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Review

Review for carrageenan-based pharmaceutical biomaterials: Favourable physical features *versus* adverse biological effects

Jingjing Liu, Xiudan Zhan, Jianbo Wan, Yitao Wang, Chunming Wang*

State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Avenida Padre Tomas Pereira, Taipa, Macau

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ABSTRACT

Carrageenan (CRG) is a family of natural polysaccharides derived from seaweeds and has widely been used as food additives. In the past decade, owing to its attractive physicochemical properties, CRG has been developed into versatile biomaterials vehicles for drug delivery. Nevertheless, studies also emerged to reveal its adverse effects on the biological system. In this review, we critically appraise the latest literature (two thirds since 2008) on the development of CRG-based pharmaceutical vehicles and the perspective of using CRG for broader biomedical applications. We focus on how current strategies exploit the unique gelling mechanisms, strong water absorption and abundant functional groups of the three major CRG varieties. Notably, CRG-based matrices are demonstrated to increase drug loading and drug solubility, enabling release of orally administrated drugs in zero-order or in a significantly prolonged period. Other amazing features, such as pH-sensitivity and adhesive property, of CRG-based formulations are also introduced. Finally, we discuss the adverse influence of CRG on the human body and then suggest some future directions for the development of CRG-based biomaterials for broader applications in biomedicine.

Contents

1. Introduction

Scientists have never stopped searching for new biomaterials with versatile properties and functions. Carrageenan (CRG), a family of natural polysaccharides derived from certain species of red seaweeds (Rhodophyta), was first extracted in 1837 and has long

* Corresponding author. Tel.: +853 8822 4696.
E-mail addresses: CMWang@umac.mo, cmwang82@gmail.com (C. Wang).

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been used as a gelling agent for food processing (van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). In the past two decades, CRG has increasingly been used as biomaterials for various pharmaceutical purposes (Liang, Mao, Peng, & Tang, 2014; Necas & Bartosikova, 2013). It demonstrates to improve drug formulation and sustained release, and has been tested for its potential use in broader biomedical applications (Li, Ni, Shao, & Mao, 2014; Pahuja, Arora, & Pawar, 2012; Prajapati, Maheriya, Jani, & Solanki, 2014). Nevertheless, researchers have also found that this class of polysaccharide may cause adverse biological effects to the living system, such as inciting unwanted immune responses and inhibiting blood coagulation, which was barely reviewed in the recent literature. As CRG-based biomaterials are increasingly used for drug delivery and other biomedical purposes—in particular for any *in vivo* application, both their desirable features and unwanted functions are of significant importance. There is a pressing demand for careful and comprehensive analysis of the properties of CRG from various angles, in order for better development and safer use of this type of natural biopolymers in broader fields of biology and medicine (Prajapati et al., 2014).

Hence, in this concise review, we will introduce the major varieties of CRG and their chemistry, summarise their unique physicochemical features which are desirable in biomedical applications, and critically analyse the potential risks CRG may pose to the living tissue. Specifically, we will attribute the current and potential applications of these polysaccharide biomaterials to their structural, gelling and functional advantages, whereas highlighting the necessity to balance such favourable material properties and possible biological risks.

2. CRG: Chemistry and gelling property

CRG consists of long linear chains of D-galactose and D-anhydrogalactose with ester sulphates, and is therefore anionic. The sulphate groups are usually neutralized by cations, such as Na^+, K^+ and Ca^{2+} (Bixler, 1994). An average molecular mass of CRG is above 100 kDa, with 15–40% content of ester-sulphate (Necas & Bartosikova, 2013). Its three major varieties are κ-, ι- and λ-CRG (Fig. 1) (van de Velde et al., 2002). Among them, κ-CRG has only one sulphate group per disaccharide repeating unit; ι-CRG has two, whereas λ-CRG has three. As a result, the three varieties show different linear charge density (λ > ι > κ) (Pavli, Baumgartner, Kos, & Kogej, 2011) and solubility (λ > ι > κ) (Bixler, 1994). κ-CRG only dissolves in hot water, while λ-CRG is soluble in both cold and hot aqueous solution (Bixler, 1994; Necas & Bartosikova, 2013). The associated cations, such as Na^+, K^+, Ca^{2+} and Mg^{2+}, could also influence the solubility (Campo, Kawano, Silva, & Carvalho, 2009). In addition, the 3,6-anhydro bridges exist in ι- and κ-CRGs, but not in λ-CRG.

Forming gel is a key property of both κ- and ι-CRG. The former can form brittle gels, and the latter forms softer, elastic gels or pastes. It is suggested that the presence of the anhydro bridges in κ- and ι-CRG molecules can be a key factor in gelation. The $^1C_4$-conformation of the 3,6-anhydro-D-galactopyranosyl units in κ- and ι-CRG allows a helical secondary structure which is necessary to form gel (Campo et al., 2009; van de Velde et al., 2002). A widely accepted theory postulates that these CRG molecules form gels *via* two steps. First, a coil-to-helix transition takes place, as is controlled by temperature and cations such as K^+ or Ca^{2+}. Then, these helices aggregate in parallel (Fig. 2) (Yuguchi, Urakawa, & Kajiwara, 2003). Sulphate groups are located at the outside of the helix. Hydrogen bonds formed between the two chains are responsible for helix stabilization. So κ- and ι-CRG could form a network of three-dimensional double helices. The gels formed by CRG are

