Review Article

Pharmaceutical Sciences and Research (PSR), 5(3), 2018, 97 - 115

Synthesis of Polymer-Drug Conjugates Using Natural Polymer: What, Why and How?

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ABSTRACT

For years, natural polymers have played a significant role in pharmaceutical field due to their biocompatibility and biodegradability. In Indonesia, most research in natural polymers focus on application of the polymers as inert pharmaceutical excipients or as drug matrix in micro- and nano- particle. Meanwhile, research about polymers in the world (mostly synthetic polymers) have been progressed to advanced drug delivery system. In this system, the polymer can act as either pharmacologically active molecules, or sophisticated carrier in targeted prodrug delivery system. The latter is called polymer-drug conjugates, a system where the drugs are covalently attached to a polymeric carrier, rather than simply entrapped in polymer matrix. Natural polymers have been one of the materials to use for the carrier due to their biocompatibility and biodegradability. This review article emphasizes the opportunity, challenges and strategies to use natural polymers as carrier in polymer-drug conjugates. Moreover, we also discuss some aspects in regards of the synthesis and analysis, to give some perspectives and encouragement for the Indonesian researcher who are interested in exploring this research field.

ARTICLE HISTORY

Received: November 2018
Revised: December 2018
Accepted: December 2018

***Keywords**: natural polymer; polymer; polymer-drug conjugates; polymer therapeutic*

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INTRODUCTION

Natural polymers have been extensively used in research and development of pharmaceutical products. Their applications are mostly on the use of the polymers as excipient in pharmaceutical dosage form. In the last 8 years, research in natural polymer in Indonesia are mainly about modifying the polymer to increase the performance as matrix in controlled release tablets and capsules (Ariani, Surini, & Hayun, 2016; Surini, Nizma, & Azizahwati, 2017; Surini, Wati, & Syahdi, 2018) gastroretentive drug delivery system (Budianto, Al-Shidqi, & Cahyana, 2017; Dessy, Siahaan, & Bangun, 2018), mucoadhesive system (Adliani & Bangun, 2016; Arianto, Bangun, Harahap, & Ilyas, 2015; Putri, Sulistomo, & Surini, 2017), microsphere (Hariyadi *et al*., 2014) and microcapsule (Halim, Arianti, & Umar, 2011). In those cases, the polymers are supposed to be inert and do not chemically interact with the loaded drugs.

Some research in Indonesia also encompassed the use of natural polymer as nanoparticle. Martien, Sa'adah, & Saifullah (2016) synthesized insulin-loaded chitosan-pectin nanoparticles via ionic gelation method for oral delivery of insulin. Pertiwi, Martien, Sismindari, & Ismail (2018) also used chitosan-pectin nanoparticle to encapsulate Ribosome Inactivating Protein isolated from *Mirabilis jalapa L.*, with particle size ~350 nm. Chitosan nanoparticles were also used to encapsulate *Phaleria macrocarpa* leaf extract for suppression of mitosis and hyperplasia in small intestine crypt epithelial cells (Kusmardi, Ramadhan Tamzir, Widiasari, & Estuningtyas, 2018).

Meanwhile, research about polymer worldwide has progressed to more advanced drug delivery system called polymer therapeutic. Unlikely application of polymers only as inert excipients in conventional dosage forms, polymer therapeutic require covalent attachment between drug and polymer to form new system which is considered as new drug. Polymer therapeutic diverse to some different systems, including polymeric drug, polymer-drug conjugates, polymer-protein conjugate, self-assembly polymeric micelles and many more (Duncan & Vicent, 2013). Some products of polymer therapeutic have reached the market and some are still in clinical trials (Atkinson, Andreu, & Vicent, 2018; Duncan, 2017). Surprisingly, two compounds of this therapeutic group, the white blood cell booster Neulasta® and the immunomodulatory drug Copaxone®, became Top 10 selling drugs in US (Atkinson e*t al.*, 2018; Duncan, 2014).

In Indonesia, research in polymer therapeutic is slightly explored. Dendrimers become the most popular "polymer" being used as carrier, either for cytotoxic drugs (Sagita, Djajadisastra, & Mutalib, 2016) or theranostic agents such as ^{198}Au (Halid, Sutriyo, Mutalib, Pujiyanto, & Gunawan, 2017) and radiogadolinium (III) (Rahmania, Mutalib, Ramli, & Levita, 2015).

Nevertheless, research in this area using natural polymer has not been found in any international publications (searching was filtered by affiliation "Indonesia").

Therefore, we would like to give some concepts about polymer therapeutics using natural polymer as carrier. We are interested in using natural polymer due to Indonesia's biodiversity of natural polymer resources that can be explored, such as dextrin, chitosan and pectin. Besides, their biodegradability and biocompatibility provide certain benefits. In this review, we focus only on polymer-drug conjugates since they are relatively simpler than other systems in polymer therapeutic cluster. This review will cover the definition of polymer-drug conjugates, design of the system and some aspects on synthesis and analysis.

Overview of Polymer-Drug Conjugates

Ringsdorf's Model

Polymer-drug conjugates (PDC) refer to systems which consist of polymer as carrier and covalently-attached small molecule drugs (Vicent & Duncan, 2006). This covalent link is able to provide clinical benefits such as alteration in the pharmacokinetics (Danson *et al*., 2004; Forrest *et al*., 2008; Šírová *et al*., 2017; Vasey *et al*., 1999) and even improve biodistribution of several cancer drugs (Ernsting, Tang, MacCallum, & Li, 2012; H. Tang *et al*., 2018). Because of some alterations in biological behavior, this system is considered as *New Chemical Entity* (NCE) and should undergo full phase of clinical trial (Atkinson *et al.*, 2018).

Ringsdorf's model is the generic concept of polymer-drug conjugates design with lysosomotropic system, which was established in 1975 by Helmut Ringsdorf (Ringsdorf, 1975). N-(2-hydroxypropyl) methacrylamide (HPMA)-doxorubicin conjugate was the first to enter Phase 1/II clinical trials in 1994 (Duncan & Vicent, 2013). This then became the prototype for HPMA-based polymer conjugates.

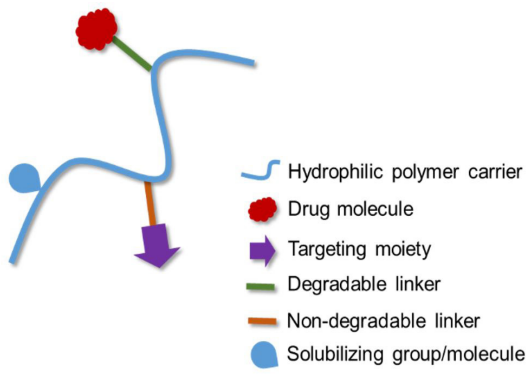


Figure 1. Ringdorf's Model for Polymer-drug Conjugates System

In Ringsdorf's model (Figure 1), the system consists of polymer carrier, linker, and conjugated molecules. The conjugated molecules can be drugs, targeting moieties, or labelling molecules such as fluorophore and radioisotope. The covalent conjugation brings superiorities such as higher drug loading, controlled drug release and minimization of undesirable drug leaking, compared to physically encapsulated polymer systems (Pang *et al.,* 2016).

