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In vitro kinetics characterisation of polymeric nanoparticles for anticancer therapy

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Abstract

World Health Organization (WHO) predicts that cancer incidence will increase in the future, thus research involving anticancer agents such as nanoparticles has gained significant importance. Nanoparticles can be made from various materials, but the focus on polymeric chitosan and/or carrageenan-based nanoparticles is significant. Research on these materials investigates dynamic parameters of *in vitro* drug release, stability under working conditions and stability under storage conditions (*in vitro* kinetics characterisations). Here, a literature review is conducted to provide in-depth insights on research methodology trends, drawbacks, suitability, suggestions for improvements and findings related to polymeric carrageenan and/or chitosan nanoparticles for anticancer therapy. Journal articles involving nanoparticles made from chitosan and/or carrageenan containing anticancer agents published between 2017 and 2022 were acquired through Google Scholar search using relevant keywords. Generally, the methods used to investigate drug release kinetics of nanoparticles can be categorised into dialysis membrane, sample and separate or direct measurement methods. Studies on the response of physiochemical characteristics towards changes in environment do not vary highly and are generalisable. Stability studies primarily measure the physicochemical changes of nanoparticles as a response measurement towards storage conditions. Both drug release selectivity and physicochemical characteristics response in different pH environments were found to be predictable *via* the ionisation of polymers and drugs used in different pH. The size of the nanoparticles formed during polyelectrolyte complexation process was found to be at its minimum at a balanced pH, possibly due to increased polymer-polymer attraction. The methods used for *in vitro* kinetics studies were generalised, and suggestions to address potential sources of errors were given in the current review. The selectivity of drug release and changes in physicochemical characteristics of the nanoparticles in different pH environments were found to largely coincide with the principles of ionisation of nanoparticle constituent.

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Introduction

The World Health Organization (WHO) has predicted that between 2008 and 2030, the incidence of cancer will increase at an alarming rate, which is a rate of 40% in high-income countries, and 80% in low-income countries. The organisation has also predicted that by the year 2030 the diagnosis of new cancer cases will reach 10-11 million (World Health Organization, 2012). In fact, from 2018 to 2040, in Malaysia alone, the yearly incidence of breast cancer and lung cancer is expected to increase by more than 70% and 100%, respectively (World Health Organization, 2020). Based on these two facts, it is expected that the demand for more efficacious and safer treatment options will increase dramatically in the upcoming years, both locally and internationally. While chemotherapy is an available treatment option, the main problem that comes with chemotherapy is the inherently unavoidable adverse effects and the emerging problem of chemotherapy-resistant cancer (Al-Samydai et al., 2019). Thus, it is important to further continue research on cancer to find new anticancer therapy improvements that are more efficacious and are minimally toxic to the human body.

Throughout recent years, nanoparticles have gained attention as possible solutions for the problems found in anticancer therapy. Nanoparticles are conventionally defined as materials that are produced at a size approximately between 1 nm and 1000 nm (Zielińska et al., 2020). Due to their properties, nanoparticles may help overcome several problems seen in conventional medicine, including in cancer medicine. Nanoparticles are beneficial due to their ability to ensure that the drugs remain in the body for a longer time. This is because instead of one bolus of drugs being administered directly into the body, nanoparticles that contain the drug will exhibit a sustained-release kinetics (Chu et al., 2019; Trousil et al., 2020). This in turn will cause an increase in virtual half-life, which thus results in prolonged presence and action in the human body. In truth, the actual degradation of the drug by the body is unchanged, but the degradation of the drug can only occur for the drugs that have been released from the nanoparticles. Therefore, the drugs that are degraded are simply replaced due to the slow drug-release kinetics (S. Wang et al., 2020). Nanoparticles also help in specific targeting of organs or sites intended for therapy. This can be achieved due to the inherent kinetics of the nanoparticles, such as by being engulfed by macrophages or adhesion due to positively-charge surface (Chu et al., 2019; Trousil et al., 2020), or due to environment-responsive nature of the nanoparticles such as magnetic or acidic stimuli (S. Wang et al., 2020; Zhang et al., 2019), or due to the incorporation of targeting moieties such as ligands (Hoshyar et al., 2016). The selectivity of the nanoparticles implies that they may enhance the pharmacodynamics effect of the drug or reduce toxicity (Chu et al., 2019; Trousil et al., 2020; S. Wang et al., 2020; Zhang et al., 2019). This can be explained by the higher proportion of drugs being sequestered and released at the site of interest rather than at other sites of the body, and hence the effect of the formulation causes more therapeutic effect and fewer side effects.

Out of all the materials that have been used in nanomedicine, hydrogel shows a very promising characteristic. The biggest reason was due to its exceptional ability to protect drugs from enzymatic degradation (Utreja et al., 2020). It is also a stable nanoparticle due to the avoidance of coalescence and reduced drug leakage (Kharkwal & Janaswamy, 2017). Lastly, its ability to hold water and swell or shrink to control the rate of drug delivery and its biocompatible characteristics have caused it to gain significant traction for use in medicine (Narayanaswamy & Torchilin, 2019). Chitosan and carrageenan are two of the materials that have been used to formulate the nanoparticles and are extensively studied.

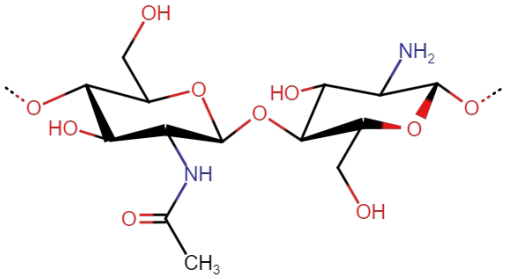

Fig. 1: The chemical structure of a chitosan monomer, which can either be in acetylated form (left) or deacetylated form (right).

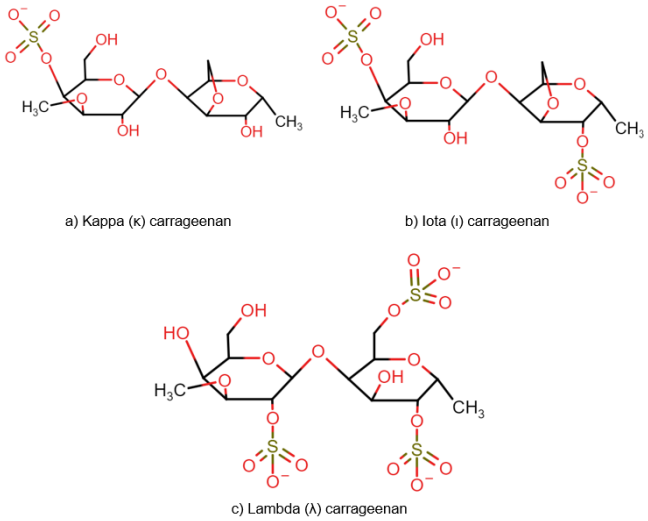

Fig. 2: The chemical structure of a carrageenan monomer. The different form of its (a) kappa, (b) iota and (c) lambda forms are delineated.

Chitosan is a polymer produced when naturally-occurring chitin undergoes deacetylation. The molecular structure consists of repeating chain units of N-acetyl-d-glucosamine and D-glucosamine, with each monomer unit having hydroxyl and amine groups (Rostami, 2020), as seen in Figure 1. Its popularity in nanomedicine can be attributed to its biocompatible, biodegradable, non-toxic and bioactive properties. To some extent, the positively charged chitosan may also impart site-specific targeting properties to chitosan nanoformulations (Ali & Ahmed, 2018). Meanwhile, carrageenan is an anionic polymer extracted from red algae that is used in pharmaceuticals, cosmetics and the food industry due to its gelling and thickening properties as well as its intrinsic antiviral, immune-activating and anticoagulant properties. Its molecular structure consists of polymer chains with alternating monomers of D-galactose and 3, 6-anhydro-galactose (Zia et al., 2017), which can be seen in Figure 2. Out of the three forms of carrageenan, only kappa (κ) and iota (ι) forms have considerable gel-forming properties, with the kappa(κ)-formed gels having a more strong and brittle properties while iota(ι)-formed gels having a more elastic and soft properties (Hotchkiss et al., 2016; Zia et al., 2017).

In the research of nanomedicine for cancer therapy involving these two polymers, the scheme of the study generally involves formulating the nanoparticle, followed by measurement of the static characteristics such as size, polydispersity index (PDI) and surface charge or zeta potential. This is followed by the measurement of dynamic characteristics such as drug release over time, stability under working conditions and stability under storage conditions. While the methodology of the first part of the study (i.e. static characteristics) has been widely researched and optimised, the second part of the study (i.e. dynamic characteristics) are highly variable in terms of their method due to differences in expected applications, objectives, focus, and understanding of the mechanics involved. Hence, there are considerable problems in regard to standardisation which therefore brings the question of accuracy, repeatability and suitability of methodology. Here, a literature review of the procedure used to measure drug release studies, stability under working conditions and stability under storage conditions in recent studies involving chitosan/carrageenan nanoparticles for anticancer therapy was conducted. In this literature review, the methods used in the measurement of dynamic characteristics were generalised, and the drawbacks and suitability of the decisions made in regard to study procedure were defined. Suggestions for consideration in future studies were also given in order to possibly improve research outcomes. Lastly, the findings of the research included in the literature

review were generalised to allow future researchers to predict the findings of their formulation more accurately.

Methodology

The current literature review involves the usage of search engines on the internet, which includes Google and Google Scholar. Our method involves two phases. The first phase (Phase I) is the acquisition and collection of journal publications and research accessible via open access or institutional access that will form the basic framework of current research practice and general key findings. In this phase, the criteria are that the articles collected must not be a review, must be a study involving nanoparticles or microparticles made from either chitosan, carrageenan and/or their derivatives, must contain substances with reported anticancer properties, and must be published within the previous 5 years (i.e. 2017-2022). The keywords used for this phase are chitosan, carrageenan, nanoparticles and anticancer.

Microparticles are included in the current phase of the literature review because, while nanoparticles have a higher propensity to be absorbed intact into cancer cells, microparticles share much of the same advantages with the nanoparticles. Primarily, it addresses the protection of drug degradation, overcoming the limitation of limited bioavailability, and selectivity in delivery is still possible when the choice of the polymer used is optimised. Macro-hydrogels are excluded due to the material being too different in physical nature from nanoparticles. Review articles are also excluded from Phase I of literature acquisition.