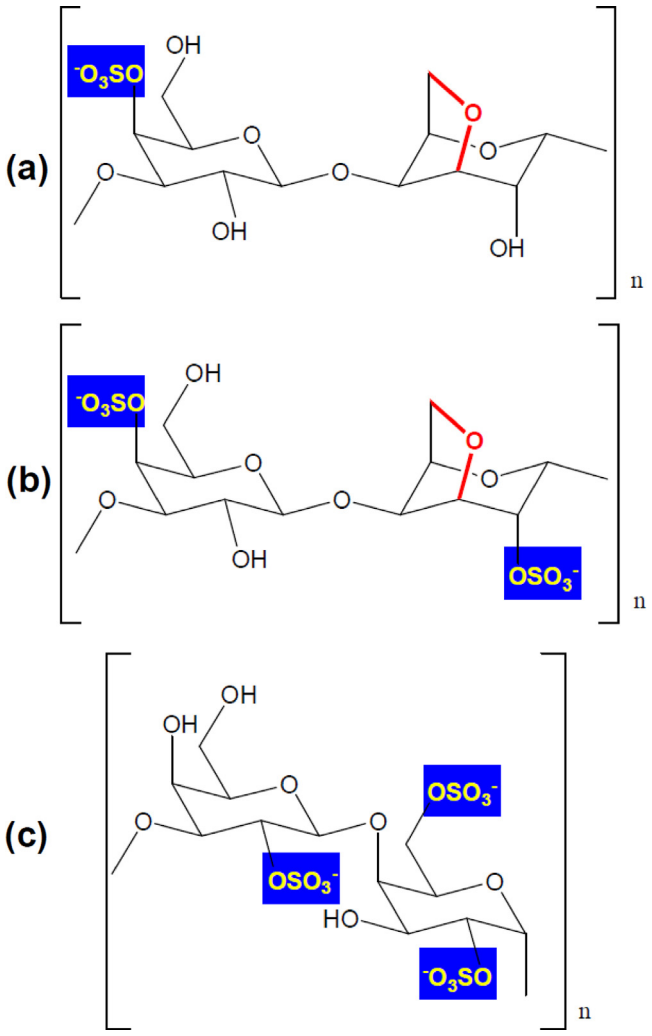

Fig. 1. Structure of the three main varieties of CRG: (a) κ-carrageenan; (b) ι-carrageenan; (c) λ-carrageenan. They differ in the presence of the 3,6-anhydro bridges and numbers of the sulphate groups.

thermally reversible. Gels start to form after cooling to about 50 °C, and melt when heated at 80–90 °C (Bixler, 1994).

On the other hand, λ-CRG that contains no 3,6-anhydro-D-galactopyranosyl units does not form gel and is thus used as a strong thickener (Bixler, 1994). The 2-sulphate group of λ-CRG, which is oriented towards the internal part of adjacent chains may prevent the formation of three-dimensional double helices (Campo et al., 2009).

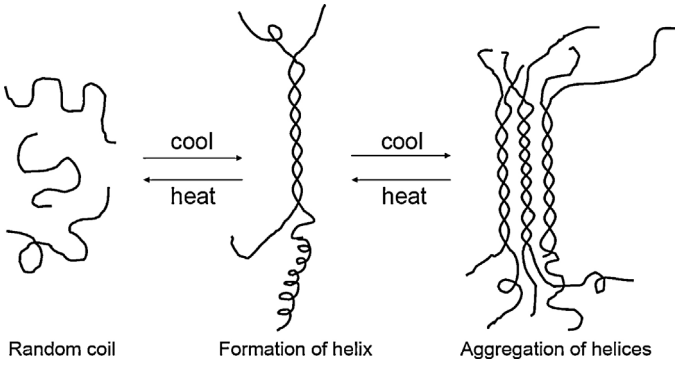

Fig. 2. Schematic illustration of the possible gelling mechanism of the CRG macromolecules.

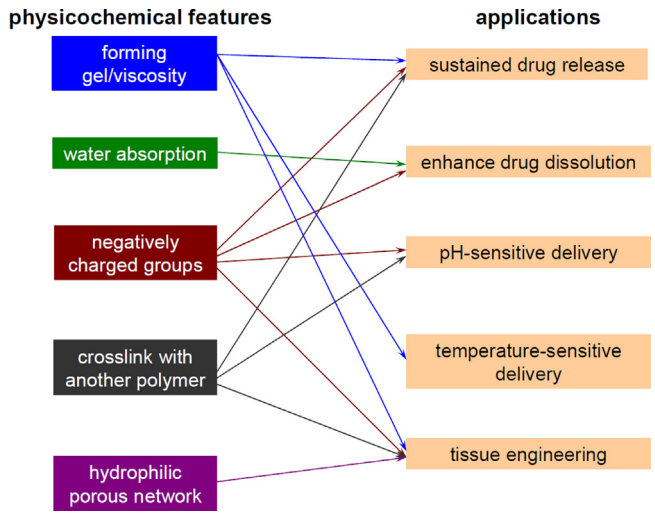

Fig. 3. Relationship between physicochemical features and applications of CRG.

In summary, the number of sulfate groups and the content of anhydro bridges affect the properties of CRG type. The varying properties of κ-, ι- and λ-CRG make these polysaccharide polymers versatile when used to develop biomaterials. Meanwhile, difference in their chemistry also brings about different biological activities, some of which may be unwanted or harmful.

3. CRG based biomaterials: Exploiting its physicochemical features

As biomaterials, CRG that shows good compatibility and consolidation behaviour (Picker, 1999a) is mainly used by pharmaceutical researchers to (i) improve drug formulation, (ii) prolong drug release and (iii) create pH-/temperature-sensitive delivery system in response to physiological environments. Notably, CRG-based formulations could enable orally administrated drugs to be released in zero-order (Nanaki, Karavas, Kalantzi, & Bikiaris, 2010; Prado, Matulewicz, Bonelli, & Cukierman, 2008) or in a significantly prolonged period–as long as 24 h (Pavli et al., 2011; Pavli, Vrecer, & Baumgartner, 2010) or 3 weeks (Grenha et al., 2010) (Table 1). Usually, κ-CRG showed the fastest drug release (Ghanam & Kleinebudde, 2011). The interaction between different CRG varieties or between CRG and other polymers are often used to achieve the ideal drug release profile. Recently, the application of CRG-based formulations also extends to ophthalmic (Bonferoni et al., 2004), buccal (Tomoda et al., 2009) and vaginal (Liu, Zhu, Wei, & Lu, 2009) drug delivery, as well as broader areas of wound healing (Boateng, Pawar, & Tetteh, 2013) and tissue engineering (Araujo, Davidenko, Danner, Cameron, & Best, 2014; Santo et al., 2009). All such promising applications of these polysaccharides can be attributed to their useful physicochemical features, as illustrated in Fig. 3 and elaborated below.

3.1. Unique matrices—For prolonged drug release

In aqueous environment, CRG can form a unique, bilayer matrix for drug release. As a consequence of gradient hydration occurring from the outside, the matrix swells and forms an outer layer, either gelated (κ-CRG and ι-CRG) or viscous (λ-CRG), surrounding an unhydrated core (Pavli et al., 2011). Such gel or viscous layer serves as a polymeric shell to control the dissolution and diffusion of the loaded drug, resisting erosion even in the extremely acidic conditions. For example, ι-CRG gels (1.5%, w/v) were found to maintain intact in the stomach and exhibited desirable *in vitro* and *in vivo* release characteristics, in comparison with agar gels (0.5%, w/v) for the oral administration of acetaminophen (Miyazaki et al., 2011).