Advantages of Polymer-Drug Conjugates: Role of Polymeric Carrier

Conjugating poorly soluble drugs to hydrophilic polymer can improve the solubility of the drugs. For example, paclitaxel-poly-L-glutamic acid conjugates could be dissolved in normal saline, while the free paclitaxel required mixture of 50:50 Cremophor-ethanol and saline (1:4), for the equivalent amount of paclitaxel (Li *et al*., 1998). Solubility of saquinavir can also be enhanced by conjugation with polyethyleneglycol (PEG) (Gunaseelan *et al*., 2004). Another example is solubility improvement of a xantin oxidase inhibitor, 4-amino-6-hydroxypyrazolo[3,4-d]pyrimidine (AHPP), when conjugated with styrene maleic acid copolymer (SMA) (Fang *et al.*, 2009).

The other advantage of PDC is prolonged plasma half-life due to their nano-sized compared to the free parent drugs. Depend on the length and shape of the polymeric carrier, the hydrodynamic size can range from 5 to 200 nm. This size is above the limit of renal filtration, which is varied by size but is around 3.5 nm (Nishiyama, 2007) and therefore reduce the excretion of the conjugates. Moreover, we can decorate the system with polyethylene glycol (PEGylation) that act as a "shield" and reduce the interaction with phagocytic cells. However, the superiority of PEG for drug delivery system is being questioned, since numerous studies found that PEGylation causes enhanced serum protein binding and anti-PEG antibody production (see review from Verhoef & Anchordoquy, 2013). A new study demonstrated increase anti-PEG antibody production in healthy wildtype mice after subsequent administration of PEGylated polymeric nanoparticles and liposomes (Grenier, de Oliveira Viana, Lima, & Bertrand, 2018) However, the linear PEG alone was less immunogenic. The authors suggested alternative PEGylation strategies for future study, for example polymer chains with comb architectures, to change the pattern of PEG layer on polymers.

Polymer-drug conjugates, as part of nanomedicine, has been extensively explored for improving cancer therapy by their well-known *enhanced permeability and retention* (EPR) characteristic. The phenomenon is due to increase permeability of tumor vasculature that makes

macromolecules larger than 40 kDa can extravasate and accumulate in tumor tissues, as well as improper lymphatic function that reduce the disposal of such materials to lymphatic system (Fabienne Danhier, Feron, & Préat, 2010; Fang, Nakamura, & Maeda, 2011). This is then popularly called passive targeting.

The EPR concept is now a controversy because of the heterogeneity of tumor cells (F. Danhier, 2016; Maeda, 2015). However, conjugation of anticancer drugs to nanoparticles or polymer has proved reduced toxicity to normal tissues (Ernsting *et al*., 2012; Lammers *et al*., 2009; H. Tang *et al*., 2018). Therefore, research in polymer-drug conjugates still need to be explored, especially with natural polymers, as they are considered as biocompatible. Moreover, current application of polymeric nanoparticles is not only for cancer therapy but also other diseases such as antiinfectives, tissue regeneration and repair, wound healing, osteoarthritis, rheumatoid arthritis, ischemia, and many more (Duncan & Vicent, 2013).

Drug Release from Conjugate

So if the drugs are covalently attached to the carrier, how can the drugs give the same pharmacological effect? This is where the "linker" or "spacer" plays a role. As we can see in Ringsdorf's model, the drug is linked to the polymer via a spacer. The linker/spacer should be made selectively degradable, which means it will not be cleaved in the systemic circulation, but it will undergo cleavage when it reaches the site of action to release the drugs. This condition may be induced by differences in the environment condition between blood circulation and site of action such as pH and the presence of specific enzyme. Kurtoglu, Mishra, Kannan, & Kannan (2010) observed different drug release characteristics from dendrimer with different linkers, and found that amide linkers were very stable at all pH, while ester and peptide linkers showed pH dependent and enzyme-activated rates, respectively.

Drug release by pH-dependent hydrolysis provides benefit for delivery of anticancer drugs, because tumor microenvironment has more acid pH (6.0-7.0) than normal plasma (7.4) (Fabienne Danhier *et al*., 2010). Furthermore, the inside of lysosomes, cell organelles that digest engulfed foreign particle, have pH 4.0-5.0 (DiCiccio & Steinberg, 2011), which facilitate rapid release of drugs inside the cells. Cisplatin was conjugated to modified PEG polymer via ester bond and showed different drug release profile *in vitro* at pH 5.0, 6.0 and 7.4 (Aryal, Hu, & Zhang, 2010). Hydrazone linkage was used to attached paclitaxel to HPMA copolymer (Etrych, Milada, Starovoytova, Blanka, & Ulbrich, 2010). The conjugates were relatively stable at the pH of blood (7.4) and release active drug in pH 5. Du, Du, Mao, & Wang (2011) also used hydrazine bond to link doxorubicin to polymer.

Enzyme-activated linker involves cleavable peptide sequences that are sensitive to specific enzymes such as cathepsins, matrix metalloproteases (MMPs), plasmin, prostate-specific antigen (PSA) and urokinase (Wong & Choi, 2015). Conjugation of paclitaxel and PEG via valine–citrulline dipeptide linker showed higher *in vitro* drug release in the existence of Cathepsin B than without the enzyme (Liang *et al*., 2012). GLFG, Gly-Leu-Phe-Gly, is a tetrapeptide that is also widely used as enzyme-activated linker (Luo, Yang, Kope Ckov, & Rich Kope, 2011; Vicent *et al*., 2005; R. Zhang, Yang, Sima, Zhou, & Kopecek, 2014). Another enzyme-sensitive linker is N-acetyl-Gly-D-ala-L-Phe-L-Lys that can be degraded by both proteases plasmin and cathepsin B (Barthel *et al.*, 2012).

Glutathione (GSH) levels in human tissue vary greatly, but they tend to be elevated in breast, ovarian, head and neck and lung tumors, compared to normal tissue (Gamcsik, Kasibhatla, Teeter, & Colvin, 2012). Due to this finding, disulfide bond can be a linker for targeted drug release which will undergo cleavage through a reduction reaction (Wong & Choi, 2015). This has been used in synthesis of various polymer drug conjugate such as PEG-co-tert butyl acrylate-paclitaxel (Chen *et al*., 2012), PEG-camptothecin (Li *et al*., 2011), and chitosan oligosacharide-doxorubicin (Su *et al*., 2015). However, there are some limitations in using disulphide linker such as low drug payload, low physical stability and possibility of change in chemical structure of the drugs after being released, and therefore requires further studies on destabilization and preclinical evaluation (Chang *et al.*, 2016).

