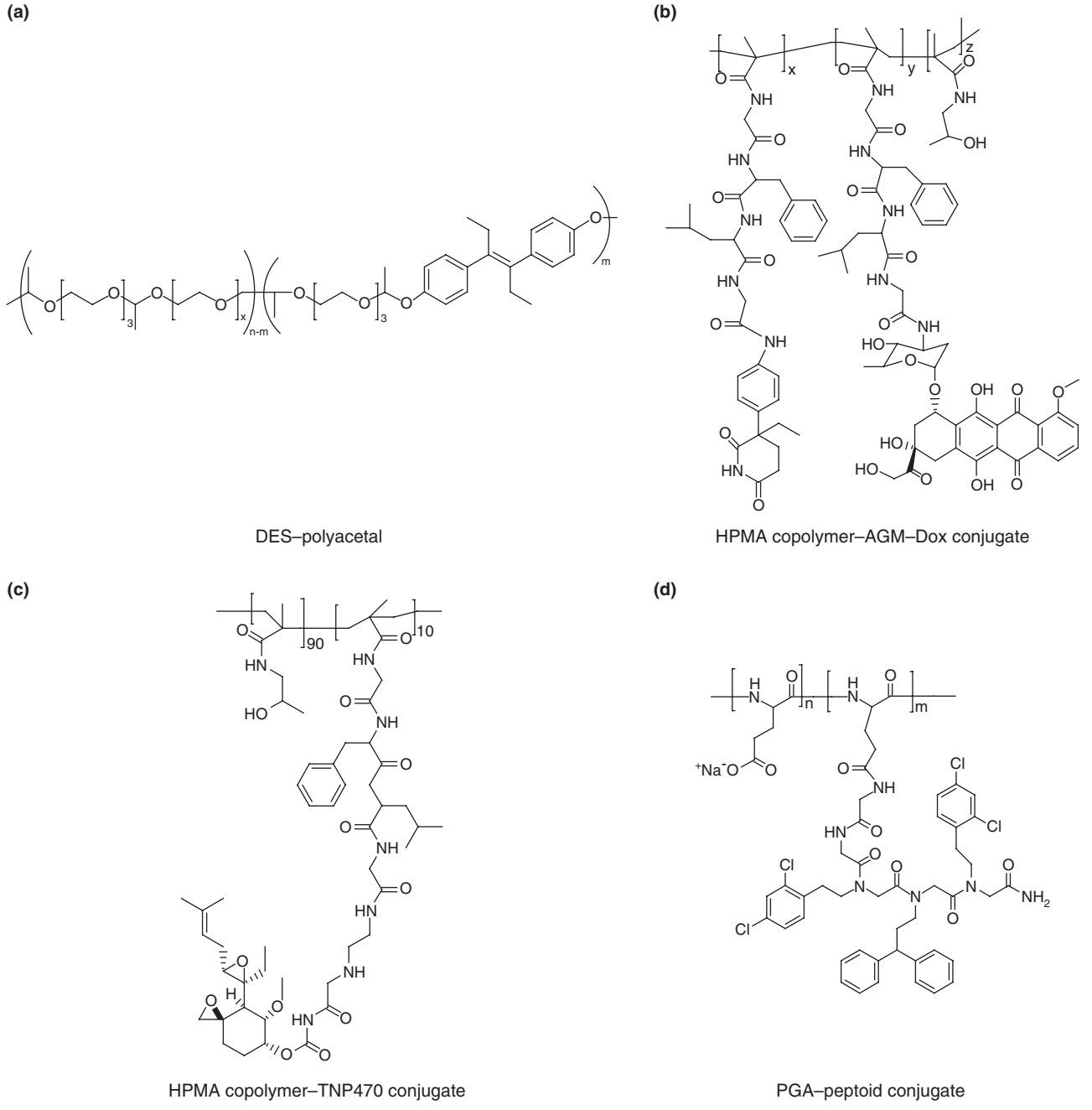

Figure 2. Second generation polymer–drug conjugates. (a) pH labile poly(acetal) containing the bioactive drug within the polymer main chain; **(b)** polymer combination therapy containing the aromatase inhibitor aminogluthethimide (AGM) and doxorubicin (Dox); **(c)** first antiangiogenic polymer drug conjugate; and **(d)** first antiapoptotic polymer conjugate to treat diseases other than cancer.
DES: Diethylstilbestrol; HPMA: *N*-2-hydroxypropyl methacrylamide.

environmental conditions. Stimuli such as changes in temperature (e.g., poly(*N*-isopropylacrylamide) (PNIPAM)-based polymers [38]) or pH (e.g., amphiphilic poly(amidoamine)s (PAAs) [39,40] or poly(2-ethylacrylic acid) (PEAAc) [41]) have been explored for protein and gene delivery, looking at endosomotropic or cytosolic delivery [8].

In contrast, a precise control of the molecular structure is needed for successful targeted delivery and, therefore, polymer uniformity, multiple functional groups for enhanced drug payload, cell targeting and monitoring are basic requirements [40,42]. A lack of polymer uniformity will potentially lead to undesirable effects at industrial level (scale-up and batch-to-batch reproducibility) and also in patients after systemic administration. As explained by Brocchini and co-workers, differences in polymer Mw, morphology and chemical structure induce a wide range of