In this process, the type of CRG affects the release rate. Some studies indicated that κ-CRG showed fast release because of fast disintegration (Ghanam & Kleinebudde, 2011) or fast (and also extensive) swelling (Picker, 1999b). A study showed that the κ-CRG tablets were eroded the fastest, reaching 100% in 8 h, followed by ι- and λ-CRG both with ~83% erosion at the same time point, while the degree of water-uptake correlates with the number of sulphate groups on CRGs (λ > ι > κ) (Pavli et al., 2010). Also, blending different types of CRG appeared a good strategy to prolong drug release, although examination of the surface with scanning electron microscopy (SEM) showed that two types of CRG in blends were not miscible (Fig. 4A) (Nanaki et al., 2010). The tablets formulated with equal amounts of λ-CRG and ι-CRG had near-zero-order release profiles of tripelennamine-HCl. A possible explanation is that λ-CRG can hold, or 'encave', the complexes of drugs and hydrated ι-CRG in λ-CR's viscous shell (Hariharan, Wheatley, & Price, 1997). Such an application represents a smart 'collaboration' between different CRG varieties in rational design of drug delivery system.

Nanoparticle blending may further help CRG prolong drug release, but different nanoparticle type exhibits different effects. For example, incorporation of Au nanoparticles could slow down the diffusion rate of the methylene blue from κ-CRG hydrogels likely due to the enhancement of the tortuosity of CRG network (Salgueiro, Daniel-da-Silva, Fateixa, & Trindade, 2013). However, addition of Fe_3O_4 nanoparticles generates opposite effects on drug release rate from κ-CRG hydrogels. Fe_3O_4 nanoparticles makes hydrogels stiffer and increases the afflux of water to balance the osmotic pressure, which in general leads to higher swelling ration (Daniel-da-Silva et al., 2012).

The adhesive property and some other interesting functions, such as positive surface charge, of CRG provide an extra advantage in prolonging drug release in the mucosal/epithelial tissues. For example, κ-CRG, in addition to its good gelation property, exhibits adhesive characteristics alone in part due to its molecular interaction with the mucosal tissues (Kianfar, Antonijevic, Chowdhry, & Boateng, 2013). Another study demonstrated that κ-CRG could interact with chitosan in the presence of tripolyphosphate (TPP) – as a cross-linker for chitosan – to form stable nanoparticles. The size of these particles, as well as their positive surface charge, is ideal for penetrating the epithelial surface (Rodrigues, Costa, & Grenha, 2012). Similarly, microspheres fabricated with κ-CRG or ι-CRG were shown to uniformly cover the inner surfaces of the oral cavity to prevent and treat oral mucositis, with high dispersing efficacy of spheres and favourable formation of a layer of membrane at the inflammation site (Tomoda et al., 2009). Taken together, the unique properties of all three CRG varieties are now benefiting both oral and parenteral drug release in various ways.

3.2. Strong water absorption—For improved drug dissolution

Another simple but useful feature of CRG polymers is their high capacity of water absorption, which improves drug dissolution and thus increases the oral bioavailability of those poorly water-soluble drugs (Tapia et al., 2004). They offer a practical alternative to microcrystalline cellulose (MCC) pellet or gelatin capsule.

In comparison with the most common pellets that are made of MCC, the pellets fabricated with κ-CRG enable water-insoluble drugs to disintegrate and then dissolve as fast as 20 min (Ghanam & Kleinebudde, 2011; Kranz, Jurgens, Pinier, & Siepmann, 2009; Thommes & Kleinebudde, 2006). Similarly, using κ-CRG as excipient could achieve a high loading rate (90%) for the drug theophylline monohydrate. All 60 pellet batches showed adequate and reproducible properties in the shape, size, size distribution and mechanical strength. The dissolution of all batches was finished

