# *Polymer-drug conjugates*

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**Abstract**

Polymer-drug conjugates are nanosized drug delivery systems, which comprise of several drug molecules covalently attached to a polymeric carrier. This chapter provides an overview of this technology in the context of drug delivery. Particular emphasis is given to different approaches and techniques used to synthesise and characterise polymer-drug conjugates. In the final part of the chapter current applications of this technology are also discussed.

## 1. Materials Chemistry

### 1.1. Definition of polymer-drug conjugates and general background to this technology

Polymer-drug conjugates are a drug delivery technology where a polymer carrier is used to improve the performance of a drug (e.g. improve drug selectivity towards the target site) as single agent or in combination regimen. Unlike other systems, such as liposomes or nanoparticles where the drug is physically entrapped within the carrier, in a polymer-drug conjugate the drug is *covalently* attached to the polymer via a linker, which is generally biodegradable (Figure 1).

The concept of conjugation of a drug to a polymeric carrier was first introduced in the 70's by Helmut Ringsdorf as a strategy to enhance the selectivity, cellular uptake and solubility of a drug [Ringsdorf, 1975]. Since then, extensive work has been carried out which has resulted in polymer-drug conjugates reaching clinical evaluation [Vasey et al., 1999; Seymour et al., 2009, also reviewed in Duncan, 2006 and Canal et al., 2011 and Ekladious et al., 2017].

The rationale for conjugating a drug to a polymer stems from the fact that the biological behaviour of the drug can be significantly altered by increasing its molecular weight (MW). Covalent conjugation to a polymer results in:

- *Prolonged circulation time of the drug*. The polymer can protect the conjugated drug from premature inactivation during its delivery to the site of action. In addition, the macromolecular size of the conjugate prevents the early elimination of the drug through renal filtration, thus typically increasing half-life and decreasing administration frequency.

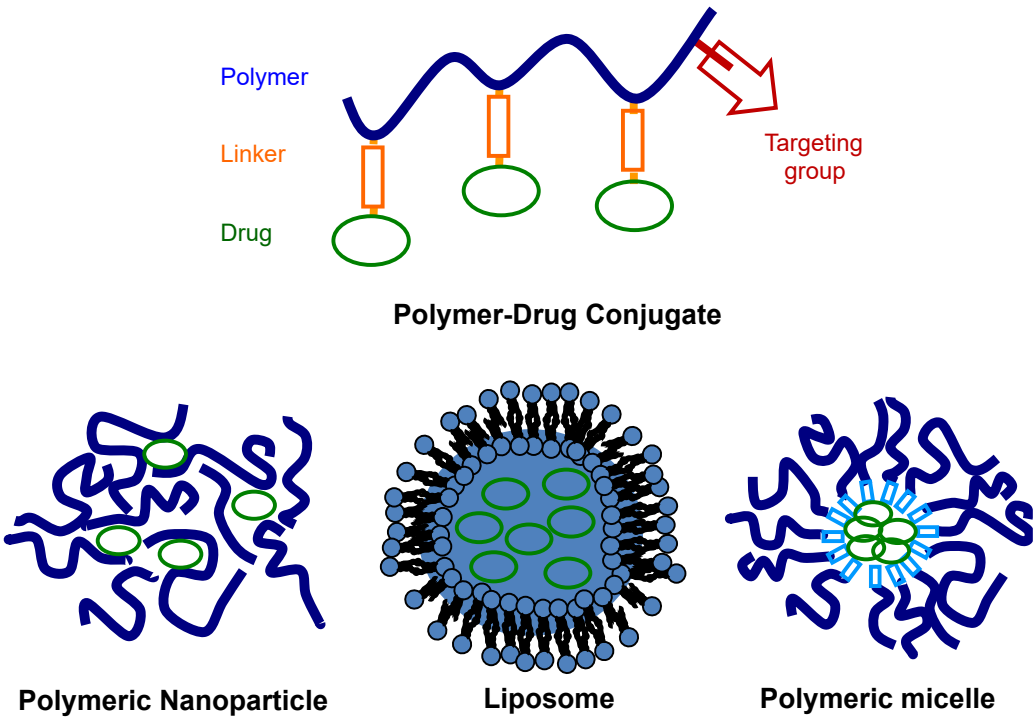



**Figure 1**. Schematic representation of a polymer-drug conjugate and other drug delivery technologies. In polymer-drug conjugates the drug is *covalently* attached to the polymer via a biodegradable linker.

- *Restricted body distribution*. An intravenously administered drug is generally able to diffuse throughout the body, with no selectivity towards the target tissue. On the other hand, the macromolecular size of a conjugate prevents extravasation of the drugs in areas where the vascular endothelium is continuous. In fact, conjugation to a polymer restricts drug access to those tissues where the vasculature presents fenestrations and gaps of appropriate size (>20 nm). The tumour tissues, for instance, is characterised by a defective vasculature which is permeable to the conjugate. This and other features (discussed in Section 4.1) make the tumour tissue a particularly good target for polymer-drug conjugates.
- *Improved drug solubility in water.* The conjugation of a hydrophobic drug to a hydrophilic carrier improves drug solubility in water [see, for example, Piao et al., 2019].
- *Selective drug release*. Polymer conjugation can also alter the cellular pharmacokinetics of the drug. Due to their large size and hydrophilicity, polymer-drug conjugates cannot enter the cell via passive diffusion across the cellular membrane as many small drugs do. Instead, they are taken up typically by endocytosis, and routed towards the lysosomes (lysosomotropic delivery). The lysosomal compartment has two peculiar characteristics: an acidic pH (4-5) and a high concentration of proteolytic enzymes (e.g. cathepsin B). This unique environment can be turned into a useful trigger for drug release from the conjugate.

Indeed, polymer-drug conjugates have been designed with biodegradable linkers either sensitive to acidic pH or selectively degraded by proteolytic enzymes. This allows the drug to be released exclusively intracellularly and to effectively reach its biological target (such as the cytosol or the nucleus depending on the drug). In addition to pH and enzymes, other environmental triggers have been explored to promote drug release, for example the reductive environment (exploited for redox-sensitive linkers) [Giraldo et al, 2021].

- *Bypassing some mechanisms of drug resistance.* Because of the polymer 'masking' the drug and also changing its cellular pharmacokinetics, polymer conjugation has been reported to bypass certain mechanisms of drug resistance [Ke et al, 2014]

The biological behaviour of a polymer-drug conjugate is strongly affected by each of its constituents and by the overall physico-chemical properties of the system (e.g. water solubility, conformation in solution). The next section will look at each component of a polymer-drug conjugate.

### 1.2. Composition of a polymer-drug conjugate.

As previously described, a polymer-drug conjugate is constituted by three (to four) components: a drug, a polymeric carrier, a linker and, optionally, a targeting group. In this section we will look at each component, individually.

*Drug(s)*

Polymer-drug conjugates that have been traditionally designed for application in cancer therapy (see Section 4), but more recently they have also been applied to diseases other than cancer (see Section 4.2). Examples of drugs that have been incorporated in polymer-drug conjugates include doxorubicin, paclitaxel and camptothecin (as anticancer agents) docosahexaenoic acid (DHA) and fasudil (for other applications).

With the help of an example (doxorubicin) we are now going to look at what characteristics make a drug suitable for conjugation to a polymeric system (also summarised in Figure 2).

Doxorubicin is a potent anticancer agent, used for the treatment of metastatic breast cancer [Paridaens et al., 2000]. The main issue with the therapeutic use of doxorubicin is its cardiotoxicity, which is a result of the lack of selectivity of this drug [Jensen 2006]. Such lack of selectivity makes it an ideal candidate for polymer conjugation, as the polymer will promote drug accumulation in the tumour tissue. The chemical structure of doxorubicin also makes it a suitable candidate for conjugation. In particular, this molecule contains a primary amino group and a hydroxyl group.