Required Characteristics of the Polymer

Water Solubility and Reactive Functional Groups

Functional groups of polymer play some important roles. They determine polymer properties in solution, including solubility, and also provide reactivity for conjugation with other molecules. Normally, natural polymers have hydroxyl (-OH) and /or carboxylic (-COOH) groups. Chitosan is one of natural polymers that has primary amine ($-NH_2$) group. Those functional groups provide hydrophilicity as well as reactivity for conjugation reaction. However, some natural polymers, such as starch and original cellulose that mainly consist of –OH groups, are not water soluble. Their insolubility in water is suspected due to strong intermolecular hydrogen bonds or hydrophobic interaction (Medronho, Romano, Miguel, Stigsson, & Lindman, 2012). Therefore, the choice of natural polymers candidate for a drug conjugate should be based on not only their available functional

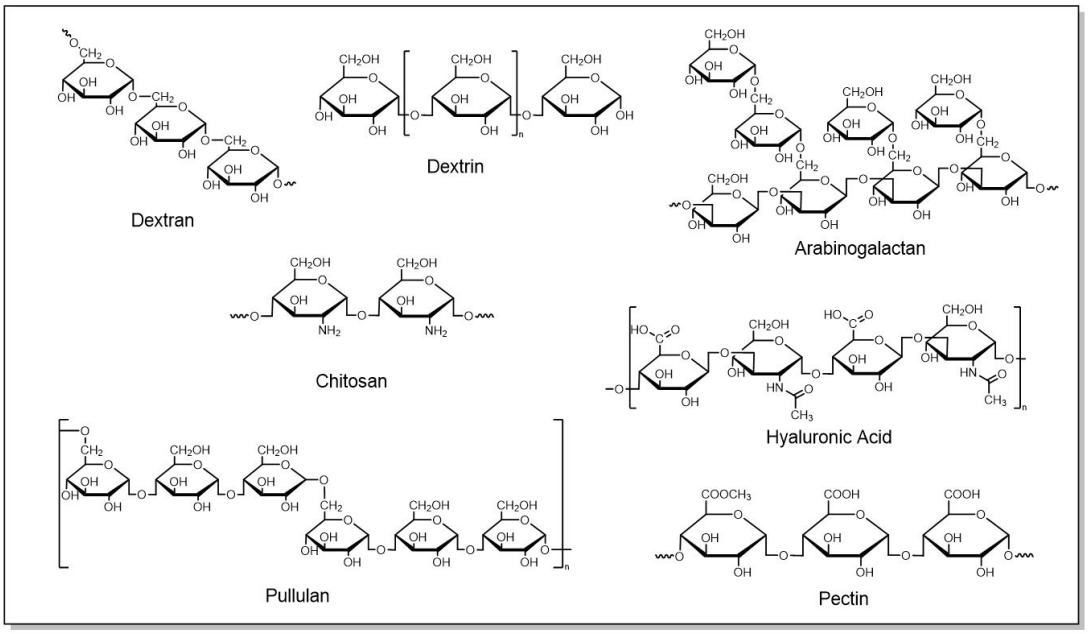


Figure 2. Structure of Some Water-soluble Natural Polymers

groups but also their solubility in water. Below are some water-soluble natural polymers that have been studied as carrier in polymer-drug conjugates. The structure of each polymer can be seen in Figure 2.

Dextran. Dextran is a polymer of α-D-glucopyranosyl with (1,6)-linked α-D-glucopyranosyl unit (BeMiller, 2003). Dextran is synthesized from sucrose by certain lactic-acid bacteria, the best known are *Leuconostoc bacteroides* and *Streptococcus mutans*. Dextran is normally used as plasma volume expander (Svens & Rodhe, 2013).

Dextran is probably the most investigated natural polymer derivate in polymer-drug conjugates, which has been developed for various types of disease. For example, daptomycin-dextran conjugates showed higher affinity for fibrinogen than free daptomycin, suggesting improvement of daptomycin efficacy in endocarditis (Muangsiri & Kirsch, 2006). Varshosaz *et al*. (2011) synthesized budenoside-dextran conjugate as colon-targeted drug delivery system for ulcerative colitis medication. Cathecin, a flavonoid, was also conjugated to dextran for the treatment of pancreatic ductal adenocarcinoma (Vittorio *et al.*, 2012).

Dextrin. Dextrins are a group of oligosaccharides which derived from partial hydrolysis of acid or amylases action (BeMiller, 2003). It is a mixture of D-glucose unit polymer linked by (1,4) or (1,6) glycosidic bonds (Y. Zhao & Tu, 2013). Corn starch is generally used as the most common sources of dextrins due to its availability in nature and its low production cost, even though starches from potato, tapioca and sago are considered easiest to be converted to dextrins (Baumann & Conner, 1994). As carrier in drug delivery system, they are usually modified into succinoylated-dextrin which undergo slower rate of degradation by pancreatic α-amylase (Hreczuk-Hirst, Chicco, German, & Duncan, 2001). Hardwicke *et al*., (2008) used succinoylated-dextrin as carrier for recombinant human epidermal growth factor (rhEGF) for wound healing. The conjugates showed elevated stability against degradation by trypsin and neutrophil elastase. Succinoylated-dextrin was also used to conjugate an antitumor protein, phospholipase A2 (PLA2), as synthesized by Ferguson & Duncan (2009). The conjugate showed lower PLA2's hemolytic activity with similar, or higher, cytotoxicity against MCF-7, HT29, and B16F10 cells, compared to free PLA2. Ferguson, Azzopardi, Roberts, Walsh, & Thomas (2014) successfully synthesized dextrin-colistin conjugates. Although the conjugates diminished the antimicrobial activity of colistin, they showed prolonged plasma retention and reduced toxicity, in comparison to colistin sulfate.

Pullulan. Pullulan is a natural-derived consisted of maltotrioses, linked by α-(1,6)glycosidic bond, which consists of three molecules of glucose linked to each other with α-(1,4) glycosidic bond (Chiellini, Piras, Errico, & Chiellini, 2008; dos Santos & Grenha, 2015; Mizrahy & Peer, 2012; Namazi, Fathi, & Heydari, 2012; Singh, Kaur, Rana, & Kennedy, 2017). This structure is suggested to give pullulan high flexibility and water solubility (Kumar, Saini, Pandit, & Ali, 2012; Trinetta & Cutter, 2016). Pullulan is an extracellular polysaccharide. It was first reported from the strains of fungus *Aureobasidium pullulans* (Bender, Lehmann,

& Wallenfels, 1959; Park & Khan, 2009). The fungus cultivation parameters strongly affect the resulting pullulan MW, which is usually ranging from 4.5 x 10^4 – 6 x 10^5 Da (Cheng, Demirci, & Catchmark, 2011; dos Santos & Grenha, 2015).

In a research by Zhang *et al*. (2011), pullulan-doxorubicin conjugate showed greater toxicity (determined by IC_{50}) toward ovarian carcinoma A2780 cells compared to free drugs after 48 hours incubation. Scomparin, Salmaso, Bersani, Satchi-Fainaro, & Caliceti (2011) demonstrated opposite result, where the polymer conjugation showed lower toxicity than free doxorubicin. However, the conjugates showed significant improvement in pharmacokinetics, with half-life ($T_{1/2\beta}$) ~4 times longer than the parent drug. Beside drug, pullulan was also used to synthesized conjugates that bring gadolinium diethylene triamine pentaacetate (Gd-DTPA) for contrast agent in MRI (Yim *et al*., 2011). The conjugates showed high accumulation in the liver, which suggests hepatocyte-specific MRI contrast agent.