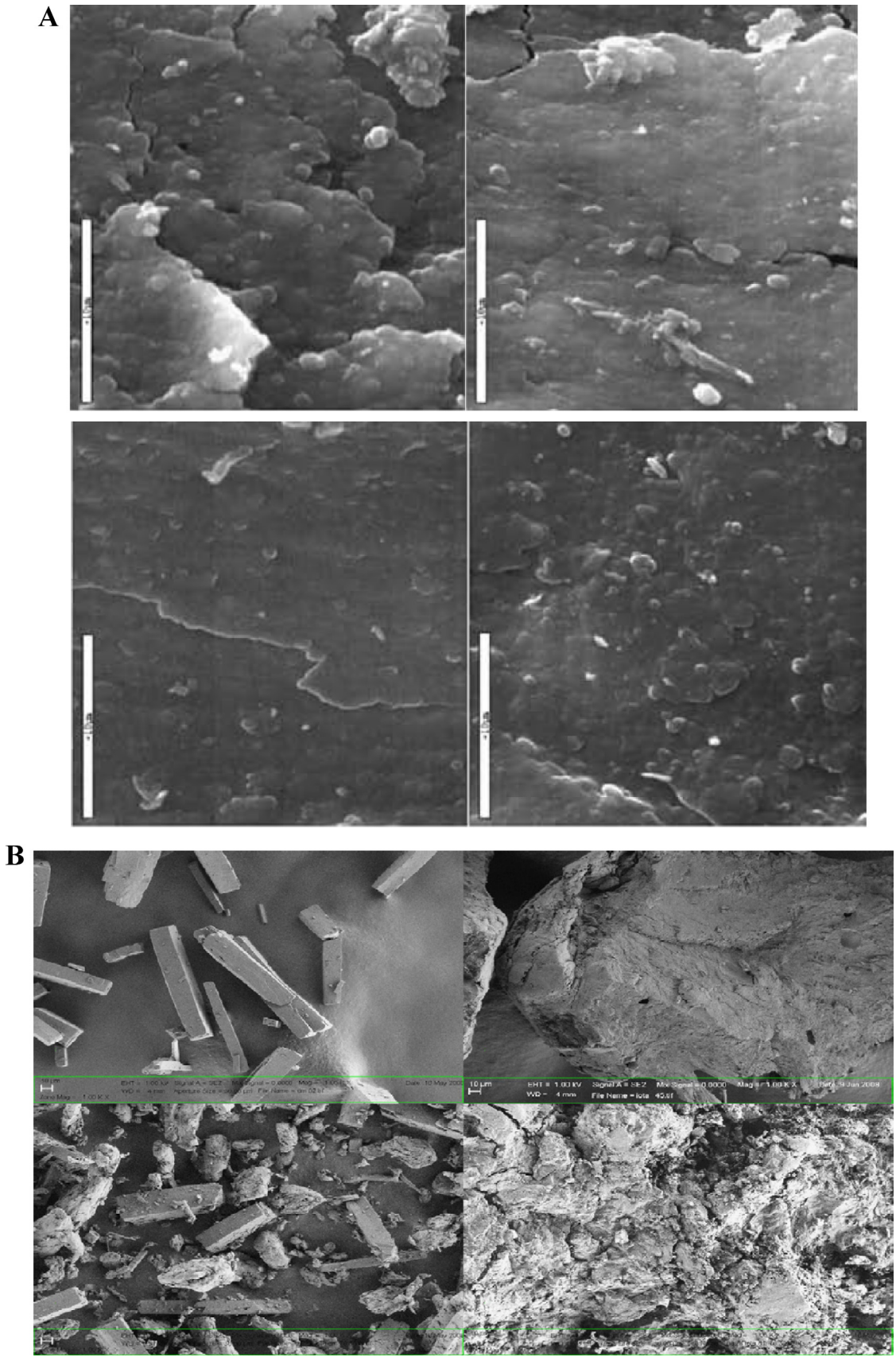

Fig. 4. The changes in structures of CRG with different treatments. (A) SEM images of liquid nitrogen fractured surfaces of ι/κ-CRG blends with ratio of 20/80 w/w (upper left), 40/60 w/w (upper right), 60/40 w/w (lower left) and 80/20 w/w (lower right) (bars are 10 μm) (Nanaki et al., 2010); (B) SEM images of doxazosin mesylate (upper left), a pure ι-CRG paste (upper right), a physical mixture of ι-CRG:DM in a 70:30 ratio (lower left) and an ι-CRG:DM paste in a 70:30 ratio (lower right) (Pavli et al., 2010); (C) SEM images of κ-CRG/carboxymethyl cellulose beads without genipin (upper left), and with 0.5 mM (upper right), 1.0 mM (lower left) and 1.5 mM (lower right) genipin as cross-linker (Muhamad et al., 2011).

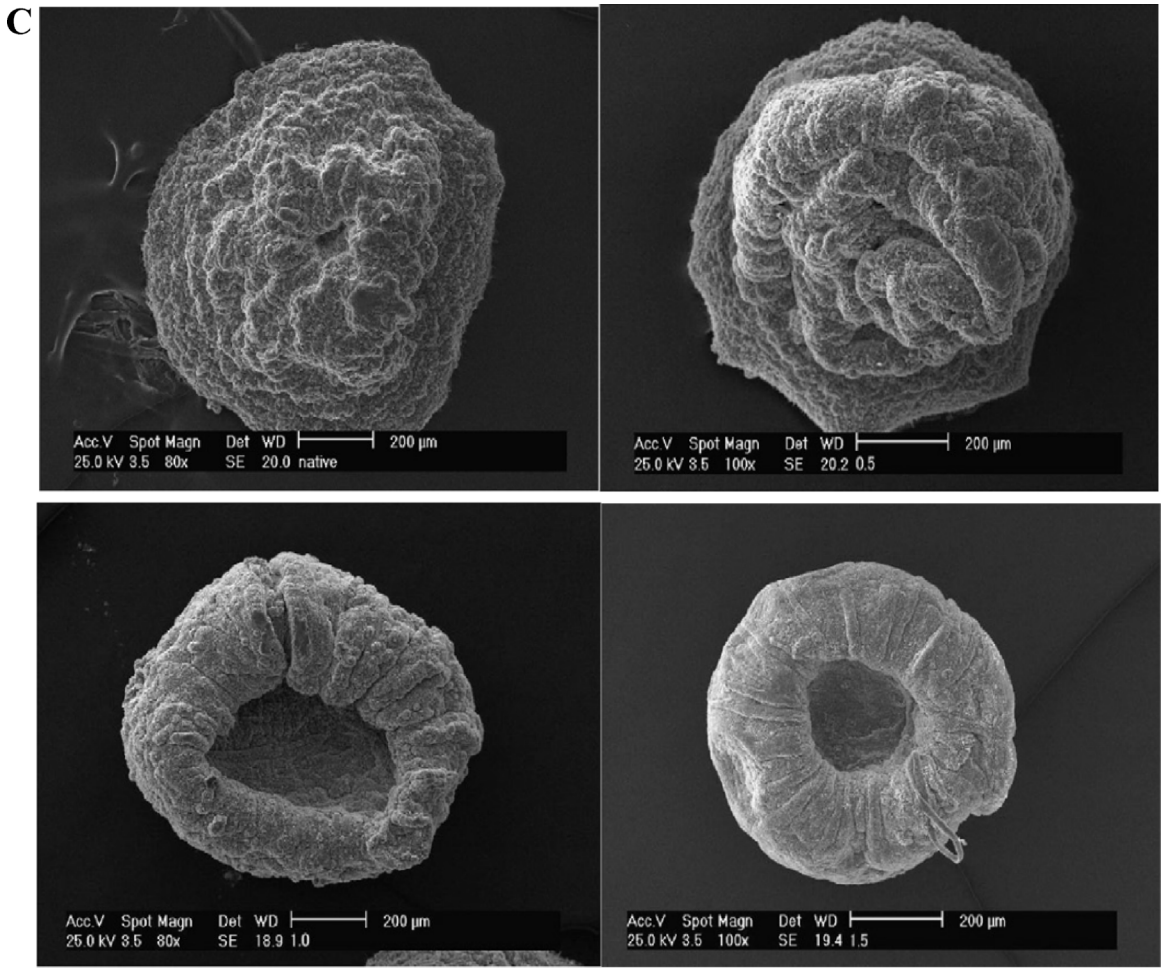

Fig. 4. (*Continued*).

within 40 min, with the average dissolution time between 8.2 and 14.7 min (Krueger & Thommes, 2013).

Moreover, the presence of CRG could also improve the drug dissolution from conventional matrices. Hypromellose capsules containing CRG as a gelling agent showed a rapid *in vivo* disintegration times in the fed and fasted state (Jones, Basit, & Tuleu, 2012; Tuleu et al., 2007). A higher and faster release of quercetin from gelatin-CRG hydrogel (1:2) was also observed compared to hydrogel made of gelatin alone, due to an increase in the porosity of the hydrogel (Varghese, Chellappa, & Fathima, 2014).

Table 1
Oral sustained-release system based on CRG.