The second phase (Phase II) involves the acquisition of publications that are supplementary to the critiques, comments and suggestions given for the current research trend and serve as evidence that certain considerations must be made in future studies. For this phase, the usage of reviews, case studies, book publications and the like are included, which may or may not be focused on carrageenan, chitosan, nanoparticles and anticancer compounds but nevertheless are relevant considerations to the discussion at hand. The requirement for publication date for this phase is less stringent, although newer studies are prioritised and publications that are more than 5 years old are included if and only if publications relevant to the current discussion are not found.

Drug Release Profiling

General procedure and considerations in drug release

Drug release studies are conducted by allowing the nanoparticles to be exposed to the drug release media which mimics the physiological conditions of the body, while a predetermined volume of samples is taken at appropriate time intervals and replaced with an equal volume of drug release media. The samples are then analysed for drug content using spectrophotometric methods such as UV-Vis spectrophotometry or HPLC. The kinetics of the drug release is then graphed and, in some cases, analysed to fit onto zero order (Gaur et al., 2022; Irani & Nodeh, 2022; Nguyen et al., 2022; Sabra et al., 2018; Shafiee et al., 2019; Yan et al., 2018), first order (Gaur et al., 2022; Nguyen et al., 2022; Sabra et al., 2018; Shafiee et al., 2019; Yan et al., 2018), Higuchi (Gaur et al., 2022; Irani & Nodeh, 2022; Nguyen et al., 2022; Sabra et al., 2018; Sahu et al., 2017; Shafiee et al., 2019; Yan et al., 2018), Hixson Krowell (Gaur et al., 2022; Nguyen et al., 2022; Shafiee et al., 2019) and Korsmeyer Peppas (Gaur et al., 2022; Irani & Nodeh, 2022; Nguyen et al., 2022; Sabra et al., 2018) kinetics of drug release in order to postulate regarding the release mechanism of the drugs that are encapsulated. Drug release methods between studies typically differ in the methods of nanoparticle separation prior to analysis which may be classified into three; (a) dialysis membrane method, (b) sample and separate method which is the most widely used, and (c) direct measurement method which is cited in one study. The studies also differ in terms of the drug release media used, the drug release study period, volume of media used and less commonly, temperature.

The drug release media used is typically a saline buffer system, usually phosphate buffer, which has been adjusted to a certain pH which mimics the environment that nanoparticles are subjected to. This includes normal extracellular

conditions of pH 7.4 (Dhavale et al., 2021; Herdiana et al., 2022; Irani & Nodeh, 2022; Nogueira et al., 2020), cancer cell environment of pH 5.0-6.0 (Herdiana et al., 2022; Nogueira et al., 2020), cell cancer endosome of pH 4.0-5.5 (Dhavale et al., 2021; Herdiana et al., 2022; Nogueira et al., 2020; Vinothini et al., 2019), gastric conditions of pH 1.2 (Nguyen et al., 2022; Sabra et al., 2018, 2019; Sun et al., 2020; Yan et al., 2018; Yusefi et al., 2021), intestinal or colonic conditions with pH ranging between 4.50 to 7.40 (Nguyen et al., 2022; Sabra et al., 2018, 2019; Shafiee et al., 2019; Sun et al., 2020; Yan et al., 2018; Yusefi et al., 2021) as well as skin pH ranging from pH 5.0-7.4 (Sahu et al., 2017).

However, drug release media are not only limited to a simple buffer system. Some studies opted for a more complex media. In some studies, the buffer system used was supplemented with pepsin or pancreatin and bile to simulate gastric and intestinal conditions respectively (K. Liu et al., 2020; Sun et al., 2020). In some cases, the drug release media used was prepared from harvested fluids from animals, such as in the case of a study which harvested caecal contents from rats' caecum directly to be used for drug release studies (Sabra et al., 2018, 2019). This approach in preparation of drug release media, while more costly, comes with the advantage of being able to more accurately reflect the physiological conditions which the nanoparticles may be exposed to. The importance of taking into account the effect of enzymes and/or biological constituents in drug release studies should not be underestimated. Chitosan, on top of undergoing slow-rate non-enzymatic hydrolysis, also undergoes enzymatic hydrolysis in the human body (Jennings, 2017). Meanwhile, bile salts secreted in the intestinal phase of digestion may help with dissolution of hydrophobic drugs (Bourbon et al., 2018). Thus, wherever possible, relevant enzymes should be included in the drug release media.

For nanoparticles prepared for oral ingestion, the nanoparticles are expected to undergo several different conditions at different segments of the digestive tract. Because of this, the approach to the drug release studies might be different compared to formulations meant for other modes of administration. Typically, this would be investigated by carrying out the procedure at different pH levels as different "runs" of the procedure (Nguyen et al., 2022; Shafiee et al., 2019; Yan et al., 2018; Yusefi et al., 2021). However, a more accurate approach would be that the nanoparticles should be exposed to gastric conditions first prior to being introduced to intestinal conditions. This is because such an approach would take into account the possibility of non-reversible changes of the nanoparticle caused by gastric conditions prior to intestinal conditions (C. Liu et al., 2020). Fortunately, some studies have shown such an approach. In some studies involving nanoparticles meant for oral administration, the nanoparticles were suspended in a media with a pH of 1-2 for up to 2 hours before the nanoparticles were added into media resembling the intestinal system (K. Liu et al., 2020; Sabra et al., 2018; Sun et al., 2020). While procedures from one study to another might slightly differ, there exists an international consensus on how to conduct simulated digestion (Mulet-Cabero et al., 2020) which has been used as a guideline where formulations are expected to pass through the gastrointestinal tract.

The temperature used to study the drug release predominantly cites the usage of 37°C as the temperature condition used due to this temperature being physiologically relevant (Arif et al., 2017; Fan et al., 2020; Gaur et al., 2022; Herdiana et al., 2022; Irani & Nodeh, 2022; Ji et al., 2017; K. Liu et al., 2020; Nguyen et al., 2022; Nogueira et al., 2020; Sabra et al., 2018; Sahu et al., 2017; Shafiee et al., 2019; Sun et al., 2020; Yan et al., 2018; Yusefi et al., 2021). The temperature of 40°C has also been cited in one study (Shafiee et al., 2019), which may be relevant in applications involving induced hyperthermia for the purpose of selective delivery (Tharkar et al., 2019). However, some studies cited the usage of other temperatures, such as 26°C (Karimi et al., 2018; Mahdavinia et al., 2017), of which the reasoning behind such a parameter is unclear.

The drug release study period also varies, but it usually is conducted for at least 24 hours (Fan et al., 2020; Sabra et al., 2018, 2019). However, shorter periods may also be possible at 4-6 hours for gastrointestinal applications (K. Liu et al., 2020;

Nguyen et al., 2022; Sun et al., 2020). Longer study periods are also reported in literature, among the longest of which included in current literature review is 96 hours (Sahu et al., 2017) and 120 hours (Shafiee et al., 2019). In one study, drug release investigation was conducted without a predefined period, and instead was conducted until complete release of drug (Irani & Nodeh, 2022). However, there are some studies which have opted for drug release studies shorter than 24 hours without intention for oral application due to complete release of drug (Gaur et al., 2022; Herdiana et al., 2022; Mahdavinia et al., 2017).

To date, the importance of assessing drug release of free or unencapsulated drugs still remains unappreciated, in which many studies were found to not have carried out this assessment as a form of control. Degradation of drugs at physiological conditions are not to be underestimated, as it has been found that such observation may be significant (Abouelmagd et al., 2015; Bourbon et al., 2018; C. Liu et al., 2020; K. Liu et al., 2020; Moradi et al., 2021). Here, it should be highlighted that drug release studies involving only the drug-loaded nanoparticles may become questionable, as degradation of drugs, if significant, may artificially cause an appearance of slow drug release. In the same extension, the sustained release potential of nanoparticles may be underestimated if the nanoparticles give some sort of protection from drug degradation such as shown in a study (Moradi et al., 2021). Other than that, the assessment of drug release of unencapsulated drugs may also give more insight into the validity of the drug release method chosen.

The volume of the drug release media is equally important to be considered. Generally, sink conditions of at least 3 times the volume required to achieve saturated concentration of drug must be achieved (Abouelmagd et al., 2015). This is because violation of sink conditions leads to the inaccurate appearance of low drug release (Yu et al., 2019). While this is easily achieved for hydrophilic drugs, problems arise with hydrophobic drugs. This is because for this class of drugs, they would conventionally require a high volume of water, leading to analytical difficulty due to low drug concentration. Alternatively, samples may be concentrated after sampling, or drug release media may be added with a dissolution aid such as detergents which increase solubility of drug (Abouelmagd et al., 2015).

By and large, it is somewhat complicated to assess whether or not the majority of studies meet the volume requirement stated above. This is because not all studies report their procedure to the full extent, and the exact saturated solubility in physiological conditions would be expected to be different than that reported with pure water at 25°C, which is more available. However, generally, reports of saturated solubility studies in studies which encapsulates hydrophobic drugs, such as mangostin and curcumin, are rather scarce. Nonetheless, the volume of media varies highly from as low as 10 mL in a sample and separate method (Nogueira et al., 2020), and as high as 500 mL (Irani & Nodeh, 2022). Most studies cited the volume between 20-60 mL to be used as a drug release media (Herdiana et al., 2022; Ji et al., 2017; Karimi et al., 2018; Mahdavinia et al., 2017; Sahatsapan et al., 2021; Sahu et al., 2017; Sun et al., 2020; Yan et al., 2018).

Here, a required validation step should be considered which may help address some uncertainties associated earlier. A drug solubility experiment may be set-up whereby excess drug is allowed to incubate and agitate in the media chosen for drug release at conditions meant to be studied (ie. PBS, 100 rpm at 37°C), at two time points (such as at 7 hours, and 24 hours). Then, the media are to be sampled, centrifuged to separate solid undissolved drug, and its supernatant is spectrophotometrically analysed for drug concentration. This will allow experimental quantification of saturated concentration, and hence, allow the determination of volume which meets sink condition. Additionally, drug degradation in the media can be assessed by comparing the drug content detected at the two different time points (Abouelmagd et al., 2015). Alternatively, confirmation of sink condition may also be partially proven by including free drug in the drug release studies in similar amounts to encapsulated drugs. To summarise this section, suggestions are tabulated as shown in Table 1.