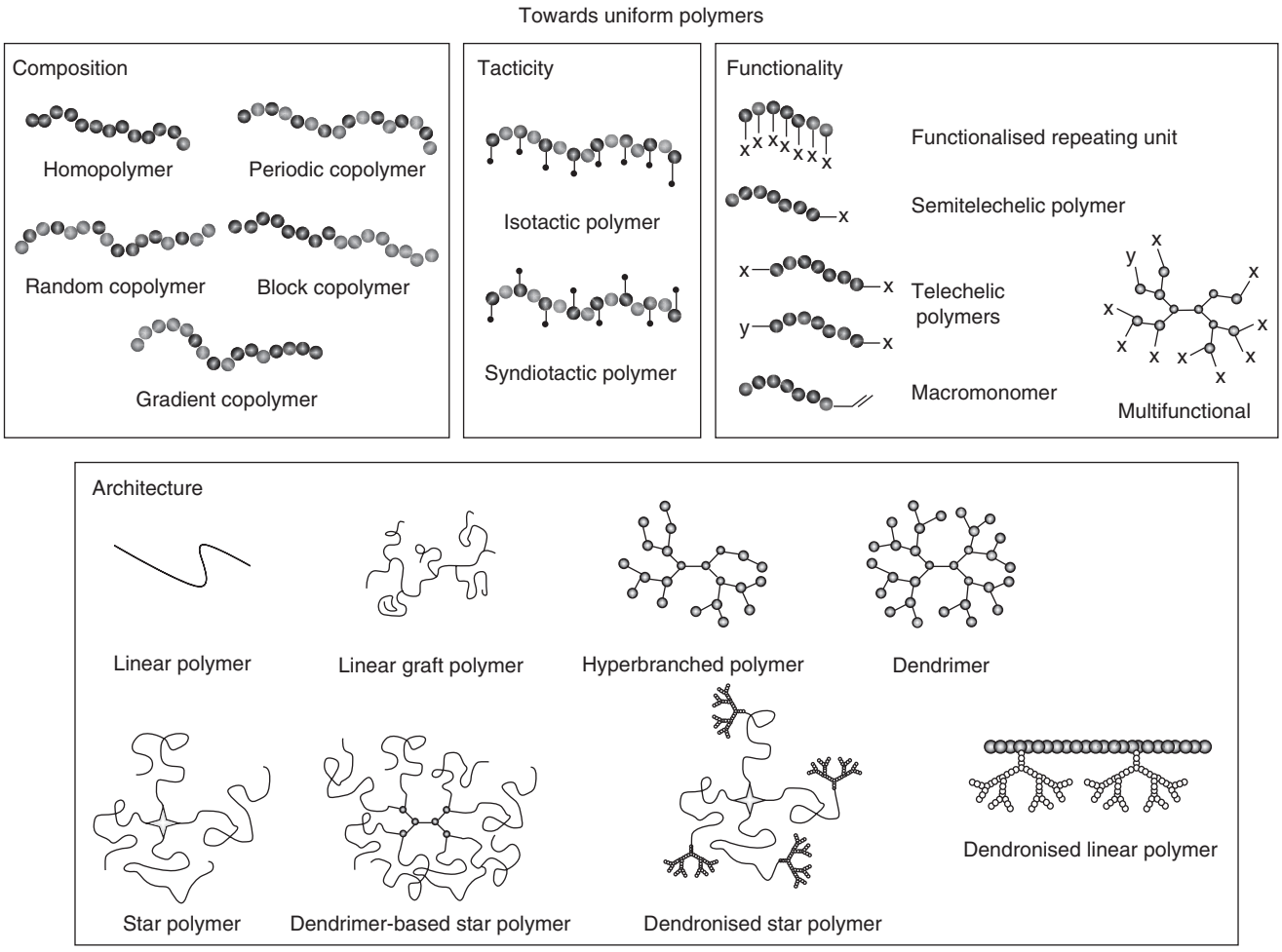

Figure 3. Representation of the different physicochemical aspects to be controlled for the synthesis of uniform polymers.
Adapted from [45,216].

differing physicochemical and biological properties of the whole polymer conjugate [43]. Although successfully transferred to the clinics, most linear polymers and their conjugates present specific challenges for pharmaceutical development, mainly due to their heterogeneity. Consequently, there is a pressing need to move towards better defined polymers where Mw distribution, chemical structure and drug loading capacity are tuneable and carefully controlled. Uniform polymers have been defined as homogeneous macromolecules in respect to their 'molecular mass and constitution' [44,45], with narrow Mw distribution and defined chemical structure regarding stereo-regularity, shape and chemical functionality (see **Figure 3**).

Novel polymeric architectures such as dendrimers [46,47], hyperbranched polymers [48] and hybrid macromolecular architectures (star polymers [49], linear graft and dendronised linear polymers [50]) (**Figure 3**) introduce new physico-chemical features (increased number of superficial endgroup functionality, monodisperse or quasi-monodisperse nanoscale geometry or decreased flexibility compared to linear random coil polymers), which could be used for the design of second generation polymer therapeutics [51].

2.1.1 Dendrimers as carriers in nanopharmaceuticals

The perfectly highly branched structure and three-dimensional (3D) globular shape of dendrimers [52-56] quickly demonstrated their attractiveness for use in the field of polymer therapeutics, as their physicochemical properties could be intelligently employed for overcoming limitations encountered with their linear polymer homologues.

Tomalia and Fréchet's groups were the pioneers of dendrimer synthesis in the late 1980s. Each of them described new methods by either the divergent [57] or the convergent [58] synthetic strategies, where step-wise procedures are employed.

Dendrimers provide many exciting opportunities for improving tissue targeting and intracellular delivery [59]. The principal advantages of dendrimers over linear polymers are

their monodispersity (which should provide reproducible pharmacokinetic behaviour), their precise 3D globular shape (which could affect their biological properties including biodistribution and cellular fate), their defined number of functional groups and their distinct inner (shell) and outer parts (surface) [60]. This last point is specially relevant as it allows the introduction of drugs in two different ways: they can either be hidden in the shell of the dendrimers (for non-covalent encapsulation of hydrophobic drugs); or can be covalently bound on the dendrimer surface (targeting and/or solubilising moieties, emergence of combination therapy) which is more applicable in the field under discussion here. The ability to tailor dendrimer properties to therapeutic needs makes them ideal carriers for small molecule drugs and biomolecules [61].

Dendrimers are key constructs within the polymer chemistry area. However, a full detailed report is beyond the scope of this review and just a short overview of dendrimer state of the art will be given. Exhaustive information on dendritic architectures can be found elsewhere [40,52,53,56,60-63].

It is important to mention two key milestones in this field: i) the transfer of the first dendrimer-based magnetic resonance imaging (MRI) agent (SH-L 643A; Gadomer-17) into clinical development by Schering [64]; and ii) StarPharma's initiation of clinical trials with the first dendrimer-based pharmaceutical, a topically applied vaginal virucide (Vivagel™) in 2005 [65].

Currently, several dendrimers such as poly(amidoamine) (PAMAM), poly(propyleneimine) (DAB), or poly(ethylene oxide) (PEO) grafted carbosilane are being studied and present research is focused on elucidating their structure–biology relationship (i.e., the effect of dendrimers' generation (G) and surface functionality on cytotoxicity and haemolytic compatibility [59,66]). Dendrimers have been explored in the field of drug delivery as anticancer, antiviral, or antibacterial drugs, MRI contrast agents and as DNA transfection agents [46].

2.1.2 *From dendrimers to other hybrid branched controlled architectures*

Apart from the symmetrically defined dendritic structures, polymer chemists have described a whole armoury of polymerisation techniques and 3D architectures with very characteristic features that will provide conjugates with valuable therapeutic outputs. Research in this area is promising but rather limited to date when compared with the extensive work done with linear polymers and dendrimers [67].

Star-shaped polymers are also considered as 3D hyperbranched structures. However, in this case, identical or distinct (in composition and/or Mw) linear arms emanate from one central body or core. Their physicochemical properties (smaller hydrodynamic radius, lower solution viscosity, less flexibility compared to their linear counterparts) and their 3D shape and hyperbranched structure (increased endgroups functionality and their distinct core and surface parts) make them perfect candidates for the design of novel polymer therapeutics. Star polymers can be obtained by living ionic polymerisation (LRP) [68], group transfer polymerisation [69] and with controlled radical polymerisation (CRP) [70-72]. Although there are still some limitations with CRP that have restricted its use on an industrial scale, namely the polymerisation rate (often slower than free radical polymerisation (FRP)), use of metal catalysts (expensive, not environmentally friendly, possible associated toxicity when used in biological systems), or the restriction to only use vinyl monomer [71], CRP is one of the most rapidly developing areas of polymer science with more than 10 papers published per week devoting their efforts to overcome the above-mentioned limitations. CRP provides living characteristics to radical polymerisation, allows the control of Mw and the synthesis of narrowly dispersed polymers (polydispersity index below 1.1 with optimal conditions), with well-defined complex architectures.

There are four main processes that have emerged to carry out CRP, namely atom transfer radical polymerisation (ATRP) (developed independently in 1995 by Matyjaszewski and co-workers [73] and Sawamoto's group [74]), nitroxide mediated polymerisation (NMP) [75], initiator-transfer-agent-terminator techniques (INIFERTER) [76] and reversible addition fragmentation transfer (RAFT) [77-79]. They all involve the same mechanistic strategy, which aims to decrease the concentration of the growing radical species by introducing dormant species, and to reach a fast equilibrium between the active radicals and the dormant species. The equilibrium has to be to a great extent on the side of the dormant species, as a result of the persistent radical effect (PRE) [71,80]. This minimises the probability of termination reactions and allows uniform growth of the polymer chains, thus narrowing polydispersity. Each of these techniques has its own associated advantages and disadvantages with regards to suitable initiators, solvents, monomers, purification, etc.