Both these functional groups can be exploited for linking the drug onto a polymer (e.g. via formation of an amide bond or an ester bond for the amino group or hydroxyl group, respectively). In addition, doxorubicin absorbs UV-Vis light and is inherently fluorescent. These are two favourable properties as the presence of the drug can be detected using appropriate instruments (see section 3). If a single polymeric chain carries more than one drug type (polymer-drug conjugates combination therapy, see Section 4.3), both drugs need to have suitable characteristics for drug conjugation. Moreover, in this case, care should also be taken when selecting relative drug ratio.

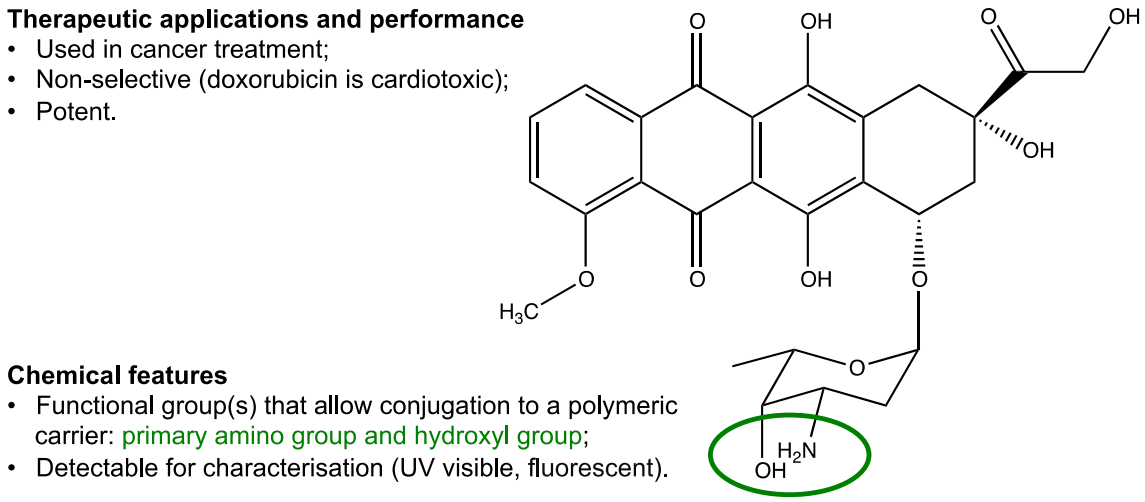



**Figure 2**. Chemical structure of doxorubicin. Key characteristics that make this drug a suitable candidate for use in the context of polymer-drug conjugates are annotated.

*Polymer*

Many polymeric carriers have been suggested for use within the context of polymer-drug conjugates. Several of them have been tested clinically: N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers, polyethylene glycol (PEG), poly-L-glutamic acid (PGA), Benzene polycarboxylic acid (PCA), Cyclodextrin- PEG, Core-cross-linked PEG, Poly (2-ethyl-2-oxazoline) (POZ), and oxidised dextran [Javia A et al, 2022].

With the help of an example (PGA) we are now going to look at what characteristics make a polymer suitable for use in a polymer-drug conjugate system (also summarised in Figure 3).

PGA is a polymer constituted by units of glutamic acid linked together via amide bonds. PGA is water-soluble and this is an important characteristic for intravenous drug delivery as biological fluids, such as the blood, are essentially water-based systems.

PGA is biodegradable, which means that in biological systems it is degraded to smaller fragments. This is an advantage for a polymeric carrier as it ensures that the carrier is eliminated

from the body after drug release. Non-biodegradable polymers, such as PEG, can also be used but their size has to be such that allows excretion via the kidneys (typically lower than 40,000 Da).

Each monomer of PGA has a pendant side chain, which terminates with a carboxyl group. These carboxyl groups can be exploited for conjugation to a drug, for instance, to an amino group to produce an amide bond or to a hydroxyl group to produce an ester bond. All polymers suggested for use as a carrier, need to have a functional group that allows conjugation to a drug. If a polymer has multiple conjugation sites (such as PGA), each polymer chain will be able to carry several drug molecules. In the case of PGA, each polymeric chain could carry one drug molecule per monomer (i.e. 200 drug molecules per chain, for 30,000 Da PGA). This property of a polymeric carrier (i.e. the ability to carry drug molecules) is called 'loading capacity'. In general, a higher loading capacity is to be preferred, as less of the carrier needs to be used in order to administer a therapeutic dose of the drug. Therefore, multivalent polymers like PGA are often a better choice for polymer-drug conjugates than monovalent polymers (e.g. mono-methoxy PEG).

Finally, PGA is non-toxic and non-immunogenic (i.e. does not stimulate an immune response). These are key properties, as toxicity from a drug delivery carrier would not be considered acceptable.

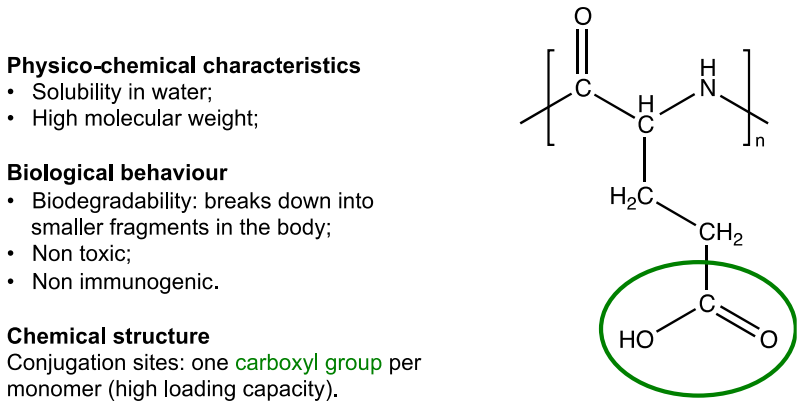



**Figure 3**. Chemical structure of PGA. Key characteristics that make it a good polymer within the context of polymer-drug conjugates are highlighted.

*Linker*

In a polymer-drug conjugate the linker is the part that connects the drug to the polymeric carrier. Many types of linkers have been suggested, which includes peptidyl linkers, redox responsive and pH-labile linkers [Duncan, 2006; Melnyk et al. 2020].

With the help of an example (the peptidyl linker Gly-Phe-Leu-Gly) we are now going to look at what characteristics make a linker suitable for use in a polymer-drug conjugate system (also summarised in Figure 4).

Early studies on a series of HPMA copolymer-doxorubicin conjugates compared different peptidyl linkers, including Gly-Phe-Leu-Gly. The conjugate containing this linker was then progressed for evaluation into clinical trials [Vasey et al. 1999; Seymour et al. 2009]. Stability tests carried out in plasma showed that the linker was stable (i.e. did not release the drug) in these conditions. This was a positive finding, as a good linker needs to be stable in the blood to avoid premature drug release. Other studies carried out on a mixture of lysosomal enzymes (mimicking the environment found by the conjugate in the lysosomes) showed drug release in these experimental settings. Biodegradability at the target site is generally an important characteristic as drug release is necessary to drug activity. Indeed, the vast majority of polymer-drug conjugates' linkers are designed to be bioresponsive and biodegradable. However, a small number of polymer conjugates have been designed with the aim of the active working while still polymer bound, and as such, in these limited cases, the linker is non-biodegradable (see section 4.4).