Table 1: Aspects of drug release profiling studies and suggestions to improve quality of data acquired.

Aspect	Suggestions	Remarks	References
The drug release media used	Simple buffer system fixed at certain pH	Drug release only affected by ionic and pH conditions	(Dhavale et al., 2021; Herdiana et al., 2022; Irani & Nodeh, 2022; Nguyen et al., 2022; Nogueira et al., 2020; Sabra et al., 2018, 2019; Sahu et al., 2017; Shafiee et al., 2019; Sun et al., 2020; Vinothini et al., 2019; Yan et al., 2018; Yusefi et al., 2021)
	Media supplemented by enzymes and biological constituent	Takes into account drug release caused by enzymatic degradation	(Bourbon et al., 2018; Jennings, 2017; K. Liu et al., 2020; Sun et al., 2020)
	Drug release media harvested directly from animal	Takes into account participation of constituents that may be present in the micro-scale	(Sabra et al., 2018, 2019)
Temperature of the drug release media throughout the procedure	37 °C	Results are more applicable/imposable for physiological therapy compared to other temperatures	(Arif et al., 2017; Fan et al., 2020; Gaur et al., 2022; Herdiana et al., 2022; Irani & Nodeh, 2022; Ji et al., 2017; K. Liu et al., 2020; Nguyen et al., 2022; Nogueira et al., 2020; Sabra et al., 2018; Sahu et al., 2017; Shafiee et al., 2019; Sun et al., 2020; Yan et al., 2018; Yusefi et al., 2021)
	40 °C (locally-induced hyperthermia)	May take into account thermal sensitivity which may be present in the nanoparticle system	(Shafiee et al., 2019)
Approach for orally-administered nanoparticles, requiring considerations of release profiles at different conditions; gastric, intestinal and colonic	Different batches of prepared nanoparticles are incubated at different conditions representing different segments of digestive tract throughout the whole study	The behaviour of nanoparticles' drug release profile at different conditions are considered separately – theoretical pH-release explanation may be more generalised	(Nguyen et al., 2022; Shafiee et al., 2019; Yan et al., 2018; Yusefi et al., 2021)
	Change in condition are simulated in sequence; gastric conditions, followed by intestinal conditions	Takes into account the nanoparticle matrix degradation caused by gastric conditions prior to exposure to intestinal or colonic conditions	(C. Liu et al., 2020; K. Liu et al., 2020; Mulet-Cabero et al., 2020; Sabra et al., 2018; Sun et al., 2020)

Study period	24 hours	The standard study period for nanoparticles meant for systemic circulation	(Fan et al., 2020; Sabra et al., 2018, 2019)
	>24 hours	May be suitable for nanoparticles with a really slow drug release	(Sahu et al., 2017; Shafiee et al., 2019)
	<24 hours	The study does not need to be prolonged more than needed in cases where all of the drugs has been released	(Gaur et al., 2022; Herdiana et al., 2022; Irani & Nodeh, 2022; Mahdavinia et al., 2017)
	4-6 hours	The standard study period for oral administration	(K. Liu et al., 2020; Nguyen et al., 2022; Sun et al., 2020)
Inclusion of free drug in the study as a means of control	Free drug needs to be included in the study	• Verifies that the results are not affected by degradation of drugs • May prove the sink conditions of the volume of media used • Able to take into account interference caused by nanoparticle separation methods	(Abouelmagd et al., 2015; Bourbon et al., 2018; C. Liu et al., 2020; K. Liu et al., 2020; Moradi et al., 2021)
Hydrophobic drug requiring high volume of media to achieve sink conditions and causing analytical difficulties due to highly diluted concentration	Concentrate the sample by means of evaporation prior to analysis	• Allows the usage of media without the presence of dissolution aid	(Abouelmagd et al., 2015)
	Add dissolution aid such as detergents	• Allows sink conditions to be achieved at low volume	(Abouelmagd et al., 2015)
Validation of sink conditions	Conduct drug solubility experiment in intended drug release media at relevant conditions (37°C etc.) for at least 24 hours	• Able to confirm that sink conditions (3 times the saturated volume) are achieved • Able to confirm that slow drug release is not due to saturation of media	(Abouelmagd et al., 2015)
	Inclusion of free drugs as one of the study groups.	• Partially proves that sink conditions are achieved	(Abouelmagd et al., 2015; Bourbon et al., 2018; C. Liu et al., 2020; K. Liu et al., 2020; Moradi et al., 2021)
Drug degradation during experiment period	Conduct drug solubility experiment in intended drug release media at relevant conditions (37°C etc.) and measured at two different time points	• Able to confirm that drug degradation is not significant factor in the study • Able to confirm that appearance of slow drug release is not caused by drug degradation	(Abouelmagd et al., 2015; Bourbon et al., 2018; C. Liu et al., 2020; K. Liu et al., 2020; Moradi et al., 2021)

Method to separate nanoparticles/adjust quantification to nanoparticle interference

As mentioned previously, the methods of drug release studies can be generally classified into three, which are the dialysis membrane method, the sample and separate method, and the direct measurement method. Of these three, the dialysis membrane method is the popular choice, followed by the sample and separate method, and then the direct measurement method. Only one study cites the direct measurement method, and it does not seem to be a widely-used method for quantification of drugs, presumably due to the questionable methodology and high uncertainty in regards to accuracy.

Dialysis membrane method

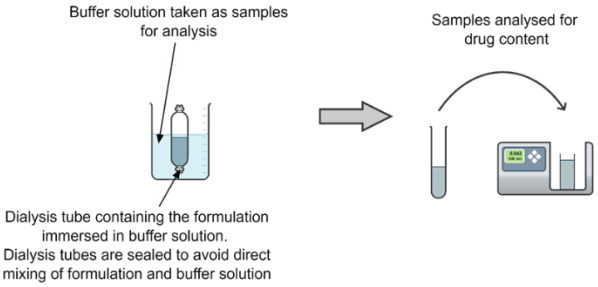

Fig. 3: The schematic diagram representing the dialysis membrane method of drug release studies.

In the dialysis membrane method, nanoparticles are put inside a dialysis bag which is then sealed and incubated in a drug release media. To this effect, the nanoparticles inside the dialysis bag are not mixed with the drug release media outside the dialysis bag. Instead, the drug has to be released inside the bag first before it can diffuse out as a free drug due to size selectivity of the membrane. This allows samples to be taken out and directly analysed without needing any particulate separation steps (Sahatsapan et al., 2021). A schematic diagram representing the dialysis membrane method is shown in **Figure 3**.

For nanoparticles that are dried, they are generally pre-dispersed in their respective drug release media prior to conducting the drug release studies (Fan et al., 2020; Gaur et al., 2022; Herdiana et al., 2022; Ji et al., 2017; Sahatsapan et al., 2021; Yan et al., 2018; Yusefi et al., 2021). For nanoparticles that are already pre-dispersed, they are generally allowed to retain their original dispersant when loaded into the dialysis membrane (Arif et al., 2017; K. Liu et al., 2020; Sahu et al., 2017; Vinothini et al., 2019). Generally, the dialysis bag is expected to be fully immersed into the drug release media. For buoyant dialysis tubes, generally, the help of a sinker may aid in ascertaining this condition (Gaur et al., 2022).

One of the strengths of the dialysis membrane method is that the separation step does not need to be carried out in this method (Modi & Anderson, 2013). This is not only more convenient, but also avoids the pitfalls related to the separation procedure that is inherent to separation methods. That is, the pressure applied in the sample and separate method inherently disturbs the equilibrium, and incomplete separations may cause data to be significantly erroneous (Modi & Anderson, 2013).

However, the usage of the dialysis tubes has its weaknesses. Mainly, those who opt to use this method are advised to properly consider the possibility of erroneous conclusion arising from the effect of the compartmentalisation of the donor phase (inside the dialysis tube) from the receiver/acceptor phase (outside the dialysis tube). It has been shown that wrongful interpretation of data can arise from the diffusion of drug through the dialysis membrane itself being a more significant rate-limiting step than the drug release from the nanoparticle, or that interactions of released drug with concentrated nanoparticle constituents significantly affecting the drug content in the donor compartment (Modi & Anderson, 2013; Moreno-Bautista & Tam, 2011; Wallace et al., 2012; Weng et al., 2020; Yu et al., 2019; Zambito et al., 2012).

To this extent, a few key decisions have to be made prior to conducting this method of study. The size selectivity of dialysis bags is often expressed as molecular weight cut-off (termed MWCO) which describes the size of the pore. While it is possible for drugs to diffuse through a

dialysis bag as long as the drug molecular weight is lower than the MWCO of the dialysis bag, it has been shown that selecting a dialysis membrane with a low MWCO may cause a lower rate of drug diffusion across the membrane despite being higher than the drug's molecular weight (Moreno-Bautista & Tam, 2011; Yu et al., 2019).

Additionally, drug diffusion across the membrane is also governed by the material of the membrane itself. While it has been suggested that the usage of membranes with 100 times higher MWCO than the size of the molecule would negate membrane resistance, a study demonstrated that a cellulose ester membrane which meets this criterion has a slower diffusion rate of doxorubicin compared to a regenerated cellulose membrane which does not meet this criterion (Yu et al., 2019). There has also been evidence suggesting that drug release may be overestimated due to destabilisation of the drug release membrane in acidic conditions which reduces efficient compartmentalisation of acceptor and donor phases of the experiment, and the interaction of the dialysis membrane with the nanoparticle, altering drug release (Weng et al., 2020).

Lastly, there have also been questions raised regarding using detergent-containing media for hydrophobic drugs. Particularly, most of the time, detergents could not pass through the dialysis membrane. Thus, the presence of detergent inside the dialysis bag may lead to the drug released to interact with the detergents, which in turn prevents the drug from passing through the dialysis bag. Meanwhile, if the detergent is only present outside the dialysis bag, then the drug may precipitate in the dialysis bag once it is released out of the nanoparticle, owing to their low solubility in water (Abouelmagd et al., 2015).

Here, to reduce the resistance of diffusion caused by the dialysis bag, dialysis tubes with higher MWCO should be selected. The study mentioned earlier has recommended that dialysis membranes with MWCO of 1000 kDa should be sufficiently small to provide a barrier to nanoparticles with a 100 nm size range. Additionally, as a means of validation, drug release from free drug and empty nanoparticles spiked with drug may be carried out to eliminate the possibility of misinterpretation due to the rate-limiting process of diffusion through the dialysis membrane, and the binding effect of the nanoparticle. Alternatively, instead of empty nanoparticles spiked with drugs, the study also suggested that repetition of the drug release studies at different drug-loaded nanoparticle concentrations may give additional insight (Modi & Anderson, 2013). Additionally, the possibility of using mathematical models proposed and investigated in one study may also be possible, although the usage of the model is not yet widely explored (Yu et al., 2019).