To some extent similar to the synthesis of dendrimers, star polymers can also be prepared by two main routes: the arm-first approach and the core-first approach. The strategy of the arm-first approach consists of synthesising living or semitelechelic linear arms of the future star and then linking them to one another. This can be achieved in radical polymerisation by: i) the synthesis of the linear arms of the star as a first step, followed by their copolymerisation with a divinyl compound, which eventually forms a small crosslinkage (corresponding to the core of the star); [81]; or ii) the synthesis of semitelechelic linear polymers as a first step, with a subsequent conjugation to the core. As an example, Kopeček and co-workers [82] described the synthesis of star PHPMA by the arm-first approach, where linear and semitelechelic PHPMA were conjugated to a PAMAM dendrimers core.

The strategy of the core-first approach is to primarily synthesise a multifunctional core, which is able, in a second step, to initiate the polymerisation of the monomer, with the arms of the star polymer directly growing from the core.

This can be achieved by ATRP using multifunctional initiators (i.e., initiators with more than two reactive carbon-halogen bonds), as reviewed by Matyjaszewski and Xia [73].

Using functional initiators which yield α-functional polymers or incorporating a range of functional groups into the monomers (such as azide, alkyne, succinimide, maleimide, aldehyde, primary amine, alcohols, carboxylic acids, etc) are the most promising strategies in CRP to achieve polymers with a variety of functional groups for bioconjugation [83] and for the preparation of interesting synthetic polymers such as glycopolymers [84,85].

The design of highly branched and high Mw architectures composed of low Mw cleavable components (that could be safely eliminated from the body) is also very attractive, and development of star-shaped polymers where the arms are linked through hydrolytically cleavable ester bonds to the core is under investigation.

Other hybrid polymeric architectures, such as linear graft polymers, dendronised linear polymers [86], dendronised star polymers or dendrimer-based star polymers [87], can be prepared by assembling linear and branched polymers into more elaborate structures. In particular, dendronised linear polymers are promising constructs as they provide an easily accessible range of Mw with control of the size of the linear polymer backbone, as well as a range of branching degrees with the control of the dendron generation. Three synthetic routes have been described in the literature: i) the polymerisation of a dendronised monomer, also known as the macromolecular route [88]; ii) the grafting route, which consists of the grafting of selected dendrons to linear polymer [89,90]; and iii) a grafting approach where dendrons are divergently added to a linear polymeric base [91]. Finally, it is important to mention the hyperbranched polymers, commonly considered as intermediates between linear polymers and dendrimers, both from a structural and biological point of view. Hyperbranched polymers can be prepared in a single-step reaction, using a more facile but less controlled procedure. It is the least preferred 3D architecture so far for drug or protein conjugation, however a few examples (e.g., hyperbranched poly(glycerol)s) have already shown *in vivo* proof of concept [48].

The birth of this new and more sophisticated chemical technology has offered many potential advantages on conjugate design, however, the structural complexity of many of these constructs represents difficult challenges regarding industrial development costs, biophysical characterisation and clinical safety. It is important to note that before any clinical evaluation, it is essential to establish the safety of new polymers, particularly in respect of general toxicity, immunogenicity and metabolic fate [66].

2.1.3 *Influence of a well-defined 3D architecture on conjugate biological fate*

While the effect of carrier Mw [30,92] and surface functionality [66,93] on *in vitro* and *in vivo* models has been studied in depth, relatively few studies have systematically investigated the effect of polymer architecture on biological properties and drug delivery efficiency (such as endocytosis, cellular internalisation and subsequent cellular fate). Here we have chosen a selected number of studies highlighting the impact of architecture on biological behaviour.

One of the first studies described showed how dextran can be internalised by endocytosis in macrophages, exocytosed and differently trafficked in and out of lysosomes in relation to Mw [94]. More recently, and also looking at more complex architecture, uptake studies of fluorescently labelled linear and star-shaped PEG containing poly(ester) dendrons from G1 to 4 in endothelial-like ECV304 cells were carried out in Professor Duncan's laboratory. It was demonstrated that the rate of uptake and cellular fate were dependant on the conjugate architecture, and also that an increase in Mw and branching density resulted in lower cellular accumulation due to an enhanced rate of exocytosis [95]. The cellular uptake of Oregon green labelled-PAMAM dendrimers (G2 – 4), branched and linear poly(ethyleneimine) (PEI) was evaluated in B16F10 murine melanoma cells, using FITC-dextran as a control [12]. These cationic polymers are internalised by 'adsorptive' endocytosis and the rate of cellular uptake obtained could be classified as follows: PAMAM G4 > > branched PEI > linear PEI > PAMAM G3 > PAMAM G2.

Architectural differences on paclitaxel-based conjugates (PEG versus PAMAM) were evaluated by Minko *et al.* [96]. As expected, the limited aqueous solubility of PTX was improved after conjugation to both polymers, with a more pronounced increase when conjugated to PAMAM G4 dendrimer. Also, both linear PEG polymer and PAMAM dendrimer enhance the internalisation degree of PTX into cancer cells, resulting in a more homogeneous drug distribution inside the single cells, proving the advantages of the polymer–drug conjugation concept. Nevertheless, it was noticed that the anticancer activity of PTX was markedly dependant on carrier architecture. PEG–paclitaxel conjugates showed an important decrease in cytotoxicity when compared to free drug (IC_{50} value 25 times higher for the PEG conjugates compared to free PTX), while PAMAM dendrimer-based PTX conjugates were 10 times more toxic than the free drug.

Similar studies were also carried out with HPMA-based polymers by Kopeček and co-workers. They compared the cytotoxicity of dendrimer-based star HPMA copolymer-Dox conjugate with their linear homologues against human ovarian carcinoma A2780 cells and showed that cytotoxicity was lower in the case of the star-shaped polymer-Dox conjugates [82]. These differences could be attributed to different rates of Dox release by lysosomal enzymes and to different rates of cell entry. Moreover, some studies showed that the reduced flexibility of hyperbranched constructs has a direct effect on biocompatibility [93,97], as rigid molecules have more difficulties interacting with cell surfaces than flexible systems [46]. Following this statement, it has been

demonstrated that the more rigid globular PAMAM dendrimers provided improved biocompatibility with increasing generation, however additional research is still needed as conflicting data were obtained when comparing the cytotoxicity of linear versus dendritic PAMAM structures [46]. As a final example, the properties and antitumour potential of antibody-targeted star-shaped HPMA copolymer-Dox conjugates were compared with linear antibody-targeted or lectin-targeted HPMA copolymer-Dox conjugates [98]. In the presence of cathepsin B, the release of Dox from polymer conjugates was determined to be twice as fast from the star structure of the targeted conjugate than from the linear structure. Although a lower *in vitro* binding activity to BCL1 cells was determined for the star, in good agreement with our prior example, the branched conjugate showed an increased antitumour activity *in vitro* as well as *in vivo* when compared with the classic linear conjugate, probably due to its different drug release kinetics.