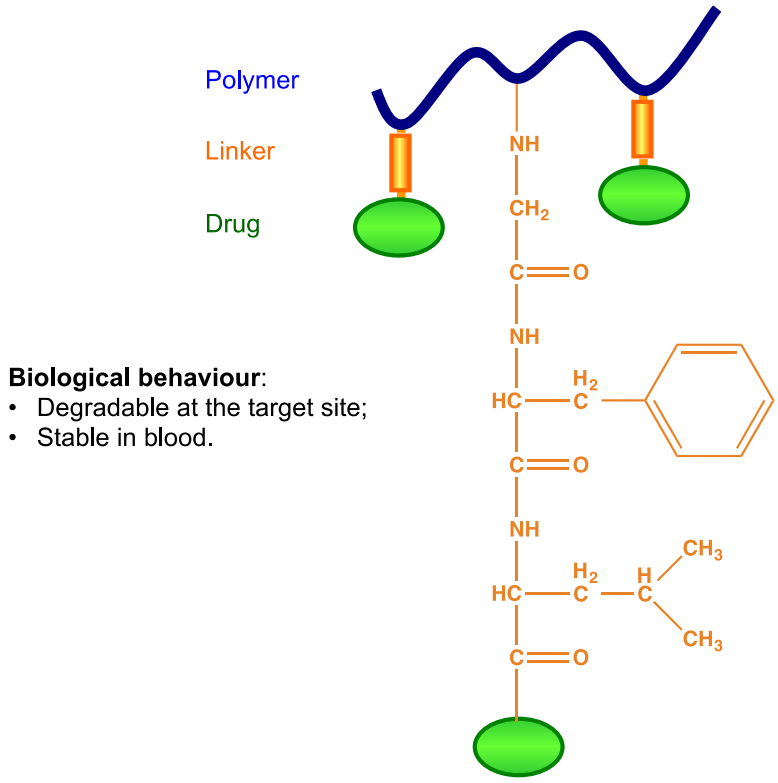



**Figure 4**. Chemical structure of the peptidyl linker Gly-Phe-Leu-Gly. Key characteristics that make this linker a suitable candidate for use in the context of polymer-drug conjugates are highlighted.

*Targeting group*

The targeting group is the fourth (but optional) structural component of a polymer-drug conjugate. Its role is to actively direct the conjugate towards the desired tissue. With the help of an example (galactosamine) we are now going to look at what characteristics make a targeting group suitable for use in a polymer-drug conjugate (also summarised in Figure 5).

Galactosamine is an amino sugar able to bind selectively to the hepatocyte galactose receptor, a liver-specific receptor [Ashwell and Harford 1982]. The ability to bind to tissue-specific markers (which are generally proteins associated to a certain tissue and not expressed elsewhere in the body) is an essential requirement for a targeting group. In addition, galactosamine presents a primary amino group, which can be used for linking this molecule to the polymer (as for the conjugated drug). These features make galactosamine a suitable targeting group to be exploited in the context of drug delivery. Galactosamine was covalently bound to an HPMA copolymer-doxorubicin conjugate designed for the treatment of liver cancer [Seymour et al., 1991; Pimm et al., 1993; Julyan et al., 1999]. This conjugate was investigated clinically (Phase I and Phase II) and clinical imaging confirmed preferential accumulation in the liver [Seymour et al., 2002].

In this chapter we have already discussed the ability of a conjugate to target the tumour tissue with a passive mechanism based on its physico-chemical properties (i.e. its molecular weight, see section 1.1). For this reason, the targeting group is an optional component in a polymer-drug conjugate designed for anti-cancer therapy. It should be noted that targeting groups can be added to actively target specific cell populations expressing a certain antigen, but also to target intracellular organelles [Han et al. 2016].

**Biological behaviour**:
Tissue-specific (binds selectively to the hepatocyte galactose receptor, liver-specific);

**Chemical features**
Functional group that allows conjugation to a polymeric carrier (primary amino group).

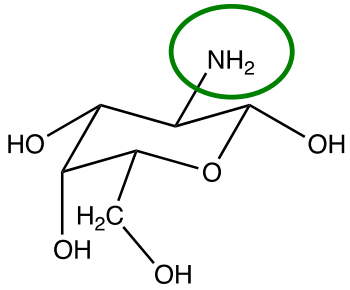



**Figure 5**. Chemical structure of galactosamine. Key characteristics that make it a good targeting group within the context of polymer-drug conjugates are highlighted.

## 2. Polymer-drug conjugates synthesis

### 2.1. Synthetic strategies

There are two main synthetic approaches that can be used to produce a polymer-drug conjugate: polymer-analogous reaction and copolymerisation of appropriate monomers (Figure 6).

- *Polymer-analogous reaction (or post-polymerisation modifications)*. This synthetic strategy starts with a polymeric precursor in which the sites of conjugation are chemically modified to increase their reactivity (e.g. a carboxylic acid converted into an ester containing an appropriate leaving group) and to produce a reaction with the drug (Figure 6a).

- *Copolymerization of appropriate monomers*. This approach involves two steps: (i) coupling of the drug to the monomer; (ii) polymerization of such drug-monomer derivatives with the monomers, to yield the conjugate (Figure 6b).

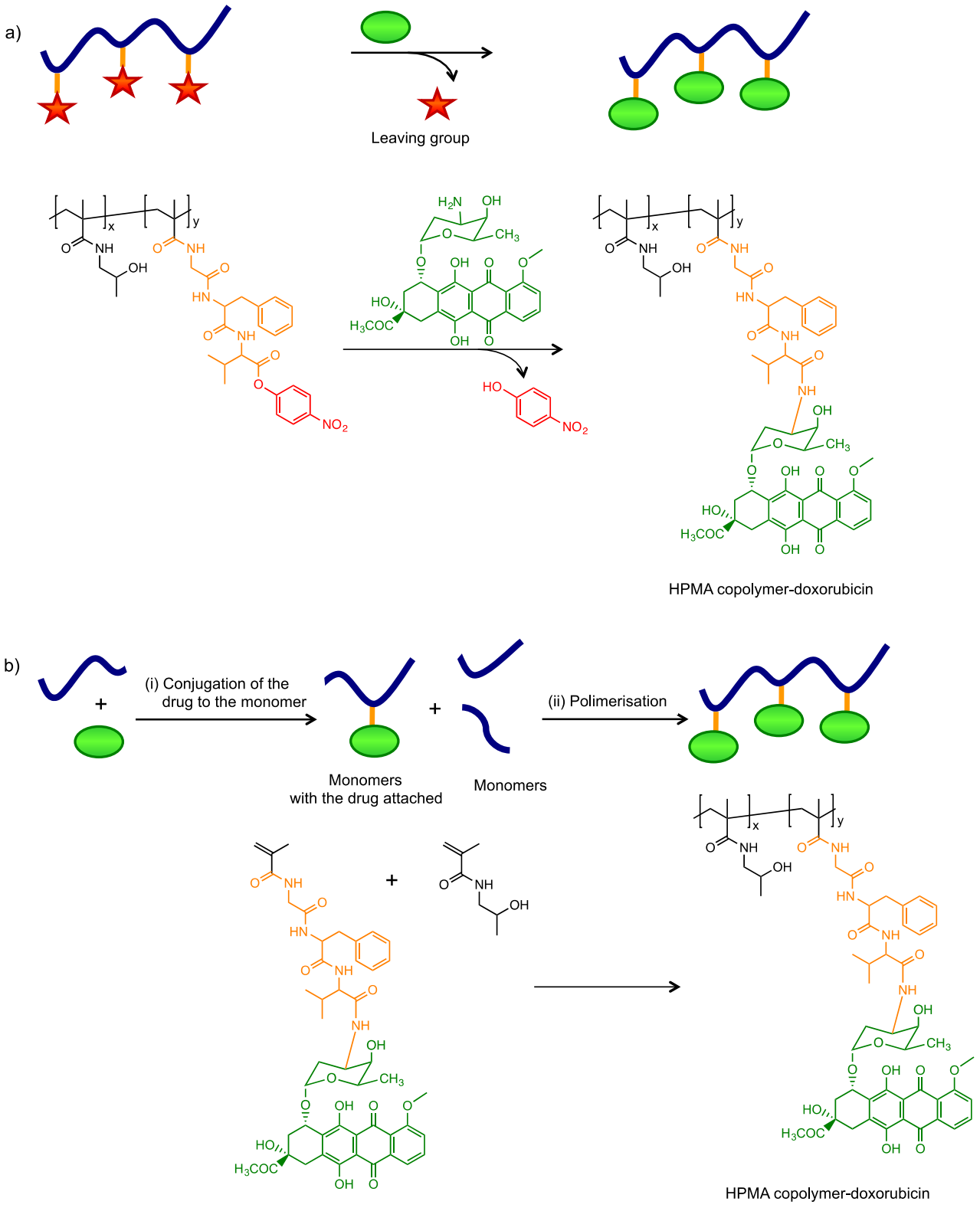



**Figure 6**. General approaches for polymer-drug conjugation: a) polymer-analogous reaction; b) conjugation of the drug to a monomer and copolymerization of the polymeric precursors.