Sample and separate method

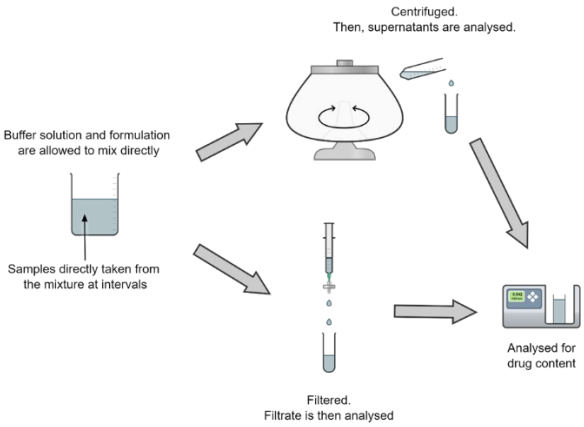

Fig. 4: The schematic diagram representing the dialysis membrane method of drug release studies.

Unlike the dialysis membrane method, this method of quantification involves allowing the nanoparticles to be directly dispersed in the drug release media in one compartment. Separation is then conducted *via* centrifugation (Mahdavinia et al., 2017; Sabra et al., 2018; Sun et al., 2020), magnetic separation (Nogueira et al., 2020), filtration, or combination, and the supernatant or filtrate are then analysed for drug content. A schematic diagram representing the sample and separate method is shown in **Figure 4**.

Unconventionally, due to the nature of this method, the drug release may be conducted either in a discontinuous method. In the continuous or conventional method, nanoparticles are suspended in drug released media. Then, the media are sampled at predetermined time points while allowing the nanoparticles to continuously release drugs without any discontinuation. The samples are put through a separation process prior to measurement (Mahdavinia et al., 2017; Sun et al., 2020). In the discontinuous method, the nanoparticles are suspended in release media and then at predetermined time points, all of the drug release media are put through the separation process. The supernatants are analysed and the pelleted nanoparticles are then resuspended in drug release media to recontinue the drug release investigation. With this approach, lower volumes of release media are required and thus concerns about excessive dilution may be addressed (Sabra et al., 2018).

Under the current scope, no filtration-based separation was chosen as the separation method. Instead, the centrifugation method was always chosen as a separation method, and all of the conditions of separation are relatively mild. Centrifugation speed only ranges from 4000-8000 rpm, for at most 10 minutes in all of the studies that fall under this category (Mahdavinia et al., 2017; Sabra et al., 2018; Sun et al., 2020). Nevertheless, in the upcoming section, widely recognised separation methods will be discussed. The goal is to provide future researchers with insights into the strengths and weaknesses of these methods, enabling them to make well-informed decisions.

The strength of this method is that drug release into the release media is not affected by the presence of a dialysis tube. As discussed previously under the dialysis membrane method, the dialysis rate may become the rate limiting step that causes an artificial appearance of slow release. Meanwhile, using the sample and separate method, such a weakness is not seen (Wallace et al., 2012). Centrifugation steps also seem to address the possibility of drug adsorption to the nanoparticle, as it has been demonstrated that drug-nanoparticle adsorption effect is reduced under this method in lieu of the pelleting of nanoparticles reducing the total surface area for adsorption (Zambito et al., 2012).

One of the weaknesses in this method lies in the effect of the separation step towards the data acquired in this study. Firstly, complete separation may not actually be achieved even in extreme conditions which thereby may cause higher apparent drug release compared to the actual value (Jung et al., 2018; Wallace et al., 2012; Weng et al., 2020). Secondly, it is possible for the separation step to disturb the equilibrium due to the force applied during the separation step (Jung et al., 2018; Modi & Anderson, 2013), which thereby might induce higher drug release from the nanoparticle. Thirdly, separation steps which take long period of time to complete associated with ultracentrifugation prohibit that data being assumed to reflect the instance defined as sampling time (Wallace et al., 2012). Fourthly, filtration-related separation steps, whether pressure-assisted or centrifugation-assisted, may yield a low volume of filtrate, which may not accurately represent the actual free drug concentration in the sample as the filter membrane needs to be saturated with the drug prior to effective filtration (Wallace et al., 2012). By the very nature of the method, several separation techniques can be used. The findings in current literature are summarised under **Table 2**.

Table 2: Separation methods used under the sample-and-separate method and their key consideration.

Methods	Key considerations	References
Centrifugation	• Long centrifugation time • Disrupts the equilibrium of nanoparticle-bound drugs Has inferior separation yield compared to other separation steps, especially if nanoparticles and surrounding media have insignificant density difference	(Jung et al., 2018; Modi & Anderson, 2013; Wallace et al., 2012; Weng et al., 2020)
Filtration	Requirement of higher volumes due to the adsorption of drug onto the filter membrane	(Wallace et al., 2012)
Centrifugation-assisted filtration	Requirement of higher volumes due to the adsorption of drug onto the filter membrane	(Wallace et al., 2012)

To overcome the low filtrate volume associated with the centrifugation-assisted filtration, one may increase the centrifugation speed and time of the nanoparticle to improve volume yield (Weng et al., 2020). However, such an approach should be done with consideration that the more these parameters are increased, the more chances for deformation to occur, disrupting nanoparticle integrity, and thus, the advantage of using this method, ie. a gentler condition compared to the centrifugation method would be lost (Wallace et al., 2012).

Here, a validation method *via* the usage of dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA) should be considered to quantify the amount of particulate seen in the filtrate or supernatant. The count rate, expressed as kilo counts per second, would then be compared to the count rate measurement of the original media, such as drug release media, to determine whether complete separation has taken place. This is the method used in several studies to ascertain the separation of the nanoparticles from the media in many studies (Jung et al., 2018; Wallace et al., 2012; Weng et al., 2020). The filtration step, which opens the possibility of low drug concentration detected due to adsorption effect, also needs to be validated. Thus, from the same studies, the usage of drug recovery validation method should be considered, whereby free dissolved drug solutions of known concentrations are filtered through the filter membrane, and the concentration of drug in the resulting filtrate is measured *via* spectrophotometric method. The measured concentration is then compared with original concentration to quantify the drug loss resulting from the process (Jung et al., 2018; Weng et al., 2020).

It was mentioned earlier in the current review that apparent drug concentration in the drug release media may be altered due to disturbance in equilibrium during separation steps. A research strategy can be implemented to validate that this effect is not significant to the quantification of drug. This strategy involves examining how drug content, nanoparticle size of filtered nanoparticle, particles detected in filtrate using DLS method, and the volume yield changes according to the changes in either centrifugal force or time. Firstly, one of the factors of centrifugal force or time is fixed, while the other factor is varied. Test samples containing nanoparticles are then tested under these conditions, and the aforementioned parameters are measured. Next, they are then compared to determine suitability of the centrifugation procedure (Weng et al., 2020).

Direct measurement method

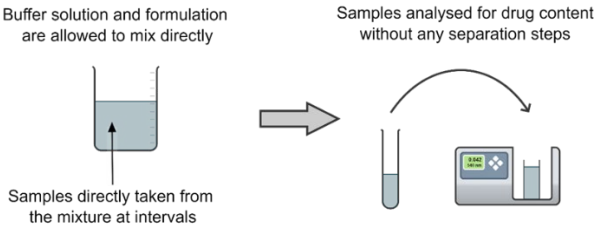

Fig. 5: The schematic diagram representing the direct measurement method of drug release studies.

Only one article was found citing the use of the direct measurement method. In this method, after sampling, the separation of nanoparticles from the sampled volume was not conducted. Instead, the concentration of the drug in the sample was analysed using UV-Vis spectrophotometry directly, using the empty nanoparticles dispersed in similar media as a blank. A schematic diagram representing the direct measurement method is shown in **Figure 5**. This accounts for the absorbance caused by the nanoparticle dispersion to be taken into account (Karimi et al., 2018). However, the usage of this technique is based on the assumption that the nanoparticle is highly reproducible in terms of size and PDI, and has low inter-batch standard deviation, as well as the assumption that loaded and empty nanoparticles have similar sizes. Here, it is erroneous to neglect proving the second assumption to be true, as it has been shown in some cases that drug-loaded and empty nanoparticles may be differ vastly in terms of size and PDI that merits ascertaining the truth of the second assumption (J. Wang et al., 2014; Yan et al., 2018). This does not take into account the possibility that changes in nanoparticle size may differ for loaded and unloaded nanoparticles. Overall, ascertaining the accuracy of this method is difficult. The usage of this approach as a potentially new method needs to be further investigated and optimised before it may be used with certain validity.

Quantification of drug release

The findings of drug release studies in the articles included are generalised here. Overall, the trends in drug release can be explained by how the ionisation of the drugs and nanoparticle constituents interact with each other. Generally, for nanoparticle complexes that encapsulate cationic drugs, more drug is released in acidic media. Meanwhile, for nanoparticle complexes that encapsulate anionic drugs, more drugs are released at higher pH. This is an observation that tends to occur under the studies included. It is possible that this is because at lower pH, protonation causes ionisation of cationic drugs and polymer, and deionisation of anionic polymer, which thus causes increased repulsion between the nanoparticle and ionic drug as well as increased passive diffusion into the hydrophilic environment. Meanwhile, the opposite is true for anionic polymer in neutral or a more basic pH. The increased repulsion force and heightened dissolution manifest themselves as increased drug release. Here, evidence that this generalisation is the most significant mechanism compared to other mechanisms is discussed.

It was found that in magnetic nanoparticles comprising of chitosan core and carrageenan outer layer, more drug is released in acidic media compared to neutral media (Karimi et al., 2018). Meanwhile, magnetic nanoparticles with carrageenan core and chitosan outer layer, despite being synthesised in the opposite manner, show the same observation (Jafari et al., 2021). Here, it should be noted that in both of these studies, sunitinib malate was the encapsulated drug, which exists as a cation at neutral pH and below (pKa = 9.8). Comparatively, chitosan and carrageenan are more protonated at acidic pH (pKa = 6.5 and pKa < 2.5 respectively), giving rise to a more charged cationic chitosan and less charged anionic carrageenan. Therefore, at lower pH, repulsive forces between the nanoparticles and sunitinib cation is more prevalent, owing up to the higher extent of cationic ionisation of the drug and chitosan, as well as the reduction of the anionic charge of carrageenan (Jafari et al., 2021; Karimi et al., 2018). Based on the two studies cited, it is possible that the configuration of which polymer constitutes the core, and which constitutes the surface is less relevant than the extent of

ionisation.