Matrix (dosage form)	Model drug	Mechanism	Reference
κ-CR (pellets coated with Kollicoat® SR)	Theophylline	Gel layer protection	Ghanam and Kleinebudde (2011)
ι-CRG (gels)	Acetaminophen	Gel layer protection	Miyazaki et al. (2011)
λ-CR, ι-CR (films)	Tolterodine ι-tartrate	Gel/viscous layer protection, CR–drug hydrogen bonds	Nanaki et al. (2010)
CR (κ-/ι-/λ-) (tablets)	Doxazosin mesylate	Outer layer protection, CR–drug interaction	Pavli et al. (2011), Pavli et al. (2010)
CR (λ-/ι-), chitosan (tablets)	Trimetazidine hydrochloride	CR–drug interaction, ionic interaction between polymers	Li et al. (2013)
CR (κ-/ι-/λ-), chitosan	Glucose oxidase	Ionic interaction between polymers	Briones and Sato (2010)
κ-CR, chitosan (nanoparticles)	Ovalbumin	Ionic interaction between polymers	Grenha et al. (2010)
κ-CR, basic butylated methacrylate copolymer (tablets)	Ibuprofen	Ionic interaction between polymers	Prado et al. (2008)
κ-CR, carboxymethyl cellulose (beads)	β-Carotene	Covalent cross-linking induced by genipin	Muhamad et al. (2011)
κ-CRG, sodium carboxymethyl cellulose (beads)	β-Carotene	Covalent cross-linking induced by genipin	Hezaveh et al. (2012)
κ-CR, polyvinyl alcohol (films)	β-Carotene	Covalent cross-linking induced by genipin	Hezaveh and Muhamad (2013a)
κ-CR, hydroxyethyl cellulose (films)	β-Carotene	Covalent cross-linking induced by genipin	Hezaveh and Muhamad (2013b)
Lectin-functionalized carboxymethylated κ-CR (microparticles)	Insulin	Covalent cross-linking induced by polyglutaraldehyde	Leong et al. (2011a,b)
polyacrylamide grafted κ-CR, sodium alginate (beads)	Ketoprofen	Dual cross-linking	Kulkarni et al. (2012)
κ-CR, gellan gum (composite polyspheres)	Simvastatin	Dual cross-linking	Kulkarni et al. (2013)

3.3. Negatively charged groups—For ionic binding

An important feature that provides CRG versatile capabilities is its overall negative charge. Because of the abundant sulphate groups on the sugar chain, CRG can bind positively charged molecules, either a small compound to deliver or another macromolecule chain to crosslink.

3.3.1. Binding a positively charged drug compound

Ionic binding between CRG and drug compound, as illustrated in Fig. 5A, provides a convenient way of drug loading, avoiding rigorous chemical routes of covalent modification or conjugation and thus the risk of changing the property of the loaded drug. In practice, drug delivery system designed in such way has demonstrated several advantages, including sustained drug release, increased drug loading and improved drug dissolution.

First, such electrostatically formed CRG–drug complex helps to achieve controlled release, which consequently improves drug bioavailability and efficacy (Bonferoni et al., 2004; Li et al., 2013; Nanaki et al., 2010). The formulation of CRG and doxazosin mesylate, a cationic compound and α_1-antagonist, has been reported to enhance sustained release, offering a new approach for designing controlled-release system against hypertension. The SEM images of the aqueous paste made from a mixture of doxazosin mesylate and ι-CRG showed that the drug was dissolved in the vehicle and thus leading to a less cohesive structure than the aqueous paste made from pure ι-CRG (Fig. 4B) (Pavli et al., 2010). In such electrostatic binding, the self-association tendency of doxazosin mesylate molecules near CRG chains has been proven important (Pavli et al., 2011). Similarly, nitrogen atoms on trimetazidine hydrochloride molecule, a drug used for the long-term treatment of angina pectoris, could also interact with sulphate groups of CRG. Such a conjugation has proven to increase the release time and half-life of the drug in physiological condition (Li et al., 2013). Again, the drug release rate highly depends on the type of CRG used. λ-CR, which has the highest linear charge density among the CRG varieties, usually binds the drug molecules most strongly and thus offers the slowest release (Pavli et al., 2010). In contrast, κ-CRG matrix showed the fastest drug release (Pavli et al., 2011) and apparently burst release (Li et al., 2013).

Second, the CRG–drug interaction increases drug loading. For instance, a study using methylene blue as model compound demonstrated that the encapsulation efficiency and loading capacity in κ-CRG nanogels doubled when the CRG content increased from 1.0 to 4.0% (w/w), also based on the interaction between the negatively charged sulphate groups and the positively charged methylene blue molecules (Daniel-da-Silva, Ferreira, Gil, & Trindade, 2011). Likewise, the loading capacity of insulin is increased in the carboxymethylated κ-CRG polymer carrier, due to the ionic interactions between CRG and the amino acids of the insulin (Leong et al., 2011a,b). As another example, the quaternization of κ-CRG particles with 3-chloro-2-hydroxypropyl trimethyl ammonium chloride generated trimethyl ammonium chloride group. Such a modification endows the particles with both negative and positive charges, consequently increasing the loading and release capacity of CRG particles for nearly 6 times (Sagbas, Butun, & Sahiner, 2012).

Third, such electrostatic interaction between CRG and drugs may increase the drug solubility. A poorly water-soluble compound consisting of piperidine and pyridine groups could complex with λ-CRG at pH 1.2. The complex increased the solubility of the free compound by 15–30 times, mainly because this compound became amorphous in the complex. The complex was further nanosized and exhibited the highest aqueous solubility and the fastest dissolution among all test groups (Dai, Dong, & Song, 2007). Similarly, the amorphization and enhancement of solubility were also observed in the complex of λ-CRG and tolterodine ι-tartrate (Nanaki et al., 2010), as well as κ-CRG based buccal wafers laden with paracetamol and ibuprofen (Kianfar et al., 2013).

3.3.2. Binding another positively charged polymer

Like small cationic molecules, polymers with positive charge can also form a polyelectrolyte complex with CRG. Such a complex may better control drug release. The most commonly used polycations is chitosan, which degrades relatively fast but has amine groups ($-NH_2$) to interact with the sulphates of CRG or drug compounds (Briones & Sato, 2010; Grenha et al., 2010; Li et al., 2013) (Fig. 5B).

All three CRG varieties have been tested to complex chitosan. Among them, the κ-CRG/chitosan gel has a molecular structure that is distinct from that of either polymer, and the binding affinity between κ-CRG to chitosan is very high. The complex also showed gastro-protective activity *via* producing the protective layer on the surface of the stomach mucosa (Volod'ko et al., 2014). Meanwhile, ι-CRG could result in a lower erosion ratio and higher swelling ratio than κ-CRG and λ-CRG, because κ-CR forms brittle gels that particularly encourage the entry of water whereas λ-CR that could not form gels lead to fast erosion (Li et al., 2013). However, once an electrostatic repulsion exists between CRG and the drug, such as glucose oxidase which is negatively charged, the lowest release rate of drug and the strongest stability were exhibited by the κ-CRG/chitosan complex (Briones & Sato, 2010).

