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Fabrication of lactobionic-loaded chitosan microcapsules as potential drug carriers targeting the liver

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ABSTRACT

This paper demonstrates a general approach for fabrication of lactobionic chitosan microcapsules using layer-by-layer assembly via click chemistry. Chitosan was selectively modified with either azide (CHI-Az) or alkyne (CHI-Alk) groups. The growth of the CHI-Az/CHI-Alk click multilayer was studied experimentally by multilayer assembly on planar supports. Linear buildup of the film was observed. The chitosan click capsules were also analyzed with confocal laser scanning microscopy and transmission electron microscopy. Capsules were found to have regular spherical shapes. In addition, (CHI-Az/CHI-Alk)-coated particles were modified with fluorescein isothiocyanate to ensure that the particles can be easily post-functionalized. Finally, lactobionic acid was conjugated onto the (CHI-Az/CHI-Alk)-coated particles and the lactobionic particles exhibited hepatoma cell (HepG2) targeting behavior.

1. Introduction

Liver cancer is a common fatal disease [1,2]. However, chemotherapy for cancer is largely limited by the toxicity of the drugs used to normal tissues [3]. Furthermore, limited circulation in the blood, poor aqueous solubility, limited stability and nonselectivity reduce the therapeutic efficacy and limit the clinical application of anticancer drugs [3]. To solve these problems, various drug carriers such as micelles, nanogels and microcapsules have been investigated [4–6]. A novel type of hollow polyelectrolyte capsule fabricated via layer-by-layer (LbL) self-assembly on sacrificial template particles, followed by core removal, has attracted a great deal of research attention. With this technique, the capsules can be engineered with a high level of control of properties such as size and shape, composition, and functionality [7,8]. Due to the toxicity to normal tissues of traditional cancer therapies, the design of drug carriers with active targeting properties is highly important. It was reported that asialoglycoprotein (ASGP) receptors were specifically abundant on the surface of hepatoma cells [9,10]. Therefore active targeting of these cells can be accomplished via introduction of galactose—which can be recognized by hepatoma cells via ASGP receptors located on their surfaces—into drug carriers for the treatment of liver cancers.

Here, we fabricated lactobionic chitosan microcapsules as potential drug carriers targeting the liver using LbL assembly via click chemistry. The click chemistry approach for LbL assembly was proposed in Refs. [11,12]. The combination of the LbL technique and click chemistry is promising mainly for two reasons.

Firstly, various kinds of materials, including those of the same charged or non-charged polymers, can be used for LbL assembly, resulting in single-component polymer, covalently stabilized, multilayer films. For traditional LbL strategies, at least two components are needed for building up the capsule wall. However, the existence of a second polymer component would limit the functionalities of the specific materials. For example, the biocompatibility and biodegradability of chitosan would be strongly influenced by a second polymer component. Thus, this technique can easily be used to fabricate single-component capsules.

Secondly, in our study, the degrees of functionalization of chitosan were kept below 10%, allowing the majority of chitosan to retain its functionality. The excess groups of chitosan, which are not modified with click functionalities in the multilayer, can be used to post-functionalize with other materials, including biomacromolecules, peptides or other functional groups. Herein, we aimed to use this method for the fabrication of biodegradable multilayered capsules with the desired properties.

Designing novel materials for biomedical applications generally requires the use of biodegradable materials. Chitosan, a renewable natural polysaccharide, is of great interest due to its good biocompatibility and biodegradability [13,14], and has been considered for various applications in modern biomedical and pharmaceutical fields [15,16]. Moreover, chitosan is a polycation and carries positive charge at low pH. Thus, it has been widely used for fabricating LbL films [17–21] and capsules [22–25] with other polyelectrolytes. Here, we demonstrate a new way to fabricate chitosan microcapsules, using click chemistry as the driving force of the LbL assembly. In this study, chitosan was selectively functionalized with either

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azide (CHI-Az) or alkyne (CHI-Alk) groups. The CHI-Alk was then modified with rhodamine dye. Chitosan click capsules were obtained after removal of the $CaCO_3$ particle template and were shown to have regular spherical shapes. In addition, (CHI-Az/CHI-Alk)-coated particles were modified with fluorescein isothiocyanate (FITC) to ensure that the particles can be easily post-functionalized. Finally, lactobionic acid was conjugated onto the (CHI-Az/CHI-Alk)-coated particles, which enable the particles to bind specifically to the ASGP receptors on the surfaces of hepatoma cells (HepG2).