2.2 Designing polymer conjugates for novel molecular targets

2.2.1 Novel target-directed anticancer therapy

Due to an increasing understanding of the molecular mechanisms that control tumour cell proliferation, motility, invasion and metastasis, the approach to treat this life-threatening disease is rapidly evolving. This has led to the identification of an unprecedented number of therapeutically important targets, and, therefore, to the discovery of target-directed anticancer agents and protein therapeutics such as tumour-selective apoptosis promoters, cell cycle modulators, signal transduction inhibitors, antiangiogenic agents and vascular targeting agents [9,99-101]. The emerging generation of polymer conjugates are using these novel molecular targets in an attempt to further enhance activity and circumvent resistance.

One example of this current trend is the first polymeric antiangiogenic conjugate, HPMA copolymer–TNP-470 (caplostatin) (**Figure 2**) [102], now under preclinical development by SynDevRx [103] in various tumour models (melanoma, glioblastoma, colon, prostate and lung carcinomas). Caplostatin does not cross the blood–brain barrier and therefore clearly reduces the neurotoxicity observed with the parent TNP-470 (fumagillol) [102]. More recently, this conjugate has shown a complete tumour ablation in a transgenic model of childhood neuroblastoma (a spontaneous murine tumour with native tumour–microenvironment interaction) [104,105]. Furthermore, when caplostatin was combined with bevacizumab (Avastin®), a humanised antivascular endothelial growth factor (VEGF) monoclonal antibody, the eradication of human colon carcinoma in mice was observed. The use of the antibody and conjugate together has a synergistic effect [106,107]. With the same idea of targeting tumour neovasculature, Mitra *et al.* [108] described a novel polymer–peptide conjugate, HPMA copolymer-RGD4C-Tc-99 m conjugate, capable of targeting tumour angiogenic vessels *in vivo* (by targeting overexpressed $\alpha_v\beta_3$ subunit) and delivering adequate radiotherapy to arrest tumour growth.

It is important to mention that these neovasculature inhibitors could be used for the treatment of angiogenic diseases other than cancer, for example diabetic retinopathy, macular degeneration, retrolental fibroplasia, trachoma, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation, capillary formation within atherosclerotic plaques, hemangiomas and excessive wound repair. For example, interferons (IFN-α and IFN-β) are multifunctional regulatory cytokines that directly inhibit proliferation of tumour cells and can also downregulate the expression of proangiogenic molecules (e.g., basic fibroblast growth factor (bFGF), interleukin IL-8, matrix metalloproteinases MMP-2 and MMP-9) [109]. Although showing a very interesting activity at low doses, IFNs have a very short plasma half-life and it has already been demonstrated that PEGylation highly enhances their therapeutic value. Clinical proof of concept has been achieved with two approved PEG–IFN-α conjugates (IFN-2a, Pegasys (Roche) and IFN-2b, PEG-Intron (Schering), see **Table 1** for details) as treatment for hepatitis C [110] and are currently under clinical evaluation in cancer, multiple sclerosis and HIV/AIDS.

Looking at selective apoptosis-inducing agents [111], it is well known that caspases as well as the Bcl-2 protein are interesting therapeutic targets in tumour pathogenesis as they play a key role in the mitochondrial-dependent apoptosis pathway. Several Bcl-2 inhibitors (e.g., HA14-1 or Bcl-2 homology 3 (BH3) peptide domain) have already been identified, and their conjugation to polymeric carriers (HPMA-HA14-1 [112], PEG-BH3 [113] and PAMAM-BH3 [96] conjugates) has shown enhanced efficacy in a variety of *in vivo* tumour models. The conjugation of pluronic F 127 to conjugated linoleic acid (CLA) significantly enhanced apoptosis in breast cancer cell models when compared to free CLA. A clear downregulation of Bcl-2 and procaspase 9 proteins were found [114].

Reactive oxygen species (ROS) induce apoptosis of many tumour cells *in vitro* via the activation of the caspase cascade. Therefore, their specific generation in tumour cells can be considered to be another interesting approach in cancer therapy. For example, PEG–zinc protoporphyrin (ZnPP) conjugate, a specific haem oxygenase (HO) inhibitor [115,116], produces a tumour-selective suppression of HO activity, as well as an induction of apoptosis, possibly by increasing oxidative stress. Within the same line, polymeric photosensitiser prodrugs (PPP) with dual activity (detection and treatment) have also been synthesised by directly conjugating the photosensitiser pheophorbide to poly-L-lysine polymer [117]. As a final example on pro-apoptotic conjugates, it is important to mention a polymer–protein conjugate that targets cell death receptors, poly-1-vinylpyrrolidin-2-one (PVP)-tumour necrosis factor (TNF)-α, currently under preclinical development for sarcoma-180 [118].

Compounds acting on signalling transduction pathways, such as kinase inhibitors (e.g., Tarceva, Gefitinib, Herceptin, Lapatinib, etc) and their combinations are being widely explored for cancer treatment [119,120]. One such compound is the PI3-kinase inhibitor, wortmaninn. *In vitro* studies with an HPMA copolymer-11-*O*-desacetylwortmanninortmannin showed that this conjugate retained the ability to inhibit type I phosphoinositide 3 (PI3)-kinase activity [121]. However, further studies are needed to evaluate its real therapeutic potential. It is believed that conjugation to a polymeric carrier would overcome mechanisms of resistance [1,20] (the main drawback in this type of compound) and would facilitate drug combination with the possibility to target different signalling pathways at once, consequently enhancing their therapeutic value [120].

2.2.2 *Targeting diseases other than cancer*

Most early products targeted cancer (see **Tables 1** and **2**), but in the last decade, clinical interest has broadened to include the development of polymer-based systems for the treatment of diseases other than cancer, for example rheumatoid arthritis (RA), nerve targeting, synthetic vaccine development, diabetes or ischaemia.

A remarkable example is Puricase® (pegloticase), a PEGylated recombinant mammalian urate oxidase currently in Phase III clinical trials for the treatment of resistant gout and hyperuricemia [122] developed jointly by scientists from Mountain View Pharmaceuticals [123] and Duke University [124]. Savient Pharmaceuticals [125] currently holds its worldwide exclusive license. A press release from Savient Pharmaceuticals in February 2008 announced additional positive results for secondary safety and efficacy endpoints in the two replicate Phase III studies for Puricase® for treatment-failure gout. Reduction in the number of tender and swollen joints and an improvement in patient reported outcomes (PRO) were also reported.

PEG-anti-TNF-α antigen binding region (Fab) conjugate (CD870, Certolizumab pegol, Cimzia™) [126] from UCB [127] is expected to be the first approved PEGylated TNF-blocker available for the treatment of RA [128]. When used in combination with methotrexate (MTX), Cimzia™ may allow more RA patients to achieve remission more quickly than treatment with currently available TNF-blockers [129]. In February 2008, UCB announced that the Food and Drug Administration (FDA) had agreed to review the Cimzia file for the treatment of this progressive autoimmune disorder. This conjugate was approved in Switzerland in September 2007 and in US in April 2008 as treatment for Crohn's disease [130] and is also being filed in the US and the European Union (EU) for rheumatoid arthritis.

Focusing on HIV treatment, PEGylation of cyanovirin-N (CV-N), a potent inhibitor of HIV and many other viruses, has been recently described by Nektar Therapeutics [131]. An optimal site-specific PEGylation had to be developed to maintain CV-N antiviral activity [132]. A number of papers have been also published regarding the development of zidovudine (AZT)–polymer conjugates and prodrugs. The aim behind them has been to produce agents with equal or higher antiretroviral potency than AZT alone and with an improved toxicological profile. However, only a few studies have investigated the potential of developing controlled release conjugates. Polymer such as kappa-carrageenan [133], sulfated alkyl laminaripentaoside [134] and α, β-poly (*N*-hydroxyethyl-DL-aspartamide) (PHEA) [135] were used to covalently conjugate AZT through a biodegradable linker. Synergism with polymer sulfate groups when present was also observed.