Chitosan. Chitosan is a polymer obtained from partial N-deacetylation of chitin, found in crustacean shells (Emeje & Anwunobi, 2011; Hamed, Özogul, & Regenstein, 2016). Chitosan and chitin structure is similar to cellulose. In chitin and chitosan, the hydroxyl at C-2 position replaced by acetamide groups (Islam, Bhuiyan, & Islam, 2017). Chitosan is composed of N-acetyl glucosamine and glucosamine residues which covalently linked by linear β-(1,4) glycosidic bonds and obtained by transforming its acetamide groups into primary amino groups (Hamed *et al*., 2016; Islam *et al*., 2017).

Chitosan has been widely explored in gene delivery. The positively charged amino group of chitosan can create electrostatic attraction with the negatively charged nucleic acid to make a complex and therefore can protect the gene from plasma nucleases (Saranya, Moorthi, Saravanan, Pandima Devi, & Selvamurugan, 2010). However, chitosan now is also used for wider application. For example, conjugates of N-succinyl-chitosan and carboxymethyl-chitin with chemotherapy drug mitomycin C (MMC) for slow release MMC (Song, Onishi, & Naai, 1992). Other example is conjugation of paclitaxel to low molecular weight chitosan, as synthesized by Lee *et al*. (2008), that showed ~42% per oral bioavailability and increased solubility, suggesting the possibility to deliver paclitaxel via oral route. Chitosan was also used to carry near infra red (NIR) dye IR820 for cancer theranostic application (Srinivasan, Manchanda, Fernandez-Fernandez, Lei, & Mcgoron, 2013). Conjugation of exendin-4, a GLP-1 mimetic peptide for treatment of type 2 diabetes, to low molecular weight chitosan showed improved stability against trypsin and better oral pharmacokinetics profile (Ahn *et al*., 2013). These findings indicate the prospect to create an orally bioavailable peptide drugs.

Hyaluronic Acid. Hyaluronan is an international nomenclature of heteropolysaccharides attributed to Endre Balazs. It encompasses the term of two different forms of the molecule e.g. the acid form, hyaluronic acid; and its salt, hyaluronate, such as sodium hyaluronate (Balazs, Laurent, & Jeanloz, 1985). At physiological pH, its carboxyl groups form anionic charge and balanced with cation such as sodium, potassium, calcium or magnesium (Fallacara, Baldini, Manfredini, & Vertuani, 2018). Hyaluronan is an extracellular matrix compound. It is a highly molecular weight glycosaminoglycans, composed of disaccharide repeats of N-acetylglucosamine and glucoronic acid linked together by alternating β-(1,4) and β-(1,3) glycosidic bonds (Necas, Bartosikova, Brauner, & Kolar, 2008). The number of repeating disaccharides in a hyaluronan can achieve 10,000 or more with molecular mass about 4 million Dalton (Cowman & Matsuoka, 2005; Necas *et al*., 2008).

Homma *et al*. (2010) optimized hyaluronic acid-methotrexate conjugates formulation to obtain best characteristic for ostheoarthritis treatment. Conjugation with hyaluronic acid can also enhance solubility of curcumin as shown by Manju & Sreenivasan (2011). Like other natural polymers mentioned before, hyaluronic acid has also been employed as carrier for paclitaxel (H. Lee, Lee, & Park, 2008; Yin *et al*., 2015; D. Zhao, Zhang, Yang, He, & Luan, 2016).

Pectin. Pectins are a member of polysaccharides which commonly present in primary cell walls and middle lamella of dicotyledonous and non-grass monocotyledonous plants (Gawkowska, Cybulska, & Zdunek, 2018). Pectins are mainly consisted of covalently linked galacturonic acid by linear chain α-(1,4) glycosidic bonds that forms the backbone of pectin, homogalacturonan (Human Metabolomic Database, 2018; Mohnen, 2008; Wishart *et al*., 2018). The fine structures of its polysaccharides are never fully known or defined, and at first were thought to be triad homopolymers i.e. homogalacturonan, arabinan and galactan. Afterward, the reports from various plant materials showed that pectic subtances is a group of complex and diversified polysaccharides, with molecular weight more than 200.000 Dalton, which correspond to polymerization degree (Flutto, 2003; Yapo, 2011).

Pectin, depending on the degree of esterification, has carboxylic groups. In this case, the degree of esterification (DE) of pectin affects many properties of pectin polymer, i.e. gelling properties, and therefore

affects its functionality (Lutz, Aserin, Wicker, & Garti, 2009; Morris, Kok, Harding, & Adams, 2010).

Tang *et al*. (2010) synthesized pectin-adriamycin conjugate which exhibited remarkable therapeutic effect on melanoma pulmonary metastasis a (B16 cell line) in C57BL/6 mice (shown by lung histology and percent of survival after 60 days tumor implant). Cheewatanakornkool, Niratisai, Manchun, Dass, & Sriamornsak (2017) also used the same drug to conjugate with thiolated pectin. The conjugates were then crosslinked by ionotropic gelation technique to produce microbeads with particle size ~1000 μm.

Arabinogalactan. Arabinogalactan is a water-soluble polysaccharide that is found in various plants such as *Cuscuta chinensis, Larix occidentalis, Larix sibirica* and many more (Paulsen & Barsett, 2005). It has some bioactive properties such as effects on macrophages, T-lymphocytes and NK-cells (Paulsen & Barsett, 2005). The (1,3)-linked β-D-galactopyranose units [→3)-β-D-Galp-(1→] build the main chain, with branching points at the C6 atom (Mikhailenko *et al*., 2016). The branching consists of 3,6-di-O-substituted and 6-O-substituted galactopyranosyl, 3-O-substituted arabinofuranosyl residues, as well as arabinofuranosyl, arabinopyranosyl and galactopyranosyl non-reducing terminal units.

In a study conducted by Pinhassi *et al*. (2010), arabinogalactan was used to create targeted delivery and target-activated release of methotrexate, with folic acid as targeting moiety. The conjugate exhibited elevated cytotoxicity against folate receptor (FR)-overexpressing cells compared to FR-lacking cells. Elgart, Farber, Domb, Polacheck, & Hoffman (2010) compared low molecular weight (L-Mw) and high molecular weight (H-Mw) arabinogalactan as carrier for amphotericin B. The result showed altered pharmacokinetics parameters of conjugates compared to free amphotericin B, where conjugation of amphotericin B with H-Mw arabinogalactan showed significant decreased in volume of distribution and clearance. Arabinogalactan was also used to carry gadolinium-diethylenetriaminepentaacetic acid for liver-specific MRI contrast agent (Li *et al*., 2008).

Molecular Weight and Dispersity

Molecular weight plays an important role in nanoparticle's behavior in the body. As mentioned before, in order to exhibit prolonged plasma circulation, a polymeric carrier should be larger than 40 kDa, which is the threshold of renal clearance (Fang *et al*., 2011). In case of hydrodynamic size, the size should be maintained 5.5 – 150 nm to avoid renal clearance (Choi *et al*., 2007) as well as to escape phagocytic uptake (He, Hu, Yin, Tang, & Yin, 2010).