The copolymerisation strategy presents the advantage that, at least theoretically, drug loading can be increased or decreased by adjusting the ratio between the monomers containing the

drug and the other monomers. However, the polymer-analogous reaction is by far the most commonly used approach, as it normally requires milder reaction conditions compared to those used in polymerisation reactions. Hence with this strategy there is generally less of a chance to degrade the drug during the conjugation process.

Conjugation of a drug to a polymeric carrier can also be defined in relation to the position of the conjugation site within the polymer chain. This is largely driven by the chemical structure of the polymeric carrier used. Two types of conjugation can be identified: terminal conjugation and pendant chain conjugation (Figure 7).

- *Terminal conjugation*: this is typical of polymers with functional groups present only at the end termini (e.g. unmodified PEG). For unmodified linear polymers, this type of conjugation results in a conjugate with a low carrying capacity, as a maximum of two drug molecules can be carried by each polymeric chain. More recently, however, polymers with regular branching at the termini have been developed (dendronised systems) to accommodate additional drug molecules in each polymer chain (see Figure 7a);
- *Pendant chain conjugation*: this refers to the conjugation to polymers with suitable functional groups present along the chain (see Figure 7b). This is the case with polymers such as the HPMA copolymer, where side chains are attached to the main polymeric backbone, or PGA that naturally contains a side carboxyl group per monomer and allows a theoretical maximum loading of one drug molecule per monomer. Pendant chain conjugation has the advantage that the loading capacity is typically higher than that achievable with terminal conjugation.

With regards to the actual conjugation reaction, the choice is primarily driven by the functional groups present on the drug and on the polymeric carrier, but standard synthetic reactions can be employed. For instance, an amino group present in the drug can be linked to a carboxylic acid group on the polymer after appropriate activation of the carboxylic acid group via standard coupling reactions (e.g. N,N'-dicyclohexylcarbodiimide (DCC) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling).

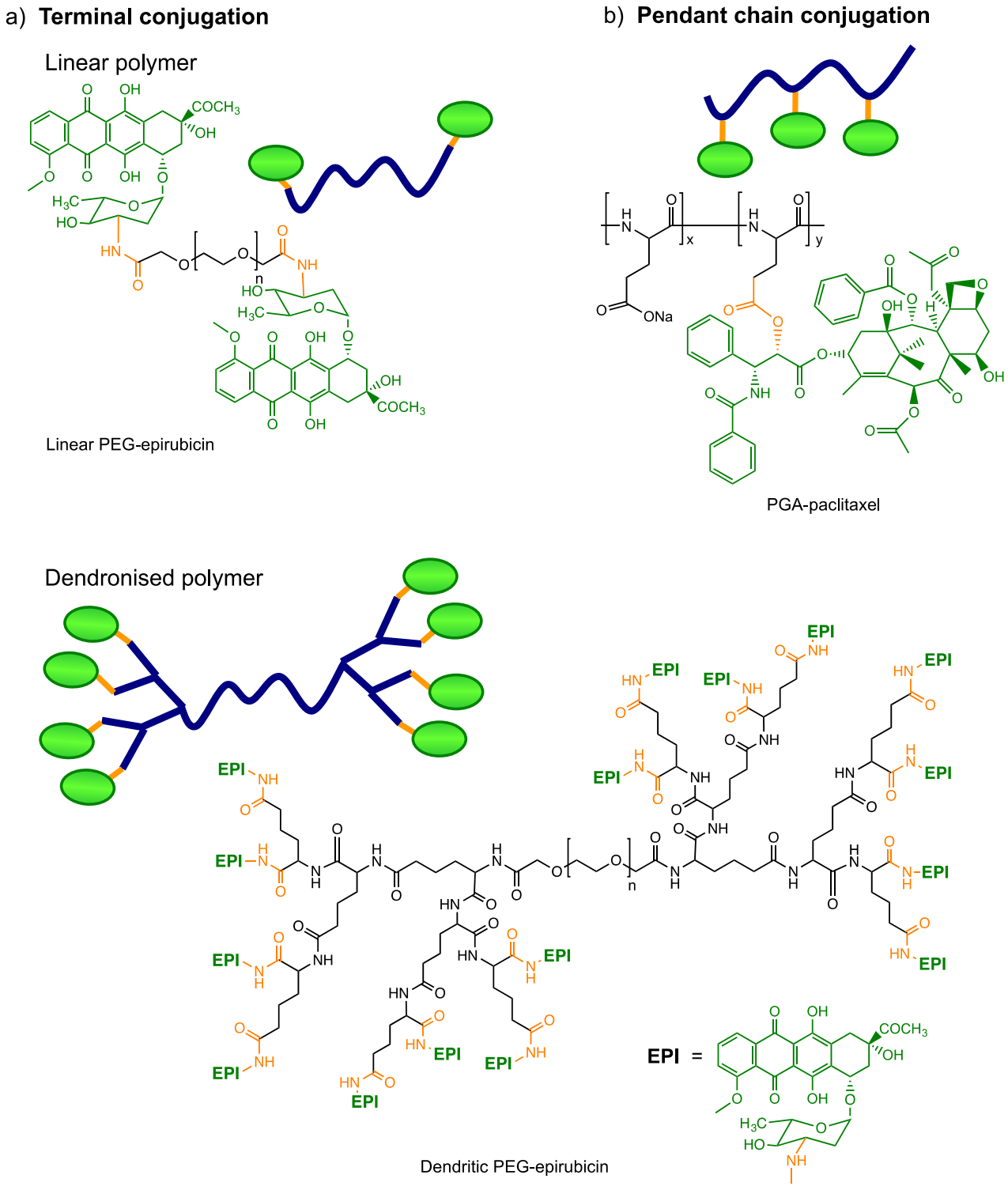



**Figure 7**. Types of conjugation according to the position of the conjugation site within the polymer chain. The drug can be attached to the polymer through (a) its terminal groups; (b) its side chains.

## 2.2. Purification

Once the conjugation reaction has been carried out, the conjugate needs to be purified from reaction by-products and from residual reagents, as these might present unwanted biological activity

(e.g. they might be toxic). Standard purification techniques routinely used for purifying small molecules can be applied to the purification of the conjugates (e.g. precipitation, ultrafiltration). In addition, as the conjugate and the reagents differ significantly in size, size exclusion chromatography is typically used in this context, or, at an industrial level, Tangential Flow Fractionation (TFF).

One type of impurity that needs particular attention is residual, unreacted free drug, as it will have a different biological activity and a different pharmacokinetic profile compared to the conjugated drug. This aspect is further discussed in Section 3.1.

## 3. Polymer-drug conjugates characterisation

After preparation, polymer-drug conjugates must be carefully characterized. Thorough characterisation prior to any testing is essential to correctly interpret biological behaviours observed for the conjugate. In addition, as conjugates progress into clinical trials, it is important to guarantee the reproducibility of their preparation and the overall quality of the final products. Because the drug is covalently attached to the polymeric carrier, polymer-drug conjugates cannot be treated simply as a new formulation of the drug (even if they carry a well-known and clinically established drug). In fact, these technologies are considered by the regulatory authorities as 'new chemical entities' (i.e. as completely new drugs) and, as such, they need to undergo extensive testing to ensure their safety and efficacy. Both European (i.e. EMA) and American (i.e. FDA) regulatory authorities have set the standards for the quality of medicines in order to guarantee their efficacy and safety (Dordevic et al. 2021).