Apart from chitosan, gelatin, with abundant amino groups, was also used to form complex with CRG *via* electrostatic interaction (Michon, Cuvelier, Launay, & Parker, 1996; Varghese et al., 2014). Because gelatin has a higher melting temperature than CRG, the composite polymer exhibited improved thermal stability than the hydrogel made of CRG alone (Michon et al., 1996; Varghese et al., 2014).

3.4. Abundant functional groups—For chemical modification and covalent crosslinking

The abundant hydroxyl/sulphate groups on the chain of CRG have provided diverse possibilities of covalent modification, in addition to the abovementioned ionic crosslinking. These modifications could endow CRG with some particularly interesting features, such as prolonged drug release and pH sensitivity.

3.4.1. Covalent crosslinking with other polymers to prolong drug release

CRG (in particular κ-CRG) can be covalently linked with other polymers, such as agar, polyvinyl alcohol or carboxymethyl cellulose, with the aid of a natural cross-linker—genipin (Hezaveh & Muhamad, 2013a,b; Hezaveh, Muhamad, Noshadi, Shu Fen, & Ngadi, 2012; Meena, Prasad, & Siddhanta, 2007; Meena, Prasad, & Siddhanta, 2009; Muhamad, Fen, Hui, & Mustapha, 2011). Such crosslinking has proven to enhance the network stability and prevent the burst release of drugs. The surface of genipin-cross-linked κ-CRG/carboxymethyl cellulose beads showed more spherical and smoother than the beads without genipin as cross-linker by SEM, indicating that genipin could lead to the change in molecular arrangement of polymers based on CRG (Fig. 4C). Also, covalent and ionic binding may be combined, by using a bi-functional cross-linking agent such as glutaraldehyde, to prepare gellan–CRG composite with even smaller swelling and slower release than the complex built upon purely ionic crosslinking (Kulkarni, Nagathan, Biradar, & Naikawadi, 2013).

A few factors can affect the release profiles, and a major one is the proportion of CRG in the complex. For many formulations in which CRG is crosslinked with agar (Meena et al., 2009), gellan gum (Kulkarni et al., 2013), polyvinyl alcohol (Hezaveh & Muhamad, 2013a), carboxymethyl cellulose (Muhamad et al., 2011), sodium

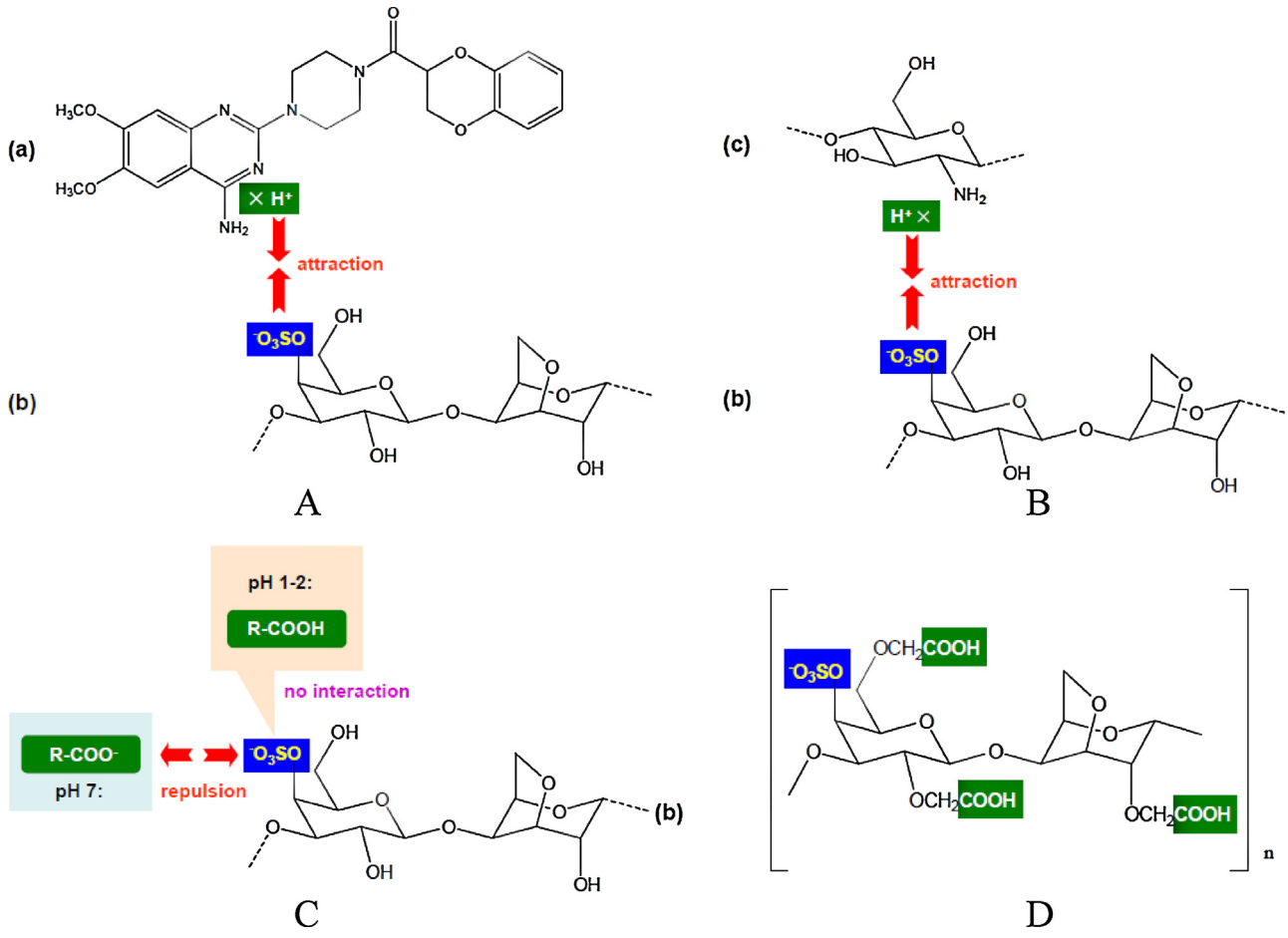

Fig. 5. CRG can interact with small-molecular drugs or polymer scaffolds, and offers abundant functional groups for modification and conjugation. (A) Ionic interaction between CRG and doxazosin mesylate [(a) doxazosin mesylate and (b) κ-CRG]; (B) ionic interaction between CRG and chitosan [(b) κ-CRG and (c) chitosan]; (C) interaction between R—COOH and CRG at different pH; (D) structures of carboxymethylated κ-CRG.

carboxymethyl cellulose (Hezaveh et al., 2012), *N*-isopropyl acrylamide (Mohanan, Vishalakshi, & Ganesh, 2011) or hydroxyethyl cellulose (Hezaveh & Muhamad, 2013b), their overall swelling increases as the proportion of CRG increases. However, increasing the κ-CR content could decrease the extent of swelling in the κ-CR/MCC network (Picker, 1999c) or produce a harder gel with suppressed swelling capacity in the κ-CR/sodium carboxymethyl cellulose complex (Hezaveh & Muhamad, 2012).