In particular, a very promising research approach looks at polymer conjugates as tools to promote tissue repair. Preliminary studies using a polyvalent dendrimer conjugate of glucosamine or glucosamine-6-sulfate to prevent scar tissue formation have been described [136]. PAMAM G3 5–glucosamine derivatives were able to inhibit the synthesis of pro-inflammatory chemokines and cytokines and to block fibroblast growth factor-2-mediated endothelial cell proliferation and angiogenesis. More importantly, combination therapy with two of these conjugates prevented scar tissue formation after glaucoma filtration surgery [136]. By applying the previously described PUMPT concept [36], the first bioresponsive polymer conjugates designed to promote tissue regeneration have been reported: a dextrin-recombinant human epidermal growth factor (rhEGF) conjugate that has showed very promising data *in vitro* as a wound healer [137].

HPMA copolymer–prostaglandin E_1 (PGE_1) conjugates have been designed for the treatment of osteoporosis, and greater plasma stability was clearly observed for the conjugated PGE_1 [138].

Moving to ischaemic diseases, another study generated from our group in collaboration with Dr Pérez-Payá suggested the use of PGA-based conjugates for the delivery of a first-in-class family of apoptosis inhibitors. Peptoid 1, an apoptotic protease-activating factor 1 (Apaf-1) inhibitor, was conjugated to PGA [139]. We were able to demonstrate that this conjugate enhances the antiapoptotic activity of the peptoid in both cellular models of apoptosis and neonatal rat cardiomyocytes under hypoxic conditions [140]. We are currently evaluating these first antiapoptotic nanoconjugates in different experimental models of myocardial infarction. If successful, we hope to open new therapeutic strategies aimed to improve the healing of damaged cardiac function.

2.3 Polymer-based combination therapy

It is becoming increasingly clear that the use of drug combination therapy will improve long-term therapeutic prognosis due to the molecular complexity of diseases. However, the use of polymer conjugates has been traditionally limited to the delivery of a single therapeutic agent alone or in combination with free low Mw drugs, radiotherapy or chemotherapy [141]. In this context, of special interest are

all FDA-approved combinations with conjugates for the treatment of chronic hepatitis C (HCV), namely: i) Pegasys plus Copegus® (ribavirin) [142] approved in Japan after obtaining a significantly higher response rate in Phase III trials when compared to the group treated with Pegasys alone (59 versus 24%, respectively); and ii) PEG-Intron plus Rebetol, the first and only PEGylated interferon combination therapy approved in the EU for retreating both HCV relapsers and nonresponders. This combination therapy was previously approved in the EU for treating HCV in naïve adult patients, including those with clinically stable HIV co-infection (see Schering-Plough press release, November 2007) [143]. A randomised Phase II trial is currently ongoing to study the safety, antiviral activity, and pharmacokinetics of the non-nucleoside polymerase inhibitor HCV-796 [144] administered in combination with Peg-Intron plus Rebetol versus Peg-Intron plus Rebetol in HCV-infected subjects [145]. Another interesting combination along this line is found for the treatment of acromegaly by combining the conjugate Pegvisomant with somatostatin analogues [146]. In cancer research, tumour-targeted generation of H_2O_2 could be achieved safely by using the combination of PEGylated-D-amino acid oxidase (PEG-DAO) with its substrate D-proline. The timing of D-amino acid infusion ensures the tumour-selective toxicity of H_2O_2 [147]. The use of thalidomide in a triple combination regime in prior nonresponders to Peg-IFN/ribavirin has also been described [148].

Similar examples are found with polymer–anticancer drug conjugates. For example, a Phase III clinical trial compared PGA–paclitaxel + carboplatin versus paclitaxel + carboplatin [141]. The same conjugate has also been tested in combination with radiotherapy in a Phase I trial for oesophageal and gastric cancer and four complete clinical responses (33%) were observed in this study [149].

In addition, combinations of two conjugates, each of them carrying a single therapeutic agent, have been suggested. The combination of PEG-DAO and PEG-ZnPP has been demonstrated to be a powerful oxidative therapy. Almost a complete tumour suppression was achieved when mice were pretreated with PEG-ZnPP followed by PEG-DAO/D-proline system [114]. Kopeček and colleagues found increased activity *in vivo* when HPMA copolymer Dox was administered together with the photo-activable HPMA copolymer-meso-chlorin e6 monoethylene diamine disodium salt (Mce6) conjugate [150]. Finally, another example of this class of combination was reported by Minko's group. They tested free CPT, CPT–PEG, CPT–PEG–BH3 domain peptide or CPT–PEG–lutenising hormone release hormone (LHRH) conjugates and the mixture of CPT–PEG–BH3 and CPT–PEG–LHRH conjugates in human ovarian carcinoma cells. An increased proapoptotic activity was demonstrated when the combination CPT–PEG–BH3 plus CPT–PEG–LHRH was used [151].

A much more recent and promising polymer-based combination approach uses polymer multivalency property to allow the conjugation of different drugs within the same carrier. With this novel approach, two different drugs are simultaneously delivered to the tumour tissue by a single polymeric platform, thus maximising their effects. At present, only a few groups have suggested the use of a polymeric carrier for the delivery of drug combinations. An HPMA copolymer carrying the aromatase inhibitor aminoglutethimide (AGM) and the chemotherapeutic agent Dox was the first conjugate that combined endocrine therapy and chemotherapy agents on a single polymeric chain [152]. This combination conjugate showed markedly enhanced cytotoxicity against human breast cancer cells *in vitro* compared to the HPMA copolymer-Dox (PK1, FCE28068) whose activity has been proven clinically [20] and to any other combination of single agents (namely, AGM + Dox or HPMA copolymer-AGM conjugate + HPMA copolymer-Dox conjugate or HPMA copolymer-Dox conjugate + AGM) [153]. A subsequent study investigating the mechanism of action of this combination polymer at a cellular level highlighted that the conjugate conformation in solution and the drug release rates are key parameters for the activity [153]. Further studies are needed to investigate these effects and define both the therapeutic potential of HPMA copolymer-AGM-Dox conjugate and the exact mechanism of action. However, it is clear that this approach offers a new opportunity for the treatment of chemoresistant metastatic breast cancer.

Research originated in Veronese's group showed the design of a series of new polymeric conjugates bearing on the same PEG chain epirubicine (EPI) and a nitric oxide (NO) releasing molecule [154]. As both compounds suffer the same cellular fate, NO is able to increase the antitumoural activity of EPI while it provides protection against apoptosis induced by oxidative stress, both in endothelial cells and in cardiomyocytes, preventing anthracycline-related cardiotoxicity [154]. By using a branched PEG polymer, Minko *et al.* were able to further enhance the therapeutic value of the previously described pro-apoptotic combination CPT–PEG–LHRH plus CPT–PEG–BH3 by means of the combination of the three active moieties within the same polymer. The multicomponent hyperbranched PEG polymer bearing an equimolecular amount of CPT, BH3 and LHRH moieties was almost 100 times more cytotoxic and displayed enhanced antitumour activity when compared with other synthetic analogues [155].