Dispersity (Đ, pronounced "*D-stroke*"), which replaces the misleading but widely used term "polydispersity index", is "dispersions of distributions of molar masses (or relative molecular masses, or molecular weights) and degrees of polymerization" (Stepto, 2010). In a simple way, dispersity is the ratio between weight average molecular weight and number average molecular weight (Mw/Mn). Dispersity index of a highly uniform polymer is 1, and this can only be obtained by protein. Other type of polymers have dispersity more than 1. For polymer-drug conjugates, the dispersity of the polymeric carrier should be as close as 1.

When using polymer as excipient in drug dosage form, dispersity index is not essential. On the other hand, for polymers that are "therapeutically active" like polymer-drug conjugates, the dispersity, which correlates with molecular weight distribution, becomes important. First, from the conjugation reaction point of view, dispersity is important to determine the stoichiometry. Broad dispersity means broad molecular weight distribution, which also means vary individual chain length. If this exists, it will be difficult to determine the molar ratio between polymer and drugs in the conjugation reaction. Second, from the therapeutic dose point of view, dispersity is essential to determine the dose. The number of drug molecules per polymer chain should be well defined to ensure the proper therapeutic dose.

The challenge of using natural polymer as carrier in polymer-drug conjugates is the wide range of molecular weight, depending on the sources. For example, arabinogalactan from gum Arabic, mango fruit exudate, and larch have Mn 71.6 kDa, 19.8 kDa, and 8.85 kDa, respectively (Nagel, Conrad, Leitenberger, Carle, & Neidhart, 2016). Chitosan sold by Sigma-Aldrich is available in wide variety of molecular weight, ranging from 5 to 375 kDa. Therefore, researcher need to pre-define the molecular weight and origin of the natural polymers prior to use it.

Technical Aspect on Synthesis and Analysis

Conjugation of Drugs and Targeting Moieties to Polymeric Carrier

Many conjugation techniques can be an option. The choice of conjugation technique depends on the functional groups present on both polymer and the small molecule (Hermanson, 2013). Moreover, the functional groups should be available and chemically compatible to make the reaction possible (Hermanson, 2013). Meanwhile, polymer chain in solution tend to form random, three-dimensional coil (Edvinsson, 2002). This conformation becomes a great challenge in conjugation reaction between polymer and small molecule because some of the reactive groups in polymer are hindered inside the conformation and inaccessible.

Different technique of conjugation are available for each kind of linkage. Generally, amide and ester are the most used for small molecule drugs, while thiol conjugation is more common for protein and peptide drugs. Therefore, in this review we will focus on the amide or ester conjugation reaction.

Amide bonds are basically created from condensation of carboxylic acids and amines. However, the reaction does not occur spontaneously at room temperature. It needs activation of carboxylic acid by converting the –OH of the acid into a good leaving group (Valeur & Bradley, 2009). Once it is activated, the amines will be easier to replace the –OH of the acid to form amide bond.

Activation of carboxylic acids needs coupling reagents and the choice of coupling reagent is essential for the success of reaction. Carbodiimides were the first coupling agents to be synthesized and are still widely used. They are called as zero-length crosslinking agent because no additional chemical is incorporated between the conjugated molecules (Hermanson, 2013).

N-substituted carbodiimides can react with carboxylic acids to form o-acylisourea derivatives (Figure 3). This o-acylisourea intermediate is a highly reactive species and can be replaced by a nucleophile such as a primary amine to form an amide bond. There are three well-known carbodiimide derivates: dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (EDC). DCC and DIC are water-insoluble reagents and thus can only be used for reaction in organic solvent. EDC is water soluble and therefore suitable for aqueous-based reaction. Addition of sulfo-NHS ester is sometimes required to reduce early hydrolysis of active carboxylates and thus increase the conjugation efficiency.

Ester bond formation uses the same principle as amide. However, it requires catalytic agent such as dimethylaminopyridine (DMAP). Addition of DMAP brings formation of acyl pyridinium intermediate which then reacts with alcohol to form ester product as shown in Figure 3 (Lutjen, Quirk, Barbera, & Kolonko, 2018).

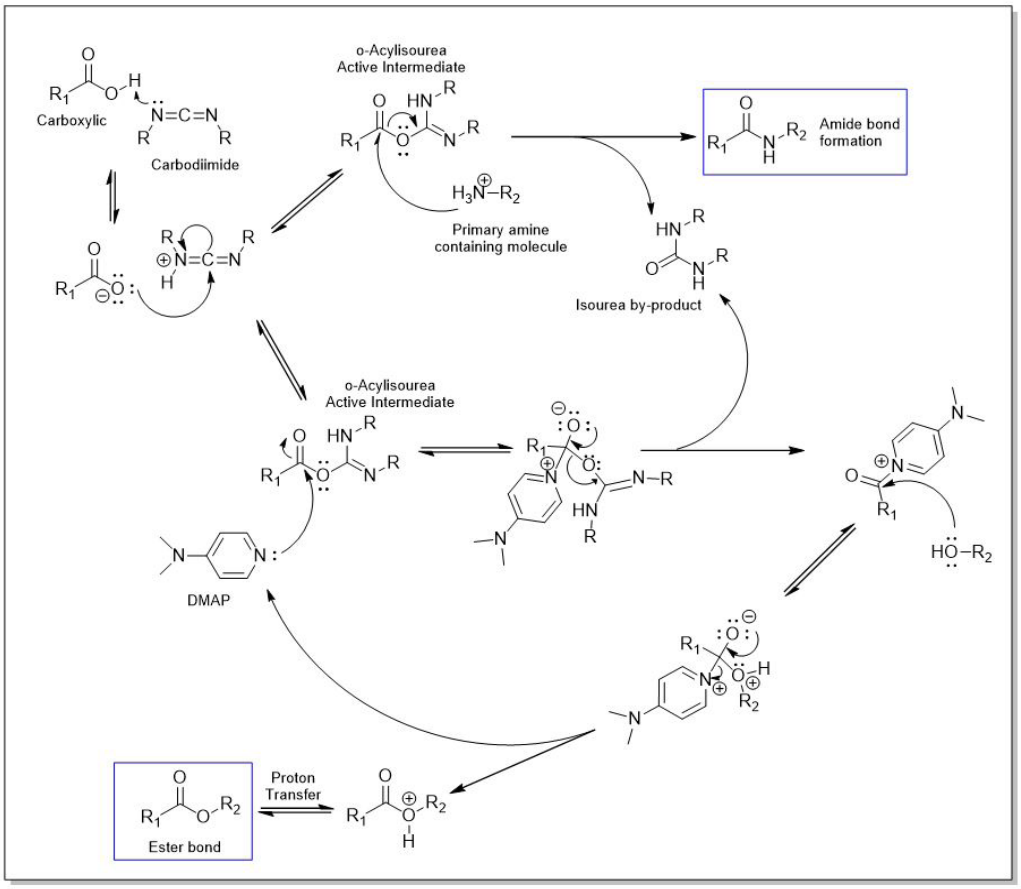


Figure 3. General Mechanism of Amide and Ester Formation Using Carbodiimide as Coupling Agent (adapted from Hermanson, (1996) & Lutjen, Quirk, Barbera, & Kolonko (2018))

Analytical Technique

Size Exclusion Chromatography. Size exclusion chromatography (SEC) or gel permeation chromatography (GPC) is basically one type of high performance liquid chromatography (LC). The fundamental difference between SEC and HPLC is in the principle of molecule separation. While HPLC separates molecules based on their hydrophilicity-hydrophobicity nature, SEC dissociates molecules solely based on their size. SEC uses columns packed with very small, round, porous particles which are made from insoluble cross-linked polymers or inorganic materials, such as spherical silicas (Agilent, 2015).