### 3.1. Characterisation parameters

*Proof of conjugation.* The initial characterisation of a polymer-drug conjugate includes finding evidence that a covalent bond between the drug and the polymer has been formed (i.e. that the drug is attached to the polymer and not physically 'entrapped' within the polymer). Several techniques have been used to achieve this purpose and, in many cases, different techniques have to be used in conjunction one with the other (see Section 3.2).

*Total drug content*. When the formation of the conjugate is confirmed, it is essential to understand *how much* drug is bound to the polymer. The total drug content can be expressed in two different ways: (i) as the percentage in weight of the whole conjugate; (ii) as the percentage of functional groups contained in the polymer that have been conjugated to the drug. (i) and (ii) are strictly related but convey different information. In particular, (i) gives an immediate measure of total drug content. For example, a PGA-paclitaxel conjugate was synthesised that contained approximately 37% w/w of paclitaxel (e.g. 100 mg of conjugate contain 37 mg of paclitaxel) [Li et

al.]. Conversely, (ii) is often used to indicate how efficient a conjugation reaction was (e.g. 80% of the functional groups available on a polymer reacted with the drug or, in other words, the drug loading was 80% of the maximum theoretical loading). Different techniques can be used to assess the drug content of a conjugate, as described in more detail in Section 3.2.

*Impurities and residual free drug content.* We have already discussed the importance of an accurate purification of the conjugate from excess reagents and reaction by-products (Section 2.2). One type of impurity that needs particular attention is residual, unreacted free drug. As the free drug will have biological activity and a different pharmacokinetic profile compared to the conjugated drug, removing it is particularly key. To this end, size-exclusion chromatography is the technique most frequently used, as the free and conjugated drug differ significantly in size. The content of residual free drug is typically expressed as a percentage of the total drug content and, in general, the purification of the conjugate should aim to achieve levels below 1%.

*Size.* Determining the size of the conjugate is very important, as this is arguably the key parameter that drives the distribution of the conjugate in the body and determines how quickly the conjugate (or the unloaded polymer) will be excreted from the body. However, it should be highlighted that most polymeric systems are polydispersed (i.e. polymers are constituted by a range of polymeric chains varying in length, depending on the number of monomers per chain). This means that most polymer-drug conjugates are polydispersed systems too. Therefore, the molecular weight stated is an average of the molecular weight of the various chain and should always be accompanied by the polydispersity value for that system.

It should be noted that depending on the conjugate type, administration route, and indication, additional parameters are needed to gain full characterisation. These may include surface properties (e.g., charge, hydrophobicity, chemical reactivity), structural attributes (e.g., solution conformation), assessment of endotoxin levels and drug release kinetics.

**3.2. Techniques employed to characterise conjugates.**

To achieve the characterisation described above, a variety of analytical techniques can be used (for physico-chemical characterisation of polymer-drug conjugates, see Dordevic et al, 2021; Melnyk et al, 2020; Gao et al, 2017; Niño-Pariente et al,2016).

*Thin layer chromatography (TLC)*

TLC is a very simple technique that is routinely used during conjugation reactions to obtain an indication of how the reaction is progressing. A sample from the reaction mixture is spotted onto a TLC plate and run with an appropriate mobile phase. Typically, the TLC plate is then viewed under a UV lamp (if, the drug absorbs at UV). Alternatively, specific stains are used to detect functional groups present in the drug and make the TLC spot visible (e.g. nynhydrin stain, which

turns the spot pink in presence of compounds containing primary amino groups). The mobile phase can be modified to ensure that the free drug and the conjugated drug have different retention times (Figure 8). As the reaction progresses, the drug starts to get detected at the retention factor of the polymer (see Figure 8).

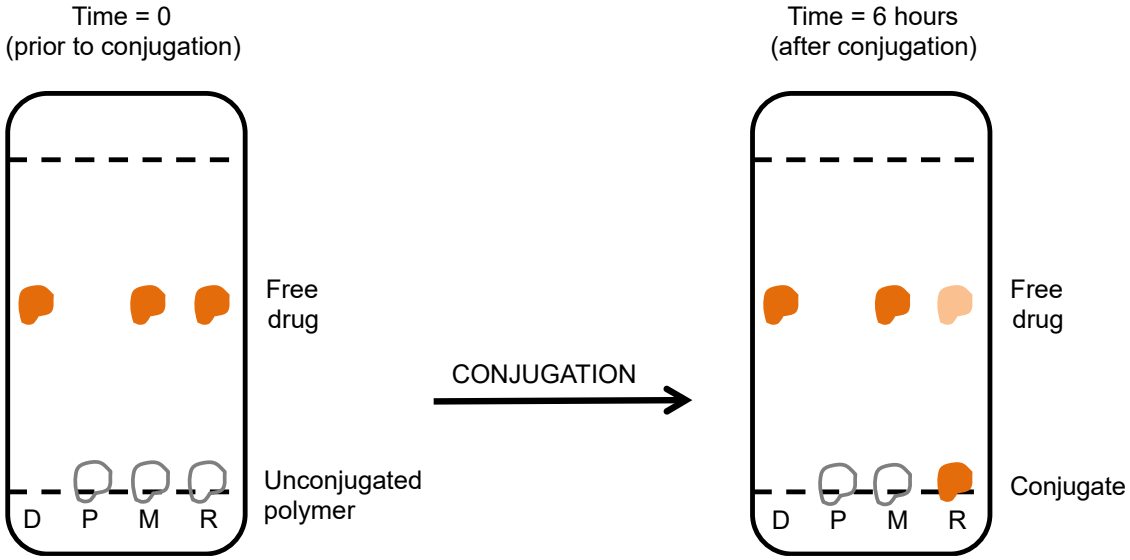



**Figure 8**. Schematic representation of how to monitor a conjugation reaction by TLC. In this example, the TLC plate is placed under an UV lamp. The drug (in D) can be detected by UV, while the polymer (in P) does not absorb UV light, therefore is not visible; lane (R) contains the reaction mixture (the change in colour of the spot on the baseline refers to the drug being conjugated to the polymer; as a consequence the spot of the free drug tends to disappear); lane (M) contains the mixture of the polymer and the drug in absence of activators of the reaction.

*UV-Vis spectroscopy*

Many drugs have a chemical structure that absorbs light in the UV-Vis region of the electromagnetic spectrum. As such, these drugs can be detected and quantified in solution by a UV-Vis spectrophotometer. On the other hand, the most common polymers used in conjugation (PEG, PGA, HPMA copolymers) do not absorb UV-Vis light. These represent very useful features when characterising a polymer-drug conjugate, as the UV-Vis spectrum of the polymer is expected to change dramatically after conjugation. However, for a correct interpretation of the conjugate's spectrum it is important to remember the following considerations:

- UV-Vis is a useful technique to identify and quantify the *total* drug content of the conjugate, but it cannot normally discriminate between *bound* and *free* drug as, typically, both absorb at the same wavelength; therefore UV-Vis spectroscopy alone is not a valid proof of conjugation and it becomes meaningful only when supported by other techniques;

- Polymer conjugation often alters the extinction coefficient of a drug (i.e. the free drug and the conjugated drug might have different extinction coefficients [Vicent et al., 2005]), which can lead to errors. In particular, if the drug content in a conjugate was estimated using a calibration curve of the free drug, this could result in an over- or under-estimation of the total drug content.

*Nuclear Magnetic Resonance (NMR) spectroscopy*

NMR spectroscopy is a technique commonly used to prove that conjugation has occurred. The presence of signals belonging to the drug in the spectrum of the conjugate is not an indication of conjugation *per se*, as they might belong to the unbound drug. However, a shift in the resonance of the protons and carbons adjacent to the conjugation site is an indication that their electronic environment has changed and this might be due to conjugation. In addition, the sharp peaks typical of the spectrum of small molecules become broad after conjugation to macromolecules, as a consequence of the slower molecular tumbling of the polymeric chains. A shift in resonance combined with the broadening of the peaks represent an indication of successful conjugation of the drug.