It is worth mentioning in this section the work carried out by Cytimmune [156] on the development of a novel multifunctional platform based on PEGylated colloidal gold (cAu). Its first patented nanoconjugate (CYT-6091) actively targets and sequesters recombinant human TNF in solid tumours, while avoiding uptake by healthy organs and clearance by the reticuloendothelial system (RES). The drug is comprised of TNF and thiolated PEG, each of which is individually and covalently bound to the surface of 26 nm cAu backbone. With CYT-6091 (Aurimune, in Phase I trials) [157], not only the anticancer therapeutic action

of TNF was observed, but also TNF served as a tumour-targeting ligand, bringing 10 times more TNF to the tumour. Building on this discovery, Cytimmune expanded its armoury to combination systems, such as CYT-21001 (Auritol) comprising both TNF and an analogue of paclitaxel (Taxol®), currently in preclinical development.

Finally, it is important to mention an alternative and very interesting two-step combination approach designed for extracellular delivery: the concept of polymer-directed enzyme prodrug therapy (PDEPT) and polymer-enzyme liposome therapy (PELT) [158]. This strategy will allow the thwarting of possible existing problems related to intracellular delivery, such as activating enzyme downregulation or endocytosis failure. The rational for PDEPT is to combine a polymer–drug and a polymer–enzyme conjugate. For example, HPMA copolymer-cathepsin B combined with HPMA copolymer-Dox and HPMA copolymer-β-lactamase combined with HPMA copolymer-cephalosporin-Dox conjugate have shown *in vivo* proof of concept [158]. PELT is a similar approach for liposomes and protein conjugates. Along this line, it has recently been shown that dextrin–phospholipase A_2 (PLA_2) [159] conjugates display significant promise as anticancer agents in their own right, and such conjugates benefit from the PUMPT concept [36].

2.4 The importance of an exhaustive physicochemical characterisation of polymer conjugates

Innovative polymer synthesis and bioconjugation techniques are leading to many new materials (see paragraph 2.1, above) but while they provide exciting opportunities, they also present challenges for careful biological and physicochemical characterisation. This is one of the main limitations in the development process of these complex macromolecules, and for that reason there is a need to know 'what do we have in the bottle' in order to secure transfer to the clinics following the regulatory authority standards. In this context, new methods applicable to polymer conjugates that are able to surmount these obstacles are urgently required. Moreover, a better understanding of the physicochemical properties and structural characteristics of polymer conjugates will also play an essential role in the design and tailoring of the therapeutic applications of these macromolecular compounds. This information is critical for the synthetic chemists to understand and hence solve the most important challenges (i.e., solubility, half-life, safety/tolerability, immunogenicity, antigenicity and toxicity) posed by polymer therapeutics. Nowadays, a wide range of biophysical techniques allowing the study of many different properties of polymer conjugates is available and waiting to be exploited. Also, a number of existing and newly emerging analytical techniques are accessible for studying drug/biopharmaceutical release processes, both *in vitro* and *in vivo*. A summary of the most useful techniques and an example of their potential in this field are described here.

In the characterisation of polymer conjugates, any information concerning the M_w and polydispersity index ($\overline{M}_w/\overline{M}_n$) is especially relevant. This data can be easily obtained from size exclusion chromatography (SEC) [160] and analytical ultracentrifugation (AUC) [161] studies. Physicochemical properties such as average size (or, alternatively, the volume median diameter; VMD) [162] can be derived from laser diffraction analysis [163], the volume median aerodynamic diameter (VMAD; important for microparticles) [164] from time-of-flight (TOF) [165] mass-spectrometry and diffusion coefficients (D) [166] or hydrodynamic radii (R_h) [167] from Taylor dispersion analysis (TDA) [168]. Properties like purity and homogeneity of water soluble polymer conjugates can be assessed by gel electrophoresis [169], a technique that is widely used in biochemistry for the routine analysis of proteins [170] and nucleic acids [171].

Matrix assisted laser desorption ionisation time-of-flight (MALDI TOF) [172] is a very powerful mass spectrometry method for endgroup analysis in polymers and for the determination of conjugate Mw distribution [173]. Each peak in the polymer spectrum represents a different degree of polymerisation, and the peak-to-peak distance reflects the mass of the repeating unit. Knowledge of the ionisation process is a prerequisite for spectrum interpretation. For example, ionisation and detection mechanisms influence the detectable mass range and the shape of the distribution curve, therefore preparation conditions, matrices, salt addition, type of analyser and acceleration voltage are of great importance. Furthermore, the influence of laser power on MALDI TOF spectra should not be underestimated [174] as excessively high power shifts the maximum distribution to lower mass values, most probably due to increased fragmentation processes. The silver lining is that higher-Mw polymers are detectable but with the drawback of a decrease in mass resolution.

Scattering techniques that have been used for characterising physicochemical properties of conjugates include small angle X-ray scattering (SAXS) [175], small angle neutron scattering (SANS) [176] and laser light scattering (LLS) [177]. The SAXS technique is often used for the characterisation of polymers, and can inform on their average radius of gyration (R_g) in solution [178]. The intensity of the scattering as a function of the angle also provides information about the arrangement of polymer segments, hence on the segment density distribution within the molecule. SAXS is particularly useful to assess low resolution structures and interactions [175]. Although SANS also gives access to R_g, this technique is able to reveal more accurate information than SAXS about the internal structure of the polymer conjugate [179]. LLS is yet another scattering technique often used in combination with SEC to determine the hydrodynamic radius (R_h) [180]. Low angle LLS can provide information about the M_w of polymer conjugates [181], and dynamic LLS is mainly used for the detection of aggregates [182]. In these scattering techniques, solution conditions such as temperature, pH and salt concentration can be adjusted to closely mimic a physiological environment. Conversely, the solutions may also be modified

to mimic extreme non-physiological conditions, for example in studies of conjugate degradation. In particular, SANS is a technique that has been recently applied to study pH-dependant conformational changes of endosomolytic poly(amidoamine)s [183,184] and to investigate the behaviour of different HPMA polymer conjugates in solution. Two studies were carried out: the first study allowed the definition of the R_g of HPMA copolymer conjugates containing both Dox and the aromatase inhibitor AGM as a combination therapy [151]. The other study was undertaken applying SANS to establish structure–activity relationships between two conjugates already in the clinic, PK1 (FCE28068) and PK2 (FCE28069), which, despite their similar chemical characteristics, displayed a significantly different maximum tolerated dose (MTD) in patients (320 mg/m^2 versus 160 mg/m^2). Therefore, the aim of this study was to use SANS to explore their solution behaviour. A larger R_g (by ~ 2.5 nm) for FCE28069 compared to FCE28068 provided a possible explanation for differences in the MTD. The particular conformation adopted by FCE28069 could lead to greater exposure of the conjugated drug to the biological environment [179]. This was the first detailed SANS analysis of structurally related polymer–drug conjugates and showed that this technique could be a valuable tool for determining structure–activity relationships of this important new class of therapeutics.

Additionally, some physical properties can be derived from the application of rheology [185], differential scanning calorimetry (DSC) [186] and dielectric spectroscopy (DS) [187]. These techniques are especially valuable in the characterisation of the polymeric carrier. Thus, rheology, and particularly dilute solution viscosimetry studies, can be used as analytical probes for the morphological structure of polymers [188]. The DSC technique is generally used to detect the glass transition temperature (T_g), a property that depends on the Mw, entanglement and chain-end composition of the polymers [189]. Moreover, molecular dynamic processes in polymers (α-, β-, γ- and δ-relaxation) can be obtained from the application of DS [190].