Another factor that determines release profile is the concentration of cross-linker. Higher cross-linker concentration could strengthen cross-linking, increase the density of polymer matrix and decrease the inner space for drug diffusion; and meanwhile decreases swelling of polymer matrix and increases the stability of the gel, making it more water resistant and slowing the release rate of drugs (Hezaveh & Muhamad, 2013a,b; Keppeler, Ellis, & Jacquier, 2009; Kulkarni, Boppana, Krishna Mohan, Mutalik, & Kalyane, 2012; Kulkarni et al., 2013; Leong et al., 2011a,b; Muhamad et al., 2011; Paşcalău et al., 2012). For example, more genipin increases the crosslinking of the κ-CRG hydrogels, of which the swelling mechanism changes from diffusion-controlled to polymer relaxation-controlled (Hezaveh & Muhamad, 2013b). In contrast, lower cross-linker concentration increases the swelling of CRG matrix whereas making the gel less stable (Keppeler et al., 2009; Kulkarni et al., 2013). However, exception exists. In a κ-CRG/agar composite, as the genipin concentration increases, the swelling ratio goes up until reaching a 0.8% (w/w) plateau (Meena et al., 2009), probably because the genipin concentration was still not high enough in this case.

3.4.2. pH sensitive drug delivery system

A pH sensitive system is particularly useful for site-specific drug release, such as that in the intestine. To date, there have been four approaches to generate a pH sensitive drug delivery system based on CRG.

The first way is to add carboxyl groups (—COOH) onto the sugar chain of CRG, which could bring the pH sensitivity to this polymer. Carboxyl groups can be added through a carboxymethylation process (Leong et al., 2011a,b) or provided by other polymer blended (Hezaveh & Muhamad, 2012, 2013a,b; Hezaveh et al., 2012; Kulkarni et al., 2012; Muhamad et al., 2011; Pourjavadi, Barzegar, & Zeidabadi, 2007). This strategy provides a promising drug delivery system for oral administration of macromolecules like β-carotene (Hezaveh & Muhamad, 2013a,b; Muhamad et al., 2011). Generally, pH-sensitive CRG polymers show lower swelling ratio and slower drug release at low pH (1.2–2.0), because the carboxylate changes to COOH (the acid form) that is less ionized and promotes the formation of hydrogen bonds with each other. However, in simulated intestinal fluid (pH 7.0–7.4) the electrostatic repulsion between COO^- and sulfonate anions induced a strong swelling force and faster drug release (Fig. 5C). Thus, in simulated gastric fluid, the carboxymethylated κ-CRG (Fig. 5D) beads have a tight network and drug is released through diffusion-control. In simulated intestinal fluid, this material has a relaxed network structure from which drug is released *via* swelling-control (Leong et al., 2011a,b).

Second, to combine CRG and chitosan could also generate pH-dependent release (Li et al., 2013). At low pH, the sulfate groups of CRG remain negatively charged and interact with the positively

charged amino groups of chitosan. At pH 6.8 or in alkaline solution, the amino groups of chitosan will be neutralized, and thus there was no electrostatic interaction. However, drug release from κ-CRG/chitosan system and ι-CRG/chitosan was more sensitive to pH change than that of λ-CRG/chitosan matrix (Li et al., 2013).

It was also found that β-carotene release from genipin-cross-linked gels based on κ-CRG was higher in neutral medium compared to acidic environment because of better swelling ratio in pH 7 (Hezaveh & Muhamad, 2013a,b; Hezaveh et al., 2012). The concentration of cross-linker could also affect the efficiency of pH sensitive system. By increasing the genipin content of κ-CRG/sodium carboxymethyl cellulose nanocomposite hydrogels containing metallic nanoparticles, drug release in pH 1.2 decreased, nevertheless the release rate in pH 7.4 increased. Silver nanoparticles could also improve the targeted release (Hezaveh & Muhamad, 2012).

Finally, the dual crosslinked hydrogel served to release drug in a pH-sensitive manner. For example, the ionically crosslinked hydrogel beads composed of κ-CRG, polyacrylamide and sodium alginate could be further treated with glutaraldehyde that covalently cross-linked sodium alginate. The dual crosslinked beads showed almost perfect control of the drug release–at most 10% of drug was released at pH 1.2, and about 90% of drug released at pH 7.4 (Kulkarni et al., 2012).

4. CRG as biomaterials: The adverse biological effects

Although CRGs have been used for a long time, the concern about their safety persists. Intake of CRG as food additive is generally safe. Experiments evaluating the oral toxicity of food-grade CRG have been conducted in rats, mice, guinea pigs and monkeys. Food-grade CRG is not significantly absorbed from the gastrointestinal tract following oral administration. Acute oral LD_{50} of food-grade CRG in rat is greater than 5000 mg/kg (Weiner, 1991). Cytotoxicity studies employing *in vitro* cultured cell lines showed that nanoparticles made of CRG/chitosan complex (0.1–3 mg/ml) were non-toxic (Grenha et al., 2010). Another study also suggested that the cytotoxicity level of the CRG/alginate hydrogels was extremely low when higher polymeric concentration (3.5%) was used. Interestingly, the hydrogels with lower polymeric concentrations (2.5%) showed relatively higher cytotoxicity. The cell viability with low polymeric concentrations (2.5%) was about 60–70% lower than that with higher concentrations (3.5%) or blank control after treatment for 3 days (Popa, Gomes, & Reis, 2011).

However, evidences have emerged that CRGs exhibit adverse activities towards immune system and blood coagulation (Li et al., 2014; Prajapati et al., 2014), probably due to their sulfate groups (Liang et al., 2014; Wang et al., 2010; Yang, Du, Huang, Wan, & Wen, 2005). The introduction of sulfate groups on G-6 causes the strongest cytotoxicity (Liang et al., 2014). Somewhat similar to heparin/heparan sulfate, the anticoagulant activity of CRG correlates with the content of sulfate groups (Silva et al., 2010). Therefore, extra attention is required when processing CRG into blood-contact biomaterials such as parental drug delivery vehicles or tissue regeneration scaffolds.