As mentioned before, when dissolved in a solvent, polymer chain will coil up to form a ball of string. Inside the SEC column, the polymer conformation will behave like spheres. The size of the sphere is molecular weight dependent, where higher molecular weight polymer will form larger spheres. When the polymer coils pass through the porous beads in SEC column, those that are bigger than the largest pores in the beads are not able to enter the pores and therefore exit the column first. The medium size polymer coils can probably enter the biggest pores, but not the small ones. As a result, these coils will exit later than the larger ones. The smallest polymer coils can occupy any pores in the beads and therefore will be retained much longer. As the fractions exit the column, they are measured by certain detector (mostly refractive index detector) and then compared with calibration standard (e.g. polystyrene). The elution profile of the sample will be displayed in chromatogram. Illustration of SEC mechanism is shown in Figure 4. With SEC, the value of M_n, M_w and dispersity can be obtained and thus, SEC becomes the most regularly used method for molecular weight determination of polymers.

Nuclear Magnetic Resonance. Nuclear magnetic resonance (NMR) spectroscopy is a well-established method for the characterization of molecules, including polymers. It has been applied for the determination of polymerization conversion and kinetics (Favier, Charreyre, & Pichot, 2004; Martin, Gody, & Perrier, 2015), monomer sequence in copolymer (Hatada & Kitayama, 2004), molecular weight of polymer (Izunobi & Higginbotham, 2011) and successful conjugation of drug to polymer (Hu *et al*., 2014; Lv *et al*., 2014). ^{1H}NMR spectroscopy is considered as a fast, reproducible, and relatively simple technique for polymer-drug conjugates structure elucidation.

Mass Spectrometry. Large molecules have triggered progress development of efficient ionization methods for mass spectrometric analysis especially in the structural analysis of biomacromolecules. These were overcome first by fast atom bombardment (FAB), and since the late 1980s more efficiently by electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Aminlashgari & Hakkarainen, 2011). The application of ESI and MALDI have been widely applied for biomolecules of oligo- and polymeric size, in combination with appropriate mass analyzers such as instruments, quadrupoles, ion traps, and time-of-flight tubes (Aminlashgari & Hakkarainen, 2011). To be more attractive, the techniques are also combined with tandem mass spectrometry.

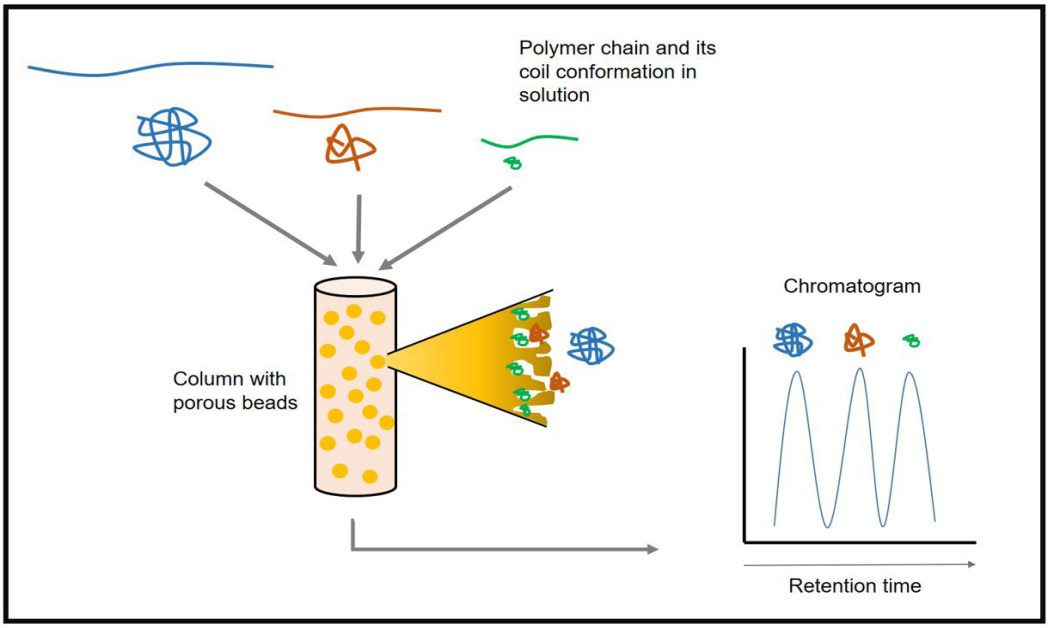
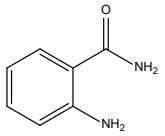
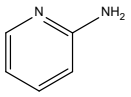
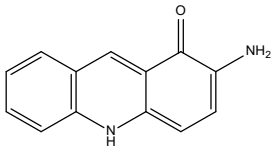
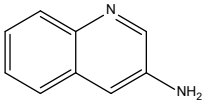
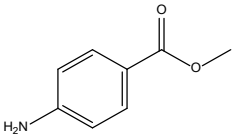
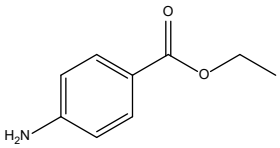
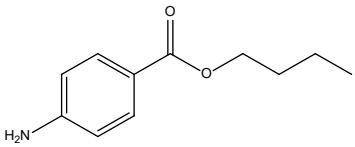
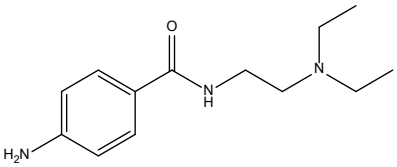


Figure 4. Illustration of Polymer Separation by GPC (adapted from Agilent Technologies Inc., (2015))

Table 1. Common Amines for labeling by Iminium Ion Formation (Harvey, 2000)

No	Amine	Abbreviation	Mass Increment	Structure
1	2-aminobenzamide	2-AB	120	
2	2-aminopyridine	2-AP	78	
3	2-aminoacridone	2-AMAC	194	
4	3-aminoquinoline	3-AQ	128	
5	4-aminobenzoic acid methyl ether	ABME	135	
6	4-aminobenzoic acid ethyl ether	ABEE	149	
7	4-aminobenzoic acid n-butyl ester	ABBE	176	
8	4-amino-N-(2-diethylaminoethyl)-benzamide	DEAEAB	219	

Analysis of polymer and biomacromolecule are suitable with soft ionization mass spectrometric techniques. There are two approaches for analyzing polymer (1) Top-down approach; polymer was analyzed for its higher-order structure. For top-down approach matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) was widely used; (2) Bottom-up approach; polymer was cut into smaller fragments of oligomer and reconstructed back into its original form. For bottom-up electrospray ionization-mass spectrometry (ESI-MS) has been excellent techniques for the analysis of the synthetic polymer. For high molecular mass polymer analysis, MALDI-MS is the best choice because of its soft ionization. Soft ionization was achieved by the interaction between the matrix and the polymer. MALDI-MS makes polymers can be analyzed in their original structure. Thus, it is possible to acquire more information about structural differences among polymers. Liquid chromatography equipped with ESI-MS is the best choice for monomer analysis of the polymer.