A similar case of magnetic nanoparticles made with carrageenan as the core constituent and chitosan as the outer layer further supports this point, which instead of encapsulating cationic sunitinib, encapsulates anionic methotrexate. The findings in this study are the opposite to the studies cited above, whereby more drug is released at neutral pH compared to acidic pH (Mahdavinia et al., 2017).

In a study involving cationic doxorubicin which was encapsulated by graphene oxide-carrageenan-biotin nanoparticles, it was generally found that higher release is observed in lower pH compared to neutral pH. This has been attributed to the ionisation of doxorubicin which breaks down the non-ionic bonding of the drug and graphene oxide substituent (Vinothini et al., 2019). Similar observations were found with cationic doxorubicin-loaded magnetic nanoparticles made from alkoxysilyl-modified κ-carrageenan (Nogueira et al., 2020) and doxorubicin-loaded chitosan-pectin silicon-dioxide nanoparticle (Ji et al., 2017).

Meanwhile, for anionic 5-fluorouracil-loaded chitosan-cellulose nanocomposite formulation, the opposite observation was found which is that the nanoparticle exhibited higher drug release at neutral pH rather than gastric pH (Yusefi et al., 2021). Similar observations are found in a study of chitosan nanoparticles complexed with carboxylic curdlan containing 5-fluorouracil, which was found to be released at a higher rate in neutral pH rather than in acidic conditions (Yan et al., 2018).

Meanwhile, in a study involving carrageenan core, chitosan outer layer and tripolyphosphate crosslinker microparticles loaded with α-mangostin, it was found that drug release is much higher in acidic conditions compared to more neutral conditions, which have been attributed to protonation of the particle constituents (Nguyen et al., 2022). In another study involving α-mangostin encapsulated in tripolyphosphate-linked chitosan nanoparticles, the drug is preferentially released in acidic media rather than neutral media (Herdiana et al., 2022). α-mangostin is estimated to dissociate *via* its carbonyl group, which would be basic. Thus, based on this estimation, the generalisation still holds true. It would be expected that the protonation of both chitosan and α-mangostin causes cation-cation repulsion in addition to increased solubility in the media, and hence enhanced release at acidic pH. However, one study involving this same drug found the opposite result. Studies involving nanoparticles made with chitosan as the core and carrageenan as the outer layer that encapsulates α-mangostin found that the drug is preferentially released in neutral media compared to acidic media. However, the effect of pH on drug release rates in this study was not significant (Wathoni et al., 2021).

Several studies observed the opposite trend as those postulated above. In a study involving anionic telmisartan encapsulated by magnetic chitosan nanoparticles, more drug is released in lower pH compared to higher pH (Dhavale et al., 2021). Meanwhile, in a study of polymalic acid surface-chitosan core nanoparticles loaded at the surface with cationic doxorubicin, the drug was found to be released at a higher rate in basic pH compared to lower pH (Arif et al., 2017). In order to rationalise this, the high likelihood the drug was adsorbed on the surface rather than sequestered into the nanoparticles as most other studies have cited should be considered, since it has been proposed to be the main drug loading mechanism. Here, the evidence of preferential drug release arising due to the breakdown of polymer-drug attractive interactions is apparent. In both of the studies cited, drugs are more deionised while the polymer constituents are much ionised in their preferential release conditions. Either of these occurrences may have led to the breakdown of stable drug-surface polymer interactions, hence causing enhanced release.

Another study also found the opposite observation than the generalised trend, involving PLGA-chitosan nanoparticle complexed with tripolyphosphate and coated with eucalyptus oil

for the controlled delivery of anionic 5-fluorouracil. It was found that maximum release was seen at lower pH compared to higher pH levels. Although results are not significant (Sahu et al., 2017), the effect is consistent enough that it warrants examination. Based on the findings of the research, it seems that despite the likelihood of 5-fluorouracil being sequestered inside the nanoparticle matrix, the current generalisation does not seem to apply here. However, this is an explainable exception as in this study, eucalyptus oil was used to coat the nanoparticle surface. Hence, due to hydrophobic-hydrophilic partitioning effect, deionised 5-fluouracil in acidic conditions partitioned more in the oily layer compared to ionised 5-fluouracil in basic conditions.

In the discussion, two studies are excluded from consideration as the drug release conditions are not equal in terms of other factors other than pH. One of the studies involves curcumin-loaded nanoparticles made from modified citrus pectin and chitosan meant for colon cancer, in which more drug release was observed in caecal conditions rather than gastric conditions (Sabra et al., 2018). The reason why this study could not be included to be generalised together with the other studies is that the caecal conditions in this study have enzymes, while the gastric condition does not. This opens up the possibility that the disparity in drug release was due to the enzymatic breakdown rather than the simple kinetics described above. A study involving a zein-Tween 80-carrageenan nanoparticle which encapsulates curcumin was also excluded from the current generalisation, whereby higher burst release was found in simulated intestinal fluid rather than in gastric fluid (Sun et al., 2020). This is because the presence of bile salts in the study may have been the major contributor to higher drug release in intestinal conditions. However, both of the studies cited above are similar in terms of their findings. Additionally, it was proposed that their findings were due to the lower solubility of curcumin, as well as the preference of tightly-knit formation over swollen formation at lower pH (Sabra et al., 2018). This theory is particularly supported by another study which reported opposite observation with curcumin-loaded chitosan-based nanoparticles, which might be caused by the opposite swelling response seen (Shafiee et al., 2019). Presence of bile salts and peptide in intestinal phase is also believed to further cause the difference in drug release in the two media (Sun et al., 2020).

In addition to the extent of ionisation, the swelling capacity of the nanoparticle might also play a major role, as it has been found that the more swellable non-magnetic chitosan-carrageenan nanoparticles have a higher rate of drug release compared to its swell-resistant magnetic counterpart (Mahdavinia et al., 2017). This is a theory that is supported in the findings and discussion of studies conducted by another nanoparticle as well (Arif et al., 2017; Dhavale et al., 2021; Nogueira et al., 2020; Sabra et al., 2018; Yusefi et al., 2021). However, similar mechanisms have also been used to explain reduced drug release rate (Yan et al., 2018), which thus makes the effect of nanoparticle swelling on drug release rate to remain inconclusive.

To summarise, the generalisation that drug release rate highly relies on drug-nanoparticle interaction arising due to ionisation state of its constituent is apparent. Most of the studies cited here are in agreement with this mechanism, and most of the findings in studies which disagree with the mechanism proposed are explainable by their nature of encapsulation *via* adsorption on the surface, as well as the hydrophobic nature of the nanoparticle surface. Future studies may be conducted to confirm this effect.

The following trends are also highlighted. It was found that when higher molecular weight chitosan is used as a core, less drug is released over time regardless of pH changes (Herdiana et al., 2022; Karimi et al., 2018). It was theorised that such an observation is seen due to the higher amount of amine group in high molecular weight chitosan which thereby allows a greater extent of interaction with oppositely charged constituent in the network, and hence allowing the nanoparticle to bind to the drug more tightly

(Herdiana et al., 2022). Under the same reasoning, the higher the degree of deacetylation, the lower the rate of degradation and erosion of nanoparticle surface, and thus the lower is the drug released (Sahu et al., 2017).

An initial burst release followed by sustained release was observed, whereby it was hypothesised that the burst release was due to the drug, which was released from the surface, while the sustained release was due to the drug which was released slowly over time from the matrix. The drug content also seems to heavily influence the drug release, as it was seen that nanoparticles with higher drug content have a slower release kinetics compared to nanoparticles with lower drug content (Nguyen et al., 2022).

In a study involving a complex electrospun polyvinyl alcohol/carrageenan/gold/pegylated polyurethane nanoparticle loaded with paclitaxel and camptothecin, it was found that drug release behaviour may differ according to not only the constituent of the nanoparticle, but also the physical structure. In this study, nanoparticles that were made into a composite had a burst release profile followed by a sustained release profile. However, nanoparticles that were made into a core-shell configuration had no observable burst release effect and provided a sustained release throughout the investigation (Irani & Nodeh, 2022).

Pharmacokinetically-Relevant Studies

Physicochemical response towards the environment

Some of the literature reviewed reports their investigations on the nanoparticle's response towards their environment, which are often characterised as changes in size, PDI and zeta potential. From this data, several characteristics of the nanoparticle can be inferred or proven, such as the relationship between physichochemical characteristics and the ionisation behaviour of the polymer used (Sabra et al., 2018, 2019; Shafiee et al., 2019; Sun et al., 2020; Yusefi et al., 2021), interactions with physiological constituent (Sabra et al., 2018, 2019), cancer selectivity (Yusefi et al., 2021) as well as stability under working, storage, transit and extreme conditions (Sabra et al., 2018, 2019; Sun et al., 2020; Yusefi et al., 2021). All of these characteristics may also be significantly related to drug release mechanism and may aid with understanding or explaining the observations seen in drug release studies, as demonstrated in some studies (Sabra et al., 2018; Shafiee et al., 2019; Yusefi et al., 2021). Other than that, response of the nanoparticles towards the environment may give insight on its storage stability (K. Liu et al., 2020). In some studies, the usage of physicochemical response towards presence of mucin may give insight to mucoadhesive properties of the nanoparticle (Sabra et al., 2018, 2019).