In some cases the overlapping of crucial peaks does not allow a complete interpretation of the spectrum. In these cases, 2D NMR spectroscopic analyses represent a valid and powerful alternative to the usual spectrum. For instance, the nuclear overhauser effect spectroscopy (NOESY) and the total correlation spectroscopy (TOCSY) have been used to prove the formation of the amide bond between HPMA copolymer and doxorubicin [Pinciroli et al., 1997].

NMR spectroscopy can also be used to determine drug content within the conjugate. This can be achieved by relative integration of the signals from the drug and those from the polymeric backbone. This allows the calculation of a molecular ratio between drug molecules and polymer monomers, which can be converted into drug content expressed as percentage in weight.

*Infrared spectroscopy (IR)*

IR is a technique very useful to identify functional groups. Polymer conjugation often results in the formation of new bonds (e.g. ester or amide bonds) between the drug and the polymer, which can be detected in an IR spectrum. A comparison of the spectrum of the conjugate with the spectra of the unbound drug and polymer can reveal the formation of the new bond. However, this might result in a challenging task when the newly formed bond is a functional group already present in the polymer. For example, PGA is constituted by monomers of glutamic acid joined together via amide bonds. If the drug is joined to the polymer via an amide bond, it might be difficult to discriminate which component of the IR signal is due to the amide bonds of the polymer and which is due to the

conjugated drug. Conversely, formation of an amide bond in a PEG-based conjugate would be easier to detect as unmodified PEG does not contain this type of bond.

High performance liquid chromatography (HPLC)

HPLC is a technique widely employed in the synthesis and characterisation of polymer-drug conjugates. The use of HPLC within the context of polymer-drug conjugates relies on the fact that the free drug and the conjugated drug are likely to interact differently with the stationary phase and the mobile phase, and therefore are likely to elute from an HPLC column at different times. Preparatory HPLC can be used to isolate and purify the conjugate from the free drug. Columns for preparative HPLC are relatively large (internal diameter: 5-20 mm) and allow loading of large amounts of a compound (up to milligrams of compounds). In a similar manner, analytical HPLC can be used to check that the conjugate is free from unconjugated drug, or to quantify the total drug content and the content of residual free drug. Columns for analytical HPLC are smaller (internal diameter typically 4.6 mm) and allow loading of small amounts of compound (up to few micrograms).

In addition, HPLC is often employed during stability studies, to verify that the conjugate is stable, and no drug is released from the conjugate during storage. Finally, HPLC can also be used to measure drug release under different physiological conditions. For example, samples from the blood or the urine of a patient can be analysed by HPLC to detect the stability of the conjugate in biological fluids and the rate of drug release.

*Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF)*

MALDI-TOF analysis is a particular type of mass spectrometry that has been employed to characterise polymer-drug conjugates [Wu and Odom 1998]. In particular, this technique can detect the shift in the mass of the conjugate due to conjugation with a drug. A mass increase in the polymer, which matches the added mass due to the presence of the drug, constitutes strong proof of conjugation. Therefore, MALDI-TOF is useful in the context of confirming covalent linkage and also in determining the molecular weight and the polydispersity of the conjugate.

*Gel permeation chromatography (GPC)*

GPC is a type of size exclusion chromatography in which compounds are separated according to their size (or, more precisely, to the volume they occupy in solution i.e. hydrodynamic volume): bigger molecules elute from the column faster (have shorter retention times) than smaller molecules. After conjugation to a drug, the hydrodynamic volume of a polymer may change significantly depending on the type of drug used (Figure 9). The conjugate could adsorb molecules

of water within its structure and adopt an extended conformation, which would result in a shorter retention time compared to the free polymer. On the other hand, the conjugate may adopt a more compact conformation and display a smaller hydrodynamic volume than that of the corresponding free polymer, which would result in a prolonged retention time.

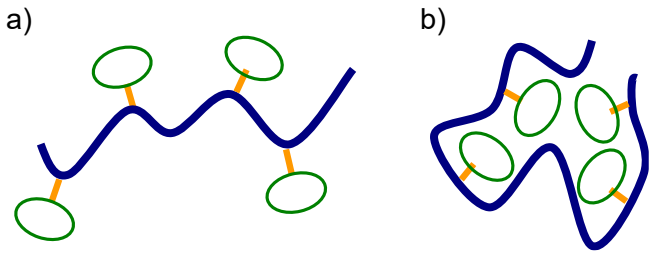



**Figure 9**. Schematic representation of the different conformations that a conjugate may assume after conjugation to a drug: extended (a) or compact (b).

*Dynamic light scattering (DLS) and Nanoparticle tracking analysis (NTA)*

These are light scattering techniques based on the Brownian motion of particles (smaller particles move faster than larger ones in a solution), therefore when particles scatter light this effect contains information on the diffusion speed and thus on the size distribution. DLS can be considered the most common size determination method, provides accurate size determination of homogeneous (quasi-monodisperse) samples, however, more heterogeneous (polydisperse) samples generally require a multimodal approach that combines DLS or NTA batch analysis in a pre-screening step with high-resolution techniques such as Asymmetric Filed Flow Fractionation (FFF; AF4), or transmission electron microscopy (TEM) in the following step.

*Asymmetric Filed Flow Fractionation (FFF; AF4)*

AF4 represents a promising method due to the gentle and sample-tailored separation inside the empty channel that maintains weak complexes such as nanoconjugates and proteins. Affinity studies of nanomedicines with long circulation times to proteins by AF4 have been reported [Dordevic, 2021]. These examples, demonstrate that coupling AF4 to MALS or DLS represents a powerful and robust analytical technique for characterizing nanoconjugates that may overcome limitations associated with batch mode light scattering techniques

*Small angle neutron scattering (SANS) and Small Angle X-Ray scattering (SAXS)*

SANS is a technique that has been used to investigate the behaviour of the conjugates in solution [Paul et al., 2007; Fernandez et al 2021]. This type of analysis can provide key information about the conformation of the polymer (e.g. radius of gyration). A comparison between the

scattering behaviour of different conjugates can also provide information about structure-activity relationships. SAXS is used for the determination of the microscale or nanoscale structure of particle systems in terms of such parameters as averaged particle sizes, shapes, distribution, and surface-to-volume ratio [Zagorodko et al 2020 and 2021].

## 4. Application of polymer-drug conjugates

This section describes the different therapeutic areas to which the concept of polymer-drug conjugates has been applied (Section 4.1. and 4.2). A recent development in the field (the use of polymer-drug conjugates to deliver drug combinations) is also reported (Section 4.3). The final section (4.4.) provides an update on the current status of polymer-drug conjugates.

### 4.1. Treatment of cancer

Traditionally, polymer-drug conjugates have been for application to cancer treatment. The tumour tissue represents an ideal target for polymer-drug conjugates. Firstly, it is characterised by a leaky vasculature, which allows extravasation of the conjugate from the capillaries feeding the tumour tissue to the tumour tissue itself. Conversely, the large size of the conjugate prevents extravasation of this system into normal tissues. In addition, the tumour tissue is also characterised by a poor lymphatic system, which is unable to effectively clear the extracellular fluid. As a consequence, the conjugate is retained in the tumour tissue for longer. The combination of these two factors (leaky vasculature and poor drainage) has been called “enhanced permeability and retention (EPR) effect” and is a phenomenon typical of solid tumours [Matsumura and Maeda, 1986; reviewed in Maeda et al., 2013 and in Maeda 2021].

The EPR effect results in the passive accumulation of macromolecules into the tumour tissue and makes polymer conjugation an ideal strategy to selectively target this disease. Unconjugated drugs are unable to take advantage of the EPR effect for the following reasons: (i) they are low MW molecules, able to diffuse throughout the body with no selectivity (i.e. size exclusion) for a specific tissue; (ii) their clearance from the biological fluids does not rely on the lymphatic system, therefore they do not accumulate in the tumour tissue.