When dealing with polymers with high molecular mass distribution, ESI-MS can create multiple charged ion adducts that can be a problem. Carbohydrates commonly form alkali adducts. Also, compared to proteins and peptides, poly- or oligosaccharides show lower surface activity, higher polarity, and are less stable.

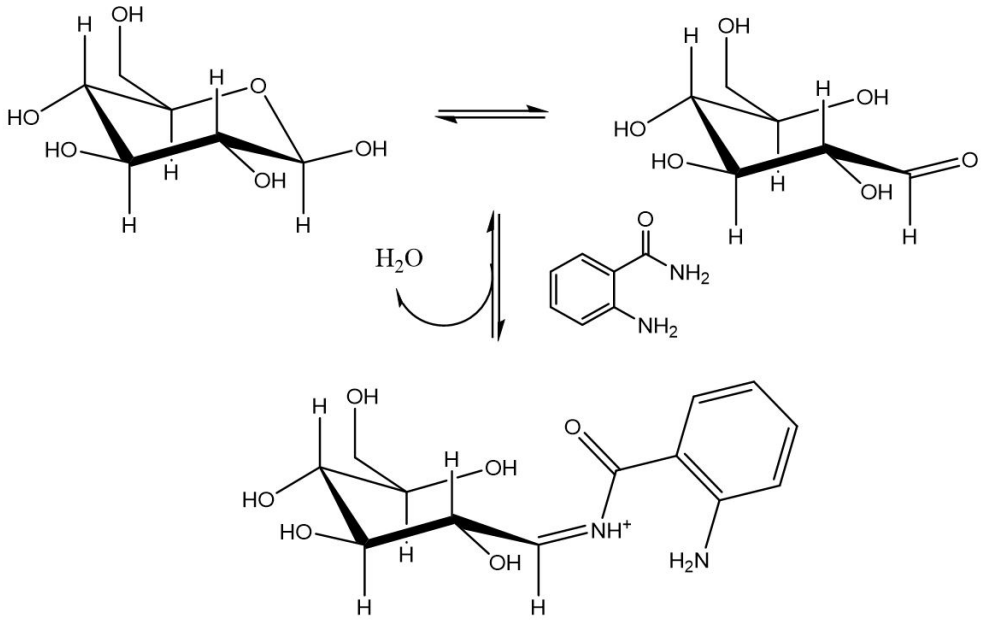


Figure 5. Iminium Ion Formation of β-glucose and 2-aminobenzamide (Unterieser, 2011)

Carbohydrates alkali adducts often show dispersity of molecular mass and chemical structure, and due to many stereochemical centers have isobaric ions, which cannot easily be differentiated by MS. The drawback with MALDI is the difficulty in studying these low mass range and often makes it impossible to detect low molecular weight compounds.

The presence of charged species determined MS analysis. Although both positive and negative ion can run simultaneously, positive mode is more common and therefore preferentially chosen. Carbohydrate is generally neutral and, apart from amino sugars, possess no basic groups that are able to be protonated. More common is, thus, the information of adducts with metal cations, mainly alkali ions, and preferentially the presence of sodium ion. Ion yield and the sensitivity of MS depend on appropriate coordination sites. The oxygen atoms achieve coordination with their nonbonding electron pairs (Crecelius, Vitz, & Schubert, 2014).

Chemical modification is the selective incorporation of a tag which has a readily ionizable functional group, often a quaternary ammonium group. Also, most of the tags are chromophores, and some of them are also fluorescent, which is useful for parallel detection in liquid chromatography. The most popular reactions are imines, and usually followed by a reduction to transfer the reversibly formed intermediate imines to the stable amines (Crotty, Gerişlioğlu, Endres, Wesdemiotis, & Schubert, 2016). Figure 5 shows the reaction between β-glucose with 2-aminobenzamide forming the iminium ion which can be detected by MS using positive ion mode. Several reagents were used to produce iminium ion as described in Table 1.

a. Linear homopolymer analysis using thermal-MS techniques

Thermal-MS techniques can decompose polymer by heat. Afterward, polymers are then analyzed by mass spectrometer to observe the degradation product of the polymer. Characterization of the temperature-dependent degradation product is useful to obtain information regarding both physical and structural properties. It is reported that simultaneous thermogravity-differential thermal analysis coupled with miniaturized ion trap MS allowed precise real-time monitoring analysis of activated organic compounds such pas pyrolysates. These features allow a better understanding of the complex thermal behavior and the precise pyrolysates material (Paine, Barker, & Blanksby, 2014; Wang, 1999).

b. Linear copolymer analysis using direct MS/MS techniques

Polymer sample can be directly injected to the mass spectrometer without preparation. The analyte ions are then fragmented to give more detailed structural and/or architectural information. The tandem MS analysis can be executed using various scanning modes such as selective ion monitoring (SIM), precursor ion scan, selected reaction monitoring, and multiple reaction monitoring (MRM). Out of all these modes, selective ion monitoring is one of the most common MS/MS modes to characterize the structure of various synthetic polymers. During this mode, a precursor analyte ion is isolated, followed by activation and fragmentation inside the

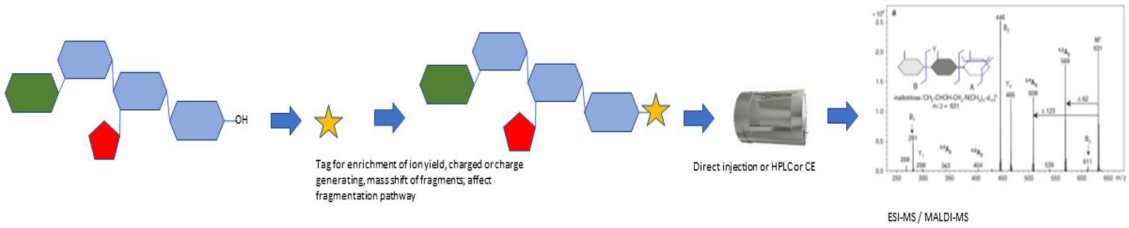


Figure 6. Analytical Workflow of Oligosaccharide-imine Conjugate

mass spectrometer. Finally, all fragmentation products are scanned and analyzed for more detailed investigation of the precursor ion structure (Crotty *et al*., 2016; Paine *et al*., 2014).

c. Complex polymer analysis using LC-MS based techniques

It has been proven that there are some cases where MS alone is insufficient for a comprehensive characterization of end groups, copolymer composition sequences, etc. Therefore, some techniques have been developed to obtain a more detailed polymer characterization, which involve hyphenation with HPLC either separating by polarity or size or 2D-LC. Many different 1D-LC systems hyphenated with MS are reported, particularly, for optimizing the transfer of the sample from a chromatographic system to the mass spectrometer. The main advantage of ESI is its compatibility with continuous hyphenation to diverse HPLC modes in comparison to MALDI, where most of the hyphenation techniques are carried out offline. Different hyphenations and different detectors are used to obtain extra knowledge about polymers, such as chemical heterogeneity and isomeric architecture (Sarrut, Crétier, & Heinisch, 2014). Therefore, enabling the chemist to improve synthetic routes.