Additionally, other than the dynamic changes of the nanoparticle that has already been formed, investigations on the effect of changes in pH and salt environments of solutions during the formation of the nanoparticle towards the physicochemical characteristics of the nanoparticles have also been reported (Yan et al., 2018). These types of investigation are relevant in terms of understanding the nature in which the nanoparticle is formed, as well as the allowing the prediction of behaviour of the nanoparticle in physiological condition (Yan et al., 2018). However, applicability other than for the aim of optimisation is not clearly established. It is also possible that while this method of characterisation can prove the role of characteristics of nanoparticle constituent better than the first approach, it does lack transferability of results to the actual response that the nanoparticle will undertake when exposed to a change in environment. For the sake of simplicity, the first approach in the paragraph above is categorised as "dynamic response" while the approach stated here is categorised as "formulation response"

Generally, the test can differ in one literature over another, but they largely follow a basic premise of measuring the changes in the characteristics of the nanoparticle mentioned above after a certain condition has been changed. In both the dynamic response and formulation response, the overall procedure may involve investigating the response of the nanoparticle to conditions resembling physiological conditions in terms of pH, salt content and temperature (Sabra et al., 2018, 2019; Yusefi et al., 2021), or the effect of the three aforementioned factors are investigated one at a time (Arif et al., 2017; Sun et al., 2020; Yan et al., 2018). However, in dynamic response, the procedure can further be described to fall under two categories, which is measurement of immediate response and continuous response, the former is the measurement in only one instance (Sun et al., 2020; Yusefi et al., 2021), while the latter is the measurement of response at multiple instances at multiple time points (Sabra et al., 2018, 2019; Yusefi et al., 2021).

The procedure for formulation response is simpler to plan yet may require more resources to complete. To conduct this study, one generally has to fix other major factors affecting nanoparticle formation, while changing the conditions of one chosen factor. For example, in one case, investigations on the effect of pH on formation of polyelectrolyte complexes requires highly controlled adjustment of pH and keeping the salt content, mass content and ratio of the constituent solutions constant (Yan et al., 2018). As mentioned before, the results of this study may give insight on the nature of the nanoparticle, but does not give sufficient information that may allow accurate predictions of the nanoparticle's response towards the environment.

Meanwhile, the procedure for dynamic response may be more complex to execute but requires smaller amount of resources. The procedure largely follows the same scheme as drug release studies, i.e. selection of media pH, salt content and temperature. However, instead of measurements of drug released, nanoparticle size, PDI and zeta potential are measured instead (Ji et al., 2017; K. Liu et al., 2020; Sabra et al., 2018, 2019; Sahu et al., 2017; Shafiee et al., 2019; Sun et al., 2020; Yusefi et al., 2021). Thus, unlike in drug release studies, separation of nanoparticles is unneeded. Measurement of size, PDI and zeta potential is conducted directly upon dilution with similar media, which allows postulations regarding how the nanoparticulate system works in different environments to be better understood.

One should be cautious when interpreting the size profile of the nanoparticles, as the nanoparticle may exhibit aggregation and deposition in response to changes to the environment, which in turn would cause size changes to be underestimated (Sun et al., 2020). Here, a procedure may be considered which may help ascertain the extent of aggregation and deposition, such as the usage of DLS to measure turbidity in terms of kilo counts per second (Jung et al., 2018; Wallace et al., 2012; Weng et al., 2020), or measurement of turbidity in UV-Vis spectrophotometry to quantify the absorbance (Yan et al., 2018). However, this comes with the assumption that in these measurements, aggregated and deposited nanoparticles is not included into the sample being measured.

Mucoadhesive properties of nanoparticles may also be assessed *via* its response to essential secretion contents. In one study, mucoadhesion was assessed by measuring changes in surface charge/zeta potential after allowing it to interact with mucin at predetermined time. The changes in zeta potential were measured in different mucin concentrations, at different pH; pH 1.2 to simulate gastric conditions and pH 7.0 to simulate colon conditions. A drop in zeta potential due to mucin interactions was

explained by the electrostatic interactions between nanoparticle surface and mucin. Due to this method, the study have successfully proven selective mucoadhesive properties of the nanoparticle to the colon mucosa rather than gastric mucosa (Sabra et al., 2019). Alternatively, mucoadhesion studies were also conducted by allowing the nanoparticles to mix with mucin and centrifuging the nanoparticles. The supernatant was then mixed with micro-BSA and was left to incubate followed by measurement of absorbance at 562 nm. In principle, this allows the measurement of free mucin which thereby allows the quantification of the extent of mucin-nanoparticle adhesion. In this variation of study on the same nanoparticles, similar observation was found (Sabra et al., 2018).

Here, the findings reported in the literature included in the review are generalised. Under dynamic response, generally, nanoparticles made from chitosan swells more at lower pH compared to higher pH (Ji et al., 2017; C. Liu et al., 2020; Sahu et al., 2017; Shafiee et al., 2019). However, some studies reported higher extent of swelling in higher pH conditions (Sabra et al., 2018; Yusefi et al., 2021). So far, there has not been a clear reasoning on why the studies reported contradictory findings. However, in general, the swelling effect is even greater where proteins with opposite charge to the surface of the nanoparticle are present in the media (Sabra et al., 2019). The extent of this swelling can be reduced by complexation with modified citrus pectin (Sabra et al., 2018), or increased by complexation with cellulose fibre (Yusefi et al., 2021).

Meanwhile, for formulation response, changes in physicochemical properties are attributable to the extent of ionisation of the nanoparticle constituent. In a study of carboxylic curdlan and chitosan nanoparticle, if the polymer solutions' pH prior to complexation is increased from pH 3.0 to 5.0, the size of the nanoparticle increases. This is believed to be due to the deprotonation of amine groups of chitosan, which decreases the extent of ionic attraction between chitosan and the anionic polymer. However, there has also been an observation whereby the chitosan-based nanoparticles may swell if it is too acidic, which is associated with the change in extent of ionisation of non-chitosan substituent which thereby decreases the extent of ionic interaction between chitosan and the ionisable anionic substituent (Yan et al., 2018). A relatively similar observation was found in a polymalic acid-chitosan nanoparticle. It was found that the nanoparticles remain in relatively similar sizes at pH 1.2-6.0 range, while a significant increase is observed at pH 7.4. This study also explains this observation as attributable to the deprotonation of chitosan which reduces the extent of complexation. However, this result is counterintuitive to the fact that the pKa of chitosan which is close to 6.5, while polymalonic acid having a pKa of 3.4-3.6. It was proposed here that instead of ionic complexation, the polymalonic acid and chitosan interact and entangle one another *via* electrostatic attractions (Arif et al., 2017).

As studies involving nanoparticle's response to environmental conditions are rather scarce for carrageenan-based nanoparticles under the pre-defined scope, a generalisable statement could not be drawn regarding their response to stimuli.

Simulated digestion

In one study involving zein-chitosan nanoparticle loaded with curcumin, the effect of digestion on size, morphology and curcumin bioaccessibility were assessed, whereby the nanoparticles are undergoing simulated digestion, which is incubation and agitation in simulated gastric fluid for 2 hours followed by simulated intestinal fluid for 2 hours, at 37°C. The mixtures are then centrifuged at 10°C and 10,000 rpm for 40 minutes, followed by filtration of the

supernatant with 0.45μm membrane filter before analysis for curcumin content. It was found that the size of the nanoparticles increased greatly in gastric conditions over time, while no changes were found in intestinal conditions. It was explained that morphological changes which occur could be due to the effect of pancreatin, pepsin and acidic or basic environments which act on the surface of the nanoparticles. Interestingly, the results of curcumin bioaccessibility indicated that free curcumin and curcumin loaded into chitosan nanoparticles is not stable in digestive conditions, but the curcumin loaded into zein nanoparticles were more stable, followed by chitosan-zein nanoparticles. This indicates that chitosan works synergistically with zein to protect curcumin from degradation (C. Liu et al., 2020).

Storage Stability Studies

Method of characterisation of stability

The storage stability studies are the least conducted study between all of the reviewed studies. Thus, due to this, reference for generalisation under this study is scarce. In the current review, it was found that the following parameters outlined in Table 3 were measured for considerations of stability.

Table 3: The parameters measured as an indication of stability in storage conditions.

Parameters	Reference
General appearance (Turbidity, deposition and/or colour)	(Gaur et al., 2022; K. Liu et al., 2020; Sun et al., 2020)
Nanoparticle size and PDI	(Gaur et al., 2022; C. Liu et al., 2020; K. Liu et al., 2020; Sabra et al., 2019; Sun et al., 2020)
Zeta potential	(Gaur et al., 2022; K. Liu et al., 2020; Sun et al., 2020)
Morphological changes	(Sabra et al., 2019)
Encapsulation efficiency	(Gaur et al., 2022; Sabra et al., 2019)
Drug loading capacity / drug content	(Gaur et al., 2022; C. Liu et al., 2020; Sabra et al., 2019)
Drug release studies	(Gaur et al., 2022)
Ease of reconstitution	(Gaur et al., 2022)
Enzyme activity	(Moradi et al., 2021)

The stability studies of all of the chitosan and/or carrageenan-based nanoparticles reported in this review were conducted either on one formulation which allows definitive interpretation to be made (Gaur et al., 2022; K. Liu et al., 2020; Sabra et al., 2019), or on multiple formulation which also allows comparative discussion (C. Liu et al., 2020; K. Liu et al., 2020; Moradi et al., 2021; Sun et al., 2020).

Generally, the length of study and measurement time points greatly differ from study to study. However, it can be classified that most stability studies reported were planned to take at least one month to be completed (a period of 30 days), while in between, regular 5 day or weekly intervals of testing were conducted. In some cases, changes in parameters are only assessed at the end of the period (C. Liu et al., 2020; K. Liu et al., 2020). In the case whereby significant changes in the parameter occur, it can be concluded that the nanoparticle is no longer stable, the stability study may be terminated in the case where only one formulation is tested (Sabra et al., 2019), or it may be prolonged to allow comparative analysis between different formulations (Moradi et al., 2021; Sun et al., 2020).

In all of the studies cited, temperature remains the focus as the factor that is controlled for the stability studies. In all studies, there are no mention of pre-defined humidity levels used to conduct the study. It should be noted that humidity effect is an important parameter to be considered as the stability of encapsulated drug may be highly affected by the addition or removal of water from the crystalline structure of drug; in some

cases, drugs may take anhydrous forms which are more unstable (Santamaría-Aguirre et al., 2018). Polymeric degradation under different moisture conditions are considerable, as changes in relative humidity leads to changes in water content of the polymer, which has been shown to affect chitosan and carrageenan stability and degradation (Friedenthal et al., 2020; Shahbazi et al., 2016). As such, where available and possible, stability studies should report on the humidity on top of temperature as it is an important consideration on stability as well.