However, by far the main interest resides in the structural characterisation, not necessarily at a high-resolution level, of the polymer conjugates. Very often, biophysical characterisation of therapeutic polymers is restricted to both far- and near-UV circular dichroism (CD), a technique that can provide a fast and convenient method for observing the secondary and tertiary (i.e., conformation) structures of proteins [191] and their polymeric conjugates [192]. In fact, a quite common approach to assess whether the conjugation affects the structural features of a peptide/protein is to compare the CD spectra before and after chemical modification. Having said that, it is important to point out that even if the secondary and tertiary structure of the native and polymeric protein species are similar, this does not ensure the conjugate will be fully functional, since the polymer can sterically interfere with the intermolecular interactions necessary for activity. Although less frequently used, Fourier-transformed infrared spectroscopy (FTIR) [193] techniques have been also employed in the conformational analysis of polymer conjugates [194]. FTIR can provide, while at a low structural resolution, valuable information about conformational details of these macromolecules. Particularly interesting is the mid-infrared region (4000 – 400 cm^{-1}) of the FTIR spectrum, where relevant information can be derived from the analysis of the fundamental vibrations and associated rotational–vibrational structure [195].

More detailed structural information can be obtained from multidimensional nuclear magnetic resonance (NMR) [196] spectroscopy. NMR is certainly the most widely used technique for the structural characterisation of macromolecules [197]. Its versatility allows NMR to cover a diverse range of applications when working with polymer conjugates. Thus NMR can be used for routine chemical analysis to confirm the nature of the conjugates, but there are also other special NMR techniques that can be applied to prove their size, morphology and structure [198]. Routine NMR analyses are especially useful during the step-by-step synthesis of polymers, even up to high generations, because they provide information about the chemical transformations undergone by the endgroups. In addition to that, NMR is an extremely useful tool for assessing the covalent attachment and location of drug/peptide/protein to the corresponding polymers [199]. In general, ^{1}H and ^{13}C NMR are the most used applications. In some cases, selective irradiation of particular resonances or the use of more complex pulse sequences (e.g., COSY, TOCSY, NOESY or EXSY) are necessary for a better characterisation of the molecule. Although examples are scarce, the potential of NMR for the characterisation of polymer–drug conjugates through these specific techniques has been clearly exemplified for several conjugates including PK1, PK2 [200] and the antiapoptotic conjugate PGA–peptoid [139]. By using NMR T_1 and T_2 relaxation time studies with three different generations of azido-terminated PEG-dendritic block copolymers, a radial decrease of density, leading to more mobile protons at the outermost periphery, and an increasingly higher compactness of the core with generation were determined [201]. In dendrimeric architectures, the branching degree can be also measured by ^{13}C NMR INADEQUATE [202]. The presence of heteroatoms (^{15}N, ^{31}P, ^{19}F, ^{195}Pt) in some polymer conjugates can be used as a source of extra information for their NMR characterisation, besides the standard ^{1}H and ^{13}C NMR experiments [203,204]. In order to investigate the size, morphology or dynamics of polymer conjugates, special NMR techniques in solution can be used. Particularly exciting is the application of diffusion-ordered spectroscopy (DOSY) NMR [205] to the characterisation of these systems. DOSY NMR is based on a pulse-field gradient spin-echo NMR experiment, in which components experience diffusion, the effect being proportional to the Mw/size of the component. The signal of each component decays with different diffusion rates either when varying the

gradient strength applied to the sample or when increasing the diffusion time. The signal decay allows the construction of a bilinear NMR data set of the component mixture. By calculating the diffusion for each component, it is possible to obtain a two-dimensional NMR spectrum: one dimension represents the conventional chemical shift, while the other yields the range of diffusion coefficients. Combining the information provided by the diffusion experiments with the Stokes–Einstein equation, an estimation of R_h can also be obtained. In a way, DOSY NMR represents an analytical technique to define the composition of polymer conjugates. Using this approach, it is possible to distinguish covalent binding from simple complexation, opening the avenue to characterise complex polymer conjugate mixtures. The first example of using NMR DOSY applied to polymer–protein conjugates (in particular dextrin–trypsin conjugates [36]) has been published very recently. The measurement and comparison of the molecular size-dependent diffusion coefficients of the free polymer/protein and the conjugates allowed the characterisation of the different species present in solution (**Figure 4**). Structural information can also be gathered from X-ray diffraction studies [206]. In principle, this technique should allow a precise determination of the chemical composition, size and shape of the polymer conjugates [207], but very frequently, they are amorphous and lack long-range order in the condensed phase.

Other techniques that have been used to get a deeper insight into the structural characteristics of polymer conjugates are atomic force microscopy (AFM) [208] and transmission electron microscopy (TEM) [209]. AFM is a modern tool for imaging nanostructures and for measuring forces between molecules. With the imaging capability of AFM, it is possible to investigate the physical properties of patterned structures. Often, the information obtained from these studies is supplemented with FTIR and TEM measurements, allowing the generation of structural models for polymer conjugates [195].

With regard to the delivery process, electron paramagnetic resonance (EPR) or electron spin resonance (ESR) [210] represents a powerful approach that could help to understand the behaviour and fate of therapeutic polymers in living systems. EPR (or ESR) is a spectroscopic tool that can be used to monitor drug release processes *in vitro* and *in vivo*. The strengths of ESR lie, in addition to the non-invasiveness of the method, in the application to heterogeneous and solid samples and in the specificity of the technique. Furthermore, spatial dissolution can be achieved by means of ESR imaging. The usefulness of ESR in the field of drug/biopharmaceutical delivery includes the measurement of microviscosity and micropolarity, the direct detection of drug release mechanisms *in vitro* and *in vivo*, the monitoring of microacidity in biodegradable polymers and the characterisation of colloidal carriers [210-212].

It should be mentioned that ESR can give information that is not assessable by NMR due to short relaxation times. For example, solid nanomaterials can be characterised *in vitro* by NMR but not *in vivo*, as the frequently necessary requirement of spinning the samples very rapidly might cause artifacts due to high shear stress. The limitations of ESR include the necessity of adding paramagnetic material and the restrictions in size for *in vivo* measurements. The majority of drug/biopharmaceutical delivery samples are diamagnetic and ESR silent, thus requiring the addition of paramagnetic molecules or groups, for example nitroxides, to allow detection. A large variety of nitroxides (also known as spin probes) with different physicochemical properties is commercially available. Larger molecules (e.g., proteins and other polymers) can be spin labelled by the covalent linking of a chemically activated nitroxide to a suitable group of a drug or polymer (e.g., amino groups). ESR is not only used to characterise delivery systems prior to their use; it is also a method to shed more light on the release mechanisms themselves. The method can give unique and important information due to the fact that different release mechanisms lead to different changes of spectral intensity and shape.

Also available are double resonance techniques [213] which combine the advantages of ESR and MRI [214]. The latter technique can also acquire non-invasively unique information about drug/biopharmaceutical delivery processes such as real-time information on pharmacokinetics, biodistribution and the delivery efficiency of the conjugates. Particularly relevant are those applications based on non-invasive visualisation of *in vivo* delivery of polymer conjugates.

Only a few groups are focusing their research on demonstrating the importance of an exhaustive biophysical characterisation to achieve clinical development of these complex hybrid macromolecules. However, we believe that in the near future the information gained from the above-described techniques will allow us to assist, guide and control the design and synthesis of optimised second generation polymer conjugates with improved therapeutic value.