A 2D (RP-LC X SEC) chromatography system can also be tandemed with MALDI and ESI. The procedure is fully automated and very versatile. The RP-LC X SEC combination appears to be one of the most important analytical methods when specific conditions are applied in the first dimension (Trathnigg, 1995). Figure 6 shows analytical workflow of oligosaccharide conjugated with amine to form imine.

d. Complex polymer analysis using ion mobility-MS (IM-MS) techniques

IM spectrometry provides an additional dimension for elucidating different conformations or architectures present in an analyte. With IM-MS, gas phase ions are separated based on their mobility and composition. Information obtained by IM separation can be used to create collision cross-sections, which are directly related to the macromolecular shape. Isobaric ions are two different chemical species with different elemental composition, having the same nominal mass (mass difference at ppm level). They often appear in polymer analysis and can only be resolved by high-resolution spectra. If isobaric species are not solved, obtaining a detailed structural characterization with MS/MS experiments can be very challenging. This can result in very complicated MS/MS data, having fragment ions from both species in the same spectrum (Crotty *et al*., 2016).

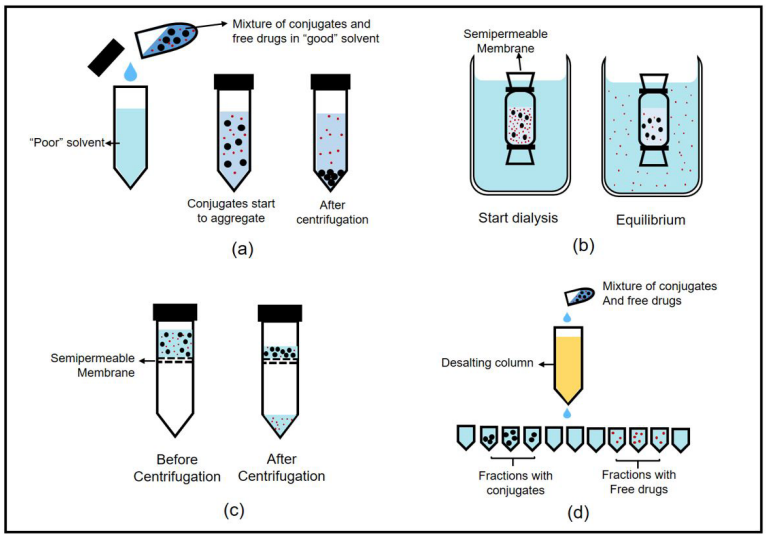


Figure 7. Some Techniques to Purify Polymer-Drug Conjugates: (a) Precipitation, (b) Dialysis, (c) Ultrafiltration by Centrifugation, and (d) Desalting Column

Purification of Conjugates

Pure conjugates are required for *in vitro* and *in vivo* studies in order to avoid false positive or negative. The conjugates must not contain free unreacted drugs that can bias the in vitro and in vivo study results. There are some purification technique that can be used to separate conjugates and the unreacted drugs, which include precipitation, dialysis and desalting column.

Precipitation is one simple way to isolate and purify polymers from small molecules. The technique needs a solvent that is "poor" for the polymer and "good" for the drug. A solution that contains a mixture of polymer and free drugs is then poured into the "poor" solvent, leading to precipitation of the polymer while leaving the free drugs in supernatant (see Figure 7a).

Dialysis involves a semi-permeable membrane with certain molecular weight cut off (MWCO). The principle is basically retention of molecules larger than MWCO in the donor compartment, while all molecules smaller than MWCO pass through the membrane into the acceptor compartment (Figure 7b). The migration of the molecules from donor to acceptor compartment will reach equilibrium, a condition where the concentration of the molecules in donor compartment is equal to that in acceptor compartment. In equilibrium state, there will be no more diffusion of the molecules through the membrane because of concentration gradient. The solvent in acceptor compartment must be regularly replaced with the new one in order to maintain the concentration gradient, and therefore allow the diffusion of the small molecules.

Other technique that adopts the principle of dialysis is ultrafiltration by centrifugation (Figure 7c). This technique requires centrifugation tube, which consists of two membrane-separated compartments. The membrane is also semipermeable and has MWCO. The solution of conjugates and drugs mixture is placed in the upper part of the tube (donor compartment), and then centrifuged. The centrifugation force will pull down the solvent through the membrane, together with the molecules smaller than MWCO and the solution residue that contains conjugates will stay in the upper part. By adding a new solvent to the donor compartment, and repeat the centrifugation for several times, pure conjugates can be obtained.

Desalting column is also a versatile tool to purify macromolecules. The principle of desalting column is basically similar to size exclusion chromatography, which is separation based on the molecular size. The solution of conjugates and drugs mixture is passed through the column and then the eluted fractions are collected (Figure 7d). All fractions are then analyzed, e.g. by HPLC, to confirm which fractions contain conjugates.

CONCLUSION AND FUTURE PERSPECTIVE

Natural polymers are potential candidate for carrier in drug delivery system. There are numerous studies in polymer therapeutic using natural polymer. However, we have not found any international publication in this field from Indonesian researcher. Therefore, we would like to encourage Indonesian researcher to explore this area, which includes production of pure natural polymer with well-defined characteristic, synthesis of polymer-drug conjugate to improve the efficacy of commercially available drugs, in vitro and in vivo study of the conjugates, as well as clinical study. Technical aspects in synthesis and analysis might become challenges due to limited facilities and analytical instruments. For example, Nuclear Magnetic Resonance (NMR) is not commonly available in every research institution. There are only few institutions in Indonesia that have NMR facility, including Research Center for Chemistry LIPI (Serpong), Universitas Gadjah Mada (Yogyakarta), Airlangga University (Surabaya), and Bandung Institute of Technology (Bandung). Therefore, collaboration between institutions, as well as government role in mapping and providing the required analytical instruments and facilities, are essential. Furthermore, collaboration between different experts, including medicinal chemists, organic chemists, pharmacists and clinicians, is also utmost important.

ACKNOWLEDGMENT

We would like to thank Dr. Joaquin Sanchis Martinez (Monash University) for his support, knowledge, and motivation. Erny Sagita and Arif Arrahman would also like to thank LPDP scholarship for Ph.D. candidature funding.

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