It can be said that protection of the active pharmaceutical ingredient (Moradi et al., 2021) and increased stability of the nanoparticle (C. Liu et al., 2020) can be seen with the usage of chitosan polymer in nanoparticle formulation. A mid-range polymer concentration showed the greatest stability in a study involving carrageenan-based nanoparticles (Sun et al., 2020).

Size changes are associated with Ostwald ripening, whereby small size of the nanoparticles causes favoured deposition on bigger particles due to higher surface area to volume ratio. Additionally, drug loading capacity and encapsulation efficiency decreased, which was explained by the autocatalytic reaction of bigger nanoparticles thereby leading to release of drugs (Sabra et al., 2019).

Conclusion

Studies involving in vitro kinetics of nanoparticle characteristics have been widespread in nanoparticle research for the objective of understanding the behaviour of nanoparticles in its application. However, due to lack of standardisation in the procedures conducted owing up to the differences in application and nanoparticle properties, differences between study results arise which makes it highly subject to inaccuracies arising due to unsuitable or incomplete study methods. The current literature review generalises the trends in the methods used in drug release profiling, pharmacokinetically-relevant studies and storage stability studies under the context of chitosan and/or carrageenan nanoparticles in anticancer application. The current review also highlights several areas which may be improved to increase accuracy in the studies conducted, which may be adapted to improve analysis in future studies. With these generalisations and suggestions, the current literature review will help researchers to be able to plan their future studies to be not only more accurate, but also potentially more comparable and similar in terms of methodology with other studies. Next up, the results of the drug release studies may also be generalisable and are explainable by the physical and chemical characteristics of constituent, particularly with the response of drug release towards changes in pH which are explainable *via* the principles of ionisation. Hence, there is a possibility that in the future, hypotheses or predictions in their kinetic behaviours can be made. In the future, these trends should be examined further under a more broadened scope to conclusively determine whether they still hold true in polymeric nanoparticles made from other materials.

Authors contributions

Conception and design of the work, A.U.; acquisition, analysis, and interpretation of articles, A.U.; writing—original draft preparation, A.U.; writing—editing, A.U.; writing—review, I.F.M.S., H.H., S.F.C.O.; writing—design and formatting, A.U., I.F.M.S.; visualization, A.U.; supervision, I.F.M.S., H.H., S.F.C.O.; project administration, I.F.M.S. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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References

Abouelmagd, S. A., Sun, B., Chang, A. C., Ku, Y. J., & Yeo, Y. (2015). Release kinetics study of poorly water-soluble drugs from nanoparticles: Are we doing it right? *Molecular Pharmaceutics*, *12*(3), 997–1003. https://doi.org/10.1021/mp500817h

Ali, A., & Ahmed, S. (2018). A review on chitosan and its nanocomposites in drug delivery. *International Journal of Biological Macromolecules*, *109*, 273–286. https://doi.org/10.1016/j.ijbiomac.2017.12.078

Al-Samydai, A., Al-Mamoori, F., Abdelnabi, H., & Aburjai, T. (2019). An updated review on anticancer activity of capsaicin. *International Journal of Scientific and Technology Research*, *8*(12), 2625–2630.

Arif, M., Raja, M. A., Zeenat, S., Chi, Z., & Liu, C. (2017). Preparation and characterization of polyelectrolyte complex nanoparticles based on poly (malic acid), chitosan. A pH-dependent delivery system. *Journal of Biomaterials Science, Polymer Edition*, *28*(1), 50–62. https://doi.org/10.1080/09205063.2016.1242460

Bourbon, A. I., Pinheiro, A. C., Cerqueira, M. A., & Vicente, A. A. (2018). In vitro digestion of lactoferrin-glycomacropeptide nanohydrogels incorporating bioactive compounds: Effect of a chitosan coating. *Food Hydrocolloids*, *84*, 267–275. https://doi.org/10.1016/j.foodhyd.2018.06.015

Chu, X., Huang, W., Wang, Y., Meng, L., Chen, L., Jin, M., Chen, L., Gao, C., Ge, C., Gao, Z., & Gao, C. (2019). Improving antitumor outcomes for palliative intratumoral injection therapy through lecithin–chitosan nanoparticles loading paclitaxel–cholesterol complex. *International Journal of Nanomedicine*, *14*, 689–705. https://doi.org/10.2147/IJN.S188667

Dhavale, R. P., Dhavale, R. P., Sahoo, S. C., Kollu, P., Jadhav, S. U., Patil, P. S., Dongale, T. D., Chougale, A. D., & Patil, P. B. (2021). Chitosan coated magnetic nanoparticles as carriers of anticancer drug Telmisartan: pH-responsive controlled drug release and cytotoxicity studies. *Journal of Physics and Chemistry of Solids*, *148*. https://doi.org/10.1016/j.jpcs.2020.109749

Fan, L., Duan, M., Sun, X., Wang, H., & Liu, J. (2020). Injectable Liquid Metal- And Methotrexate-Loaded Microsphere for Cancer Chemophotothermal Synergistic Therapy. *ACS Applied Bio Materials*, *3*(6), 3553–3559. https://doi.org/10.1021/acsabm.0c00171

Friedenthal, M., Eha, K., Kaleda, A., Part, N., & Laos, K. (2020). Instability of low-moisture carrageenans as affected by water vapor sorption at moderate storage temperatures. *SN Applied*

Sciences, *2*(2). https://doi.org/10.1007/s42452-020-2032-9

Gaur, P. K., Puri, D., Singh, A. P., Kumar, N., & Rastogi, S. (2022). Optimization and Pharmacokinetic Study of Boswellic Acid–Loaded Chitosan-Guggul Gum Nanoparticles Using Box-Behnken Experimental Design. *Journal of Pharmaceutical Innovation*, *17*(2), 485–500. https://doi.org/10.1007/s12247-020-09527-0

Herdiana, Y., Wathoni, N., Shamsuddin, S., & Muchtaridi, M. (2022). Cytotoxicity Enhancement in MCF-7 Breast Cancer Cells with Depolymerized Chitosan Delivery of α-Mangostin. *Polymers*, *14*(15). https://doi.org/10.3390/polym14153139

Hoshyar, N., Gray, S., Han, H., & Bao, G. (2016). The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine*, *11*(6), 673–692. https://doi.org/10.2217/nnm.16.5

Hotchkiss, S., Brooks, M., Campbell, R., Philp, K., & Trius, A. (2016). The Use of Carrageenan in Food. In L. Pereira (Ed.), *Carrageenans - Sources and Extraction Methods, Molecular Structure, Bioactive Properties and Health Effects* (1st ed., pp. 229–243). Nova Science Publishers.

Irani, M., & Nodeh, S. M. (2022). PVA/κ-carrageenan/Au/camptothecin/pegylated-polyurethane/paclitaxel nanofibers against lung cancer treatment. *RSC Advances*, *12*(25), 16310–16318. https://doi.org/10.1039/d2ra02150a

Jafari, H., Atlasi, Z., Mahdavinia, G. R., Hadifar, S., & Sabzi, M. (2021). Magnetic κ-carrageenan/chitosan/montmorillonite nanocomposite hydrogels with controlled sunitinib release. *Materials Science and Engineering C*, *124*. https://doi.org/10.1016/j.msec.2021.112042

Jennings, J. A. (2017). Controlling chitosan degradation properties in vitro and in vivo. In *Chitosan Based Biomaterials* (Vol. 1, pp. 159–182). Elsevier Inc. https://doi.org/10.1016/B978-0-08-100230-8.00007-8

Ji, F., Li, J., Qin, Z., Yang, B., Zhang, E., Dong, D., Wang, J., Wen, Y., Tian, L., & Yao, F. (2017). Engineering pectin-based hollow nanocapsules for delivery of anticancer drug. *Carbohydrate Polymers*, *177*, 86–96. https://doi.org/10.1016/j.carbpol.2017.08.107

Jung, F., Nothnagel, L., Gao, F., Thurn, M., Vogel, V., & Wacker, M. G. (2018). A comparison of two biorelevant in vitro drug release methods for nanotherapeutics based on advanced physiologically-based pharmacokinetic modelling. *European Journal of Pharmaceutics and Biopharmaceutics*, *127*, 462–470. https://doi.org/10.1016/j.ejpb.2018.03.010

Karimi, M. H., Mahdavinia, G. R., & Massoumi, B. (2018). pH-controlled sunitinib anticancer release from magnetic chitosan nanoparticles crosslinked with κ-carrageenan. *Materials Science and Engineering C*, *91*, 705–714. https://doi.org/10.1016/j.msec.2018.06.019

Kharkwal, H., & Janaswamy, S. (Eds.). (2017). *Natural Polymers for Drug Delivery*. CAB International.

Liu, C., Yuan, Y., Ma, M., Zhang, S., Wang, S., Li, H., Xu, Y., & Wang, D. (2020). Self-assembled composite nanoparticles based on zein as delivery vehicles of curcumin: Role of chondroitin sulfate. *Food and Function*, *11*(6), 5377–5388. https://doi.org/10.1039/d0fo00964d

Liu, K., Huang, R. L., Zha, X. Q., Li, Q. M., Pan, L. H., & Luo, J. P. (2020). Encapsulation and sustained release of curcumin by a composite hydrogel of lotus root amylopectin and chitosan. *Carbohydrate Polymers*, *232*. https://doi.org/10.1016/j.carbpol.2019.115810

Mahdavinia, G. R., Mosallanezhad, A., Soleymani, M., & Sabzi, M. (2017). Magnetic- and pH-responsive κ-carrageenan/chitosan complexes for controlled release of methotrexate anticancer drug. *International Journal of Biological Macromolecules*, *97*, 209–217. https://doi.org/10.1016/j.ijbiomac.2017.01.012

Modi, S., & Anderson, B. D. (2013). Determination of drug release kinetics from nanoparticles: Overcoming pitfalls of the dynamic dialysis method. *Molecular Pharmaceutics*, *10*(8), 3076–3089. https://doi.org/10.1021/mp400154a

Moradi, R., Mohammadzadeh, R., & Akbari, A. (2021). Kappa-Carrageenan Crosslinked Magnetic Folic Acid-Conjugated Chitosan Nanocomposites for Arginase Encapsulation, Delivery and Cancer Therapy. *Nano LIFE*, *11*(03), 2140005. https://doi.org/10.1142/S1793984421400055

Moreno-Bautista, G., & Tam, K. C. (2011). Evaluation of dialysis membrane process for quantifying the in vitro drug-release from colloidal drug carriers. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *389*(1–3), 299–303. https://doi.org/10.1016/j.colsurfa.2011.07.032