Degraded CRG (<50 kDa) could lead to a series of immune reactions linked to the activation of NF-κB, including the inhibition of THP-1 cell proliferation *in vitro*, increase of ICAM-1 expression, aggregation of monocytes, and up-regulation of TNF-α expression and secretion (Benard et al., 2010). The immune response may be influenced by the molecular weight and structure of CRG (or CRG fractions) (Stephanie, Eric, Sophie, Christian, & Yu, 2010). At high concentrations, all types of CRGs up-regulated the expression of interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α), which are notable pro-inflammatory cytokines with strong immunostimulatory effects, while down-regulating of anti-inflammatory interleukin 10 (IL-10) in a dose-dependent manner (Yermak et al., 2012). CRGs can induce paw oedema and pleurisy *in vivo* using rats (Silva et al., 2010). In laboratories, CRGs are employed to create pathological models for exploration of the process and treatment of inflammation (Albayrak et al., 2013; Bonet, Fischer, Parada, & Tambeli, 2013; Ghosh et al., 2013; Shao et al., 2013).

Furthermore, CRG may induce adverse effects on human intestinal epithelial cells. Cell death was found in human colonic epithelial cells–both primary cells and a cell line, after the treatment of CRG (1–10 mg/l) for 1–8 days. It was further suggested that the cells died through necrosis instead of apoptosis (Bhattacharyya, Borthakur, Dudeja, & Tobacman, 2008). Exposure to un-degraded and degraded CRG was associated with the occurrence of intestinal ulcerations and tumour (Tobacman, 2001).

CRG may also impair the blood vessel formation. Oligosaccharides derived from both κ-CRG and λ-CRG could inhibit the growth of new vessels significantly in chicken chorioallantoic membrane model (Chen, Yan, Lin, Wang, & Xu, 2007; Yao, Wu, Zhang, & Du, 2014). They could also inhibit the invasion, proliferation, migration and tube formation of human umbilical vein endothelial cells (Chen et al., 2007; Yao et al., 2014) probably *via* down-regulation of intracellular MMP-2 expression (Chen et al., 2007).

5. Perspectives

The increasing use of CRG for biomedical purposes has motivated us to explore more possibilities in design and application of these macromolecules, In particular, future studies may be focused on the following three directions.

The first one is to explore more possibilities of chemical modification. Chemical modification is among the most practical ways to engineer polysaccharides, and has indeed been extensively applied to CRG. For example, it has been demonstrated that carboxymethylated κ-CR could generate pH-sensitive delivery system as detailed in the above sections (Leong et al., 2011a,b) and exhibit a series of good properties (such as antibacterial activity and moisture-retention capacity) (Fan et al., 2011). Multi-step carboxymethylation was also studied to produce CRG derivatives with high degree of substitution (Tranquilan-Aranilla, Nagasawa, Bayquen, & Dela Rosa, 2012). However, the art of chemistry is expected to offer more. An exciting question to ask is whether we can lower the adverse biological effects of CRG by modifying functional groups on the CRG chain. Specifically, is it possible to minimise the anticoagulant activity from sulfate groups while retaining the good properties of the gel? Such and similar questions invite more investigations into both design of new chemistry routes and understanding of the relationship between structure and function.

The second interesting focus is to devise temperature-sensitive matrices based on CRG. In theory, this is possible, because a few studies have indicated that temperature could affect the drug release property of CRG gels through two major mechanisms. First, temperature may affect the swelling. For example, methylene blue release from κ-CRG nanogels achieved a plateau at 60% at both 25 °C and 37 °C, increasing to 95% at 45 °C. It is probably because CRG based gels swell as temperature increases (Daniel-da-Silva et al., 2011). The hydrogel composed of κ-CRG and poly(*N*-isopropyl acrylamide) was observed to exhibit a significant change in water content as temperature changes. Higher temperature removes more water and thus decreases the swelling of CRG (Mohanan et al., 2011). Second, higher drying temperature (>80 °C) of κ-CRG pellets could decrease the disintegration time and mean dissolution time due to the fragmentation of the κ-CRG molecule (Thommes,

Blaschek, & Kleinebudde, 2007), also suggesting that drying temperature may control the drug release from CRG matrix. These preliminary findings provide practical information for design of a CRG-based system from which drug can be released in response to temperature.

The third direction ongoing is to broaden the use of CRG in the areas of tissue engineering. Although this is still a relative new field to explore, a few studies have started to show the desirable properties of CRG-based hydrogels, such as their interconnected porous network (Varghese et al., 2014), stability (Mihaila et al., 2013), elastic behaviour (Mihaila et al., 2013) and viscoelastic property (Mihaila et al., 2013), for tissue engineering applications. For instance, a murine chondrocytic cell line in the alginate/κ-CRG hydrogel beads/fibres demonstrated high viability and proliferation during a 21-day long-term culture (Popa et al., 2011). The combination of physical and chemical crosslinking of CRG also resulted in the formation of hydrogels permitting maintenance of viable encapsulated cells (Mihaila et al., 2013). Inflammatory response in body may lower local pH. The pH sensitive scaffolds made of CRG and chitosan which were demonstrated to be stable at acidic environment could work in response to inflammation (Araujo et al., 2014). Moreover, κ-CRG could be developed to encapsulate platelet derived growth factor that plays key roles in various physiological processes; such a system has the potential to be used for wound healing and other regenerative purposes (Santo et al., 2009). Furthermore, κ-CRG hydrogel also showed potential for bone-like apatite formation which will be helpful to the reconstruction of damaged bone (Kim et al., 2011). Clearly, these explorations shed light on the use of CRG for a broad range of regenerative medicine applications, while the current research should focus on how different types of cells interact with these natural polymers.

To sum up, although there have been a number of literatures analysing the advantages and disadvantages of CRG in biomedical applications, a comprehensive cost-/risk-benefit evaluation in a scientific perspective is still absent, for which the lack of systematic understanding of adverse biological effects may be an important reason. More comprehensive toxicology studies, both *in vitro* and *in vivo*, with specific models and under proper regulatory guidelines are highly demanded.

6. Conclusion

In summary, CRG, in its three major variant forms, possesses favourable physicochemical features, including unique gelling mechanisms, strong water absorption capacity, overall negative charge and abundant function groups to modify/crosslink. These properties have prepared this natural polysaccharide a versatile material for biomedical applications, extending from food processing to drug delivery to broader purposes such as the ongoing trials of tissue regeneration. Meanwhile, though widely accepted to be safe as a food additive, CRG, as biomaterial components, may cause adverse effects to blood coagulation, immune system or mucosa cells. Balancing their outstanding physicochemical properties and potential biosafety concerns is critical for the increasing use of these polysaccharide polymers in the pharmaceutical and medical industry. More comprehensive toxicology studies, both *in vitro* and *in vivo*, with specific models and under proper regulatory guidelines are highly demanded.

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