As the EPR effect promotes an improved selectivity towards the tumour tissue, the toxicity of polymer-drug conjugates compared to their parent free drug is generally lower. For example, the maximum tolerated dose (a parameter indicating the toxicity of a therapeutic agent) of free doxorubicin is 60-80 mg/m$^2$ [Muzykantov and Torchilin, 2003], while that of an HPMA copolymer-doxorubicin is 320 mg/m$^2$ [Vasey et al., 1999]

Within the context of cancer treatment, many established anticancer agents (e.g. paclitaxel, and doxorubicin) have been proposed in the form of polymer-drug conjugates. More recently, the concept of polymer-drug conjugates has also been applied to experimental anticancer agents that had shown promising anticancer activity but that had failed to progress into the market due to toxicity or other unfavourable properties. For instance, the antiangiogenic agent TNP-470 has been conjugated to an HPMA copolymer with a view to maintaining the anticancer activity while reducing its toxicity (TNP-470 is neurotoxic when administered as a free drug) [Saatchi Fainaro et al., 2004; Saatchi-Fainaro et al., 2005]

**4.2. Polymer-drug conjugates in diseases other than cancer**

Traditionally polymer-drug conjugates have been developed for the treatment of cancer, but over the last fifteen years or so the versatility of this approach has been exploited also in other therapy areas, underpinned by different rationales [Natfji et al., 2017]. Representative examples of different therapeutic areas to which the concept of polymer-drug conjugates has been applied are described here.

*Inflammation.* The application of polymer-drug conjugates for the delivery of drugs to inflamed tissues is not surprising because these tissues present similar characteristics of hyperpermeability to those observed in tumours. As such, conjugates have been proposed with the idea of having maximised extravasation in the inflamed tissues. As expected, these conjugates contained anti-inflammatory drugs like corticosteroids (e.g. PEG-dexamethasone Liu et al., 2010; linear cyclodextrin-α-methylprednisolone Hwang et al., 2008).

*Antimicrobial (antibacterial, antifungal, antiviral, antiprotozoal).* Polymer-drug conjugates have been explored for the delivery of agents targeting different types of microrganisms including viruses, bacteria, fungi and protozoa. Different rationales have been suggested for applying polymer-drug conjugates to this therapeutic area, including obtaining sustained release and increasing half-life. For some micro-organisms it has also been suggested that the conjugate can promote a better targeting (e.g. polymer drug conjugate of 8-aminoquinoline for Leishamniasis, Roy et al., 2012; Nan et al., 2004; Nan et al., 2001).

*Cardiovascular diseases.* Covalent conjugation of drugs to a polymeric carrier has also been explored preclinically for various cardiovascular diseases (e.g. ischemia and hypertension). Depending on the study, the use of a polymeric carrier was proposed for different reasons, like increasing solubility of a drug molecule, endothelial wall protection from reperfusion damage [Tejedor S et al., 2021] or for a more effective intracellular delivery [Vicent and Perez-Paya 2006].; or for the simultaneous co-delivery of different therapeutics agents (reviewed in Natfji et al. 2017).

*Central nervous system.* The application of polymer-drug conjugates in relation to the CNS is particularly interesting because attempts have been made to use this technology both to target the CNS as well as to prevent penetration in the CNS. In the case of targeting of the CNS, LRP1 receptor is one of the most widely used to implement active moieties such as Angiopep-2 (a 19-aminoacid peptide) in the design of polymer conjugate to bypass the blood-brain barrier (BBB) [Duro-Castano et al., 2021]. Conversely, in other cases, conjugation to a polymer has been suggested to prevent a drug from crossing the BBB, thus restricting a drug's action to the peripheral tissues (see, e.g. Heath et al 2016; Natfji et al 2020). One such cases is particularly important as it relates to PEG-naloxone, the only polymer-drug conjugate on the market (further discussed in Section 4.4.)

### 4.3. Combination therapy

Many diseases (e.g. cancer and HIV) are treated with cocktails of drugs rather than with a single therapeutic agent. The overall aim of this type of therapeutic regimen (combination therapy) is to maximise efficacy while decreasing toxicity. For instance, in the case of anticancer treatment, different chemotherapeutic agents are administered jointly over repetitive treatment cycles. The development of drug delivery platforms able to carry multiple types of drugs could allow the simultaneous administration of the drugs in combination, resulting in improved patient compliance and simplified therapies. To this purpose, in recent years, polymer-drug conjugates carrying two types of drugs have been developed and tested pre-clinically. Examples of this approach are reported in Table 1.

**Table 1. Examples of polymer-drug conjugates containing combination therapy**

| Conjugate | Type of combination | Reference |
|---|---|---|
| Xyloglucan-doxorubicin-Mytomicin C | Chemotherapy; Antibiotic | Xu et al., 2021 |
| PGA-Selumetinib(SLM)-Dabrafenib (mDBF) | MEK1/2 inhibitor (selumetinib, SLM) and a modified BRAF inhibitor (Dabrafenib) | Pisarevsky et al, 2020 |
| PGA-doxorubicin-aminoglutethimide | Chemotherapy; Endocrine therapy. | Arroyo-Crespo et al., 2018 |
| HPMA copolymer-doxorubicin-aminoglutethimide | Chemotherapy; Endocrine therapy. | Vicent et al., 2005; Greco et al., 2007. |
| PEG-NO-epirubicin | Chemotherapy; Cardioprotective agent. | Pasut et al., 2009; Santucci et al., 2006. |
| HPMA copolymer-TNP470-alendronate | Anti-angiogenic agent; Bisphosphonate drug. | Segal et al., 2011. |

| | | |
|---|---|---|
| HPMA copolymer-paclitaxel-alendronate | Chemotherapy; Biphosphonate drug. | Miller et al., 2009. |
| HPMA copolymer-doxorubicin-dexamethasone | Chemotherapy; Anti-inflammatory and anti-proliferative agent. | Kostkova et al., 2011. |
| PEG-paclitaxel-alendronate | Chemotherapy; Biphosphonate drug. | Clementi et al., 2011. |

### 4.4. Current status of polymer-drug conjugates

Many polymer-drug conjugates have entered and are progressing through clinical evaluation (Table 2). Despite many clinically tested conjugates were designed for cancer applications, the first polymer-drug conjugate to enter the market (PEG-naloxone, Movantik) is approved for the treatment of opioid-induced constipation. In this case the rationale for polymer-conjugation is to change the body distribution of the active ingredient in order to uncouple desired peripheral effects from un-desired central effects. Specifically, conjugation of naloxone to PEG allows selective antagonism of some opioids peripheral side effects (GI tract, constipation) while allows to maintain opioid central effects, as the PEG-naloxone conjugate is unable to cross the blood brain barrier (BBB).

**Table 2. Clinical status of polymer drug conjugates that have entered clinical evaluation.**

| Status | Conjugate | Name | Reference |
|---|---|---|---|
| Market | PEG-naloxone | NKTR-118; Movantik | Floettmann et al., 2017; Neumann et al., 2007; Webster and Dhar, 2009 |
| | PEG-APL-2* | Empaveli | El Mehdi et al., 2017 |
| Phase III | PEG-Loxenatide* | PEX168 | Wang et al, 2019 |
| Phase II | HPMA-DACH-platinate | AP5346; ProLindac® | Campone et al., 2007. |
| | Dextran-Alendronate | OsteoDex | https:// dextechmedical.com/ en/candidatemedications/ osteodex; https://dextechmedical. com/en/dextechsphase-iib-study-forosteodex-ends-withpositive-follow-upresults/ |
| | Benzene polycarboxylic acid-Cis-diammineplatinum | BP-C1 | Anisimov et al., 2017; Butthongkomvong et al., 2019 |