3. Expert opinion

The enormous potential of the field of polymer conjugates in clinics offers a wide range of research approaches within the scientific community. The high versatility of these macromolecular drugs allows the design and development of effective treatments for a variety of human pathologies. From early macromolecular prodrugs of established anticancer agents, their applications have expanded dramatically in recent years. Delivery of new anticancer agents using novel molecular targets such as specific signalling pathways or cellular apoptosis [111,120], combination therapy [151,153], novel polymer architectures (including dendrimers) [46,67] and the treatment of diseases other than cancer, in particular ischaemia and tissue repair [137,139] are the most exciting and promising areas, with many challenges still to address and future opportunities for developing this technology further.

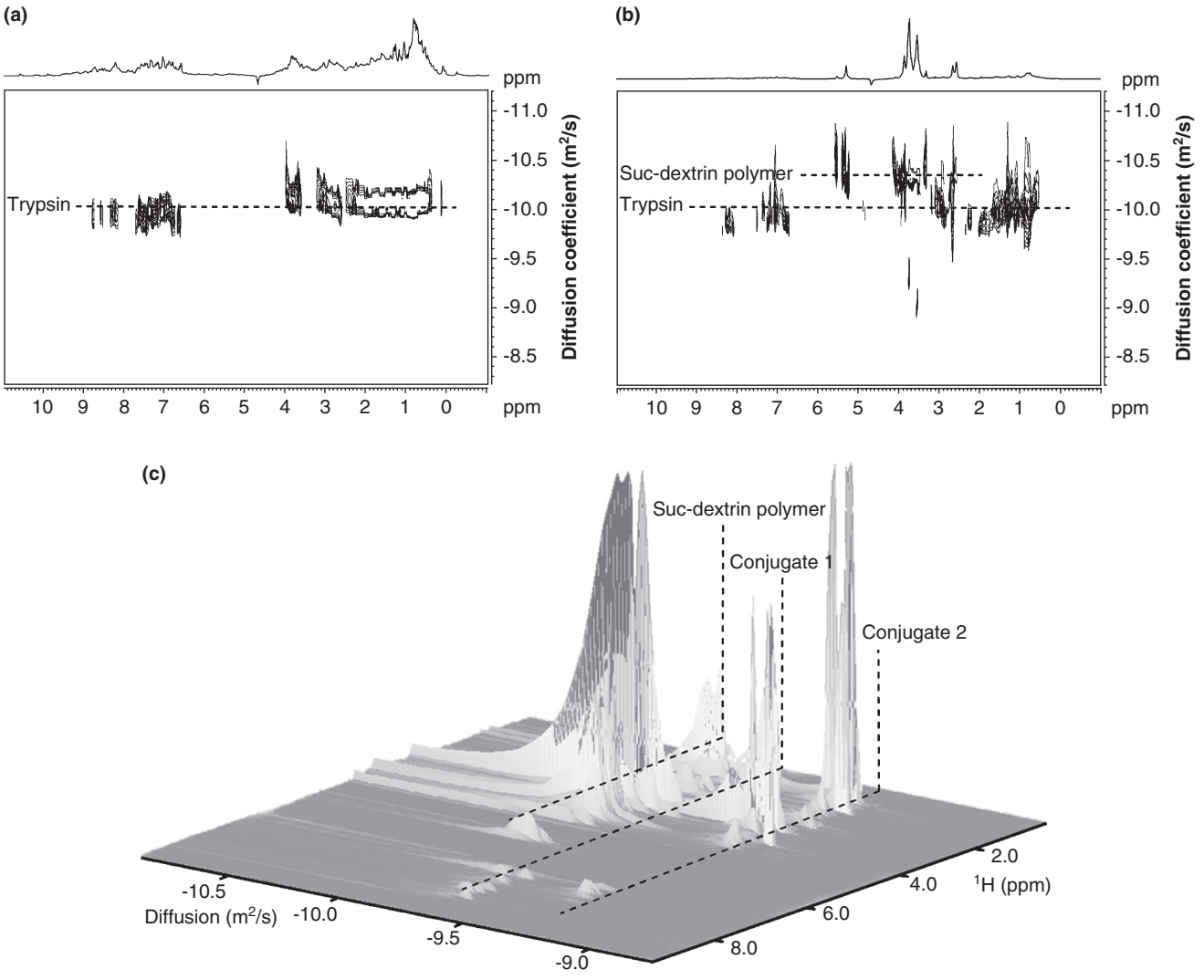

Figure 4. DOSY spectra of: (a) trypsin, (b) a mixture of succinoylated dextrin and trypsin and (c) dextrin–trypsin conjugate (3D view). The different molecular weights for trypsin (24 kDa), succinoylated dextrin (47 kDa) and the conjugates allow the separation of signals in the DOSY spectrum for each component. Typical experimental parameters: diffusion time 50 ms, gradient time 5 ms, gradient strength 5 – 100%. The horizontal axis projection shows the ^{1}H spectrum of the corresponding sample.

With the recent progresses made in synthetic and polymer chemistry, it is now possible to move forward towards novel, well-defined 3D macromolecular architectures, with an increased number of endgroup functionality, where biodegradability can be tailor-made for an enhancement of the EPR-mediated targeting, which can in turn be intelligently used for the synthesis of well-defined, uniform polymer conjugates. However, sufficient safety and toxicological data as well as a defined biophysical characterisation should be gained for complex macromolecules before they transfer to the clinics. More exhaustive efforts are needed to establish pharmacokinetics and the cellular fate of these highly branched architectures in order to achieve regulatory approval.

Achieving successful active targeting by using specific moieties (peptides, antibodies, etc) on the polymer main chain has been more difficult to demonstrate, mainly due to the lack of awareness for the appropriate overexpressed disease cell receptor to target, but, for example, with the novel VEGF targeted systems [100], this limitation is readily to be surpassed. Furthermore, in the context of cancer and other diseases, polymer-based systems are now being explored as components of combination therapy as well as, for the first time, carriers of combination therapy, which represents an important opportunity to enhance tumour response rates. Combination therapy may seem costlier than monotherapies in the short-term, but means significant savings in terms of lower treatment-failure rate, lower case–fatality ratios, a slower development of resistance and consequently less money needed for development.

Realisation of the full therapeutic potential of these novel nanomedicines is only possible through multidisciplinary

research involving collaboration on such a variety of different preclinical and clinical skills, including knowledge of molecular targets, polymer chemistry, analytical techniques, animal models, medicine and the awareness of industrial development and regulatory approval processes [215]. The feasibility of industrial scale manufacture and characterisation of these complex macromolecular entities have been already demonstrated and over the last years many new nanotechnology companies or specific industrial lines of research focusing on the development of these types of nanopharmaceuticals have emerged.

This is a rapidly emerging field with exponentially growing opportunities to apply new polymer chemistry and our improved biological understanding of disease progression to achieve medical treatments with highly enhanced therapeutic value.

Declaration of interest

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Affiliation

María J Vicent[†1] PhD, Lucile Dieudonné[1] PhD, Rodrigo J Carbajo[2] PhD & Antonio Pineda-Lucena[2] PhD

[†]Author for correspondence

[1]Centro de Investigación Príncipe Felipe, Polymer Therapeutics Laboratory, Avenue Autopista del Saler 16, E-46012 Valencia, Spain

Tel: +34 963 289680; Fax: +34 963 3289701; E-mail: mjvicent@cipf.es

[2]Centro de Investigación Príncipe Felipe, Structural Biology Lab, Avenue Autopista del Saler 16, E-46012 Valencia, Spain