Mulet-Cabero, A. I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., Le Feunteun, S., Sarkar, A., Grundy, M. M. L., Carrière, F., Golding, M., Dupont, D., Recio, I., Brodkorb, A., & Mackie, A. (2020). A standardised semi-dynamic: in vitro digestion method suitable for food-an international consensus. *Food and Function*, *11*(2), 1702–1720. https://doi.org/10.1039/c9fo01293a

Narayanaswamy, R., & Torchilin, V. P. (2019). Hydrogels and Their Applications in Targeted Drug Delivery. *Molecules*, *24*(3), 603. https://doi.org/10.3390/molecules24030603

Nguyen, T. H., Nguyen, T. C., Nguyen, T. M. T., Hoang, D. H., Tran, D. M. T., Tran, D. T., Hoang, P. T., Le, V. T., Tran, T. K. N., & Thai, H. (2022). Characteristics and Bioactivities of Carrageenan/Chitosan Microparticles Loading α-Mangostin. *Journal of Polymers and the Environment*, *30*(2), 631–643. https://doi.org/10.1007/s10924-021-02230-2

Nogueira, J., Soares, S. F., Amorim, C. O., Amaral, J. S., Silva, C., Martel, F., Trindade, T., & Daniel-Da-Silva, A. L. (2020). Magnetic driven nanocarriers for pH-responsive doxorubicin release in cancer therapy. *Molecules*, *25*(2). https://doi.org/10.3390/molecules25020333

Rostami, E. (2020). Progresses in targeted drug delivery systems using chitosan nanoparticles in cancer therapy: A mini-

review. *Journal of Drug Delivery Science and Technology*, *58*, 101813. https://doi.org/10.1016/j.jddst.2020.101813

Sabra, R., Billa, N., & Roberts, C. J. (2018). An augmented delivery of the anticancer agent, curcumin, to the colon. *Reactive and Functional Polymers*, *123*, 54–60. https://doi.org/10.1016/j.reactfunctpolym.2017.12.012

Sabra, R., Roberts, C. J., & Billa, N. (2019). Courier properties of modified citrus pectinate-chitosan nanoparticles in colon delivery of curcumin. *Colloid and Interface Science Communications*, *32*, 100192. https://doi.org/10.1016/j.colcom.2019.100192

Sahatsapan, N., Rojanarata, T., Ngawhirunpat, T., Opanasopit, P., & Patrojanasophon, P. (2021). Doxorubicin-loaded chitosan-alginate nanoparticles with dual mucoadhesive functionalities for intravesical chemotherapy. *Journal of Drug Delivery Science and Technology*, *63*. https://doi.org/10.1016/j.jddst.2021.102481

Sahu, P., Kashaw, S. K., Jain, S., Sau, S., & Iyer, A. K. (2017). Assessment of penetration potential of pH responsive double walled biodegradable nanogels coated with eucalyptus oil for the controlled delivery of 5-fluorouracil: In vitro and ex vivo studies. *Journal of Controlled Release*, *253*, 122–136. https://doi.org/10.1016/j.jconrel.2017.03.023

Santamaría-Aguirre, J., Alcocer-Vallejo, R., & López-Fanárraga, M. (2018). Drug Nanoparticle Stability Assessment Using Isothermal and Nonisothermal Approaches. In *Journal of Nanomaterials* (Vol. 2018). Hindawi Limited. https://doi.org/10.1155/2018/3047178

Shafiee, S., Ahangar, H. A., & Saffar, A. (2019). Taguchi method optimization for synthesis of Fe3O4 @chitosan/Tragacanth Gum nanocomposite as a drug delivery system. *Carbohydrate Polymers*, *222*. https://doi.org/10.1016/j.carbpol.2019.114982

Shahbazi, M., Rajabzadeh, G., Ettelaie, R., & Rafe, A. (2016). Kinetic study of κ-carrageenan degradation and its impact on mechanical and structural properties of chitosan/κ-carrageenan film. *Carbohydrate Polymers*, *142*, 167–176. https://doi.org/10.1016/j.carbpol.2016.01.037

Sun, X., Pan, C., Ying, Z., Yu, D., Duan, X., Huang, F., Ling, J., & Ouyang, X. kun. (2020). Stabilization of zein nanoparticles with k-carrageenan and tween 80 for encapsulation of curcumin. *International Journal of Biological Macromolecules*, *146*, 549–559. https://doi.org/10.1016/j.ijbiomac.2020.01.053

Tharkar, P., Varanasi, R., Wong, W. S. F., Jin, C. T., & Chrzanowski, W. (2019). Nano-Enhanced Drug Delivery and Therapeutic Ultrasound for Cancer Treatment and Beyond. In *Frontiers in Bioengineering and Biotechnology* (Vol. 7). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2019.00324

Trousil, J., Pavliš, O., Kubíčková, P., Škorič, M., Marešová, V., Pavlova, E., Knudsen, K. D., Dai, Y.-S., Zimmerman, M., Dartois, V., Fang, J.-Y., & Hrubý, M. (2020). Antitubercular nanocarrier monotherapy: Study of In Vivo efficacy and pharmacokinetics for rifampicin. *Journal of Controlled Release*, *321*, 312–

323. https://doi.org/10.1016/j.jconrel.2020.02.026

Utreja, P., Verma, S., Rahman, M., & Kumar, L. (2020). Use of Nanoparticles in Medicine. *Current Biochemical Engineering*, *6*(1), 7–24. https://doi.org/10.2174/2212711906666190724145101

Vinothini, K., Rajendran, N. K., Munusamy, M. A., Alarfaj, A. A., & Rajan, M. (2019). Development of biotin molecule targeted cancer cell drug delivery of doxorubicin loaded κ-carrageenan grafted graphene oxide nanocarrier. *Materials Science and Engineering C*, *100*, 676–687. https://doi.org/10.1016/j.msec.2019.03.011

Wallace, S. J., Li, J., Nation, R. L., & Boyd, B. J. (2012). Drug release from nanomedicines: Selection of appropriate encapsulation and release methodology. *Drug Delivery and Translational Research*, *2*(4), 284–292. https://doi.org/10.1007/s13346-012-0064-4

Wang, J., Ni, C., Zhang, Y., Zhang, M., Li, W., Yao, B., & Zhang, L. (2014). Preparation and pH controlled release of polyelectrolyte complex of poly(l-malic acid-co-d,l-lactic acid) and chitosan. *Colloids and Surfaces B: Biointerfaces*, *115*, 275–279. https://doi.org/10.1016/j.colsurfb.2013.12.018

Wang, S., Pi, L., Wen, H., Yu, H., & Yang, X. (2020). Evaluation of novel magnetic targeting microspheres loading adriamycin based on carboxymethyl chitosan. *Journal of Drug Delivery Science and Technology*, *55*, 101388. https://doi.org/10.1016/j.jddst.2019.101388

Wathoni, N., Meylina, L., Rusdin, A., Abdelwahab Mohammed, A. F., Tirtamie, D., Herdiana, Y., Motoyama, K., Panatarani, C., Joni, I. M., Lesmana, R., & Muchtaridi, M. (2021). The potential cytotoxic activity enhancement of α-mangostin in chitosan-kappa carrageenan-loaded nanoparticle against mcf-7 cell line. *Polymers*, *13*(11). https://doi.org/10.3390/polym13111681

Weng, J., Tong, H. H. Y., & Chow, S. F. (2020). In vitro release study of the polymeric drug nanoparticles: Development and validation of a novel method. *Pharmaceutics*, *12*(8), 1–18. https://doi.org/10.3390/pharmaceutics12080732

World Health Organization. (2012). *Cancer - Key Statistics*. https://www.who.int/cancer/resources/keyfacts/en/

World Health Organization. (2020). *Malaysia - Cancer Country Profile 2020*. https://www.who.int/cancer/country-profiles/MYS_2020.pdf

Yan, J. K., Qiu, W. Y., Wang, Y. Y., Wu, L. X., & Cheung, P. C. K. (2018). Formation and characterization of polyelectrolyte complex synthesized by chitosan and carboxylic curdlan for 5-fluorouracil delivery. *International Journal of Biological Macromolecules*, *107*(PartA), 397–405. https://doi.org/10.1016/j.ijbiomac.2017.09.004

Yu, M., Yuan, W., Li, D., Schwendeman, A., & Schwendeman, S. P. (2019). Predicting drug release kinetics from nanocarriers inside dialysis bags. *Journal of Controlled Release*, *315*, 23–30.

https://doi.org/10.1016/j.jconrel.2019.09.016

Yusefi, M., Chan, H. Y., Teow, S. Y., Kia, P., Lee-Kiun Soon, M., Sidik, N. A. B. C., & Shameli, K. (2021). 5-fluorouracil encapsulated chitosan-cellulose fiber bionanocomposites: Synthesis, characterization and in vitro analysis towards colorectal cancer cells. *Nanomaterials*, *11*(7). https://doi.org/10.3390/nano11071691

Zambito, Y., Pedreschi, E., & di Colo, G. (2012). Is dialysis a reliable method for studying drug release from nanoparticulate systems? - A case study. *International Journal of Pharmaceutics*, *434*(1–2), 28–34. https://doi.org/10.1016/j.ijpharm.2012.05.020

Zhang, E., Xing, R., Liu, S., Li, K., Qin, Y., Yu, H., & Li, P. (2019). Vascular targeted chitosan-derived nanoparticles as docetaxel carriers for gastric cancer therapy. *International Journal of Biological Macromolecules*, *126*, 662–672. https://doi.org/10.1016/j.ijbiomac.2018.12.262

Zia, K. M., Tabasum, S., Nasif, M., Sultan, N., Aslam, N., Noreen, A., & Zuber, M. (2017). A review on synthesis, properties and applications of natural polymer based carrageenan blends and composites. *International Journal of Biological Macromolecules*, *96*, 282–301. https://doi.org/10.1016/j.ijbiomac.2016.11.095

Zielińska, A., Carreiró, F., Oliveira, A. M., Neves, A., Pires, B., Venkatesh, D. N., Durazzo, A., Lucarini, M., Eder, P., Silva, A. M., Santini, A., & Souto, E. B. (2020). Polymeric Nanoparticles: Production, Characterization, Toxicology and Ecotoxicology. *Molecules*, *25*(16), 3731. https://doi.org/10.3390/molecules25163731