| | | | |
|---|---|---|---|
| | PEG-Irinotecan | NKTR-102 | Hamm et al., 2009; Borad et al, 2008. |
| | HPMA copolymer-carboplatinate | AP5280 | Rademaker-Lakhai et al., 2004 |
| | PEG-SN38 | EZN-2208 DFP-13318 | Guo et al., 2008; Fontaine et al , 2020 |
| | Cyclodextrin PEG-camptothecin | CRLX101 | Oliver et al., 2008, Keefe et al, 2016 |
| | PEG-TRL7/8 agonist | NKTR-262 | Diab et al, 2019 |
| Phase I/II | Carboxymethyldextran-exatecan camptothecin | DE-310 | Soepenberg et al., 2005. |
| | PEG- docetaxel | NKTR-105 | Calvo et al., 2010. |
| Phase I | PHF-camptothecin | XMT-1001 | Sausville et al., 2010 |
| | Poly(2-ethyl-2-oxazoline)-Rotigotine | SER-214 | Olanow et al, 2020 |
| | PEG-camptothecin | Pegamotecan | Scott et al., 2009 |
| | HPMA copolymer-doxorubicin | PK1; FCE28068 | Vasey et al., 1999; Seymour et al., 2009. |
| | PEG-paclitaxel | - | Beeram et al., 2002 |
| Discontinued | HPMA copolymer- camptothecin | MAG-CPT | Schoemaker et al., 2002 |
| | HPMA copolymer- paclitaxel | PNU166945 | Meerum et al., 2001 |
| | Oxidized dextran-doxorubicin | DOX-OXD | Danhauser-Riedl et al., 1993 |
| | HPMA copolymer-doxorubicin-galactosamine | PK2; FCE28069 | Seymour et al., 2002 |
| | PGA-camptothecin | CT-2106 | Homsi et al., 2007; Daud et al., 2006. |
| | PGA-paclitaxel | CT-2103; Opaxio® | O'Brien et al., 2005; Albain et al., 2006; Langer et al., 2008; O'Brien et al., 2008; Paz-Ares et al., 2008. |

***** note the drug is a peptide, literature on polymer-protein conjugates is also relevant

## 5. Conclusions

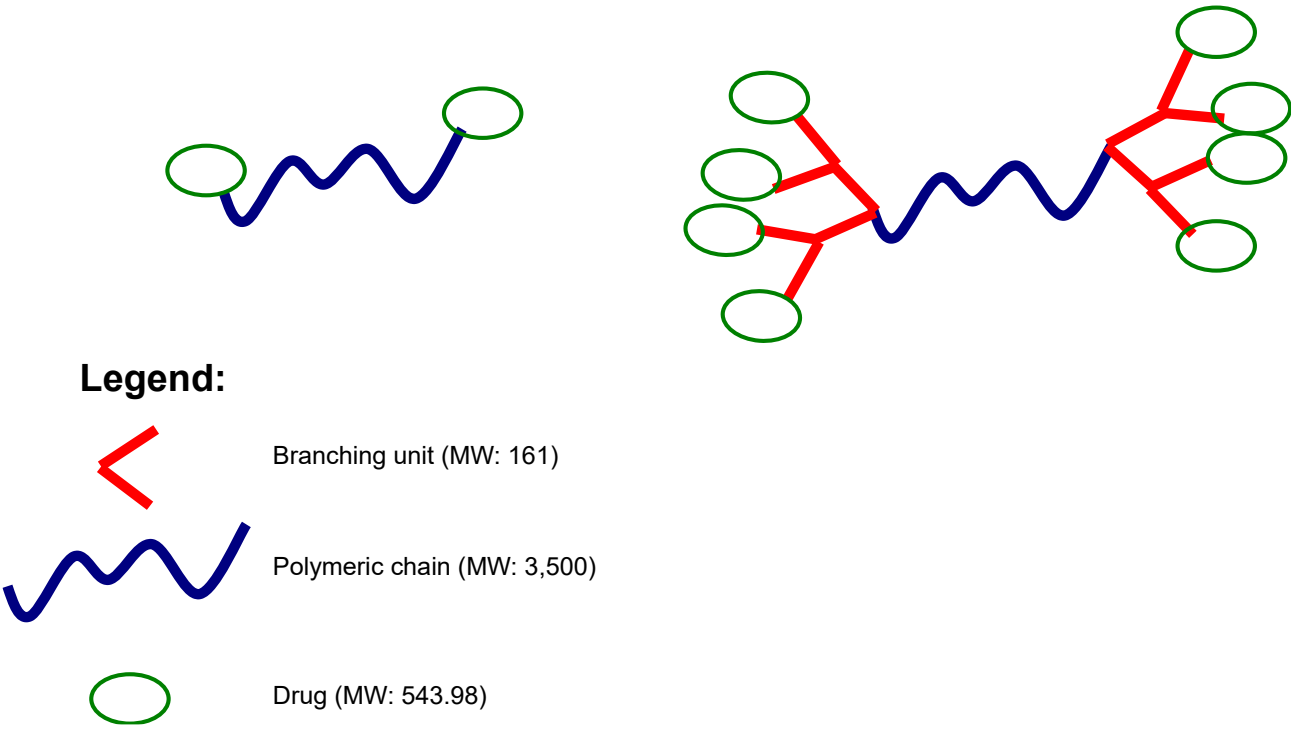
Polymer-drug conjugates are a drug delivery technology based on the covalent conjugation of drug molecules to a polymeric carrier. Conjugation to a polymer prolongs the circulation time of the drug, increases its selectivity for the target tissue (e.g. the tumour tissue) and allows selective drug release (if the system has a bioresponsive, biodegradable linker). The development of a polymer-drug conjugate requires a careful design and an accurate choice of its components (polymer, linker, drug and optional targeting moiety). Optimised synthetic conditions and extensive characterization are necessary to make a reproducible and "high quality" polymer-drug conjugate. The application of this concept has initially focused on the treatment of cancer, but has expanded to a multitude of other therapeutic areas, although in many cases still preclinically. The last decade has seen the first marketed polymer-drug conjugate and more research is ongoing to further develop this concept. Progress in polymer chemistry is leading to innovative polymer architectures and new molecular targets are being investigated to extend this technology to new applications.

**Problem box**

**Question 1.**

Calculate the drug loading (expressed as % w/w) for a linear polymer-drug conjugate and for its dendronised derivative (schematic representation of the structures and the MWs of the various components are reported in figure below). Briefly discuss the importance of drug loading within the context of polymer-drug conjugates.

Important note. In your calculation assume that conjugation of the drug to the polymer or to the branching unit results in the loss of a molecule of water (loss of a H from the drug and loss of an OH from the polymer/branching unit). Addition of each branching unit also results in a loss of a molecule of water.




**Answer 1.**

Step 1. Calculate the % w/w of the linear polymer-drug conjugate.

(a) Calculate the drug content:

(543.98*2) – 2 = 1085.96

(b) Calculate the total weight of the conjugate

(543.98*2) + 3,500 – (18*2) = 4551.96

(c) Calculate the % w/w:

(1085.96/4551.96) * 100 = 23.8%

Step 2. Calculate the % w/w of the dendronised polymer-drug conjugate.

(a) Calculate the drug content:

(543.98*8) – 8 = 4343.84

(d) Calculate the total weight of the conjugate

(543.98*8) + 3,500 + (161*6) – (18*14) = 8565.84

(e) Calculate the % w/w:

(4343.84/8565.84) * 100 = 50.7%

**Question 2.**

The anticancer agent paclitaxel has been considered a promising candidate for delivery via polymer-drug conjugate technology. Indicate what characteristics of this drug molecule make it suitable for applications with this technology.

**Answer 2.**

(a) Paclitaxel is an anticancer drug with toxicity due to non-selective delivery (conjugation to a polymer can improve selectivity); (b) paclitaxel is poorly water soluble and has to be administered in a oil-based formulation (Cremophor), which has a certain toxicity; conjugation to a polymer can improve solubility in biological fluids; (c) paclitaxel contains an hydroxyl group (this can be used for conjugation via, for instance, and ester linkage); (d) paclitaxel has a short plasma half-life (which can be prolonged by conjugation to a polymer).

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