2. Experimental

2.1. Materials

Chitosan (M_w = 50 kDa, deacetylation degree = 85.3%) was purchased from Haidebei Marine Bioengineering Co. Ltd. (Jinan, PR China). Poly(sodium 4-styrene-sulfonate) (PSS) (M_w = 70 kDa) was obtained from Sigma–Aldrich. 2-Bromo-2-methylpropionic acid (98%), fluoresceinamine isomer (FITC) and lactobionic acid were purchased from ACROS. Rhodamine B was obtained from Alfa Aesar. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sodium azide (NaN_3), potassium carbonate (K_2CO_3), calcium chloride ($CaCl_2$), copper(II) sulfate, sodium ascorbate, sodium hydroxide (NaOH), dimethyl formamide (DMF) and disodium ethylenediaminetetraacetate (EDTA) were purchased from Shanghai Reagent Chemical Co. (PR China). All the reagents were used directly unless specifically indicated. 4-Oxo-4-(prop-2-ynyloxy) butanoic acid used was synthesized according to our previous report [26]. Fetal bovine serum albumin (FBS), Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin and phosphate-buffered saline (PBS) were purchased from Sigma–Aldrich. All other chemicals were obtained from Shanghai Reagent Chemical Co. (PR China) and used as received.

2.2. Synthesis of alkyne-modified chitosan

Chitosan with alkyne functionality (CHI-Alk) was synthesized via amide bond formation between the amine groups of chitosan side-chains and carboxyl groups of 4-oxo-4-(prop-2-ynyloxy) butanoic acid. 1.0 g chitosan was dissolved in acetic acid solution (2 wt.%). Then 0.9 g 4-oxo-4-(prop-2-ynyloxy) butanoic acid and 2.3 g EDC were added after adjusting pH of the solution to 5–7. The clear solution was stirred at room temperature for 24 h. The crude product was dialyzed extensively against de-ionized (DI) water for 3 days and freeze-dried to obtain white polymer in a yield of 0.8 g.

2.3. Synthesis of azide-modified chitosan

Chitosan with azide functionality (CHI-Az) was synthesized via EDC-mediated coupling of the amine moieties of chitosan with 2-bromo-2-methylpropionic acid. Chitosan (1.0 g) was dissolved in 50 ml acetic acid solution (2 wt.%). After adjusting the pH of the solution to 5–7, 1.0 g 2-bromo-2-methylpropionic acid and 2.3 g EDC were added. The clear solution was then stirred at room temperature for 24 h. The crude product was dialyzed and freeze-dried. The product was then dissolved in acetic acid solution (2 wt.%). 0.6 g NaN_3 was added after adjusting the solution to neutral. The reaction was carried out for 24 h at room temperature. The CHI-Az was obtained by dialyzing and freeze-drying for 3 days, yielding 0.7 g.

2.4. Fluorescent labeling of alkyne-modified chitosan

CHI-Alk was subsequently fluorescently labeled with a dye, rhodamine B. All CHI-Alk was dissolved in distilled water. Then rhodamine B (10 mol.% based on amine groups of the chitosan) and EDC (1.2-fold molar excess compared to rhodamine B) was added. The mixture was stirred for 24 h at room temperature. Rhodamine B-labeled CHI-Alk was obtained by dialyzing against DI water and freeze-drying.

2.5. Fourier transform infrared spectroscopy

The samples were analyzed by Fourier transform infrared spectroscopy (FT-IR) using a Perkin–Elmer Spectrum One spectrophotometer. Before the measurements, the samples were pressed into potassium bromide (KBr) pellets.

2.6. ^1H nuclear magnetic resonance

The ^1H nuclear magnetic resonance (NMR) spectra of the polymers were recorded on a Mercury VX-300 spectrometer at 300 MHz (Varian, USA) using D_2O as a solvent and TMS as an internal standard.

2.7. Multilayer assembly on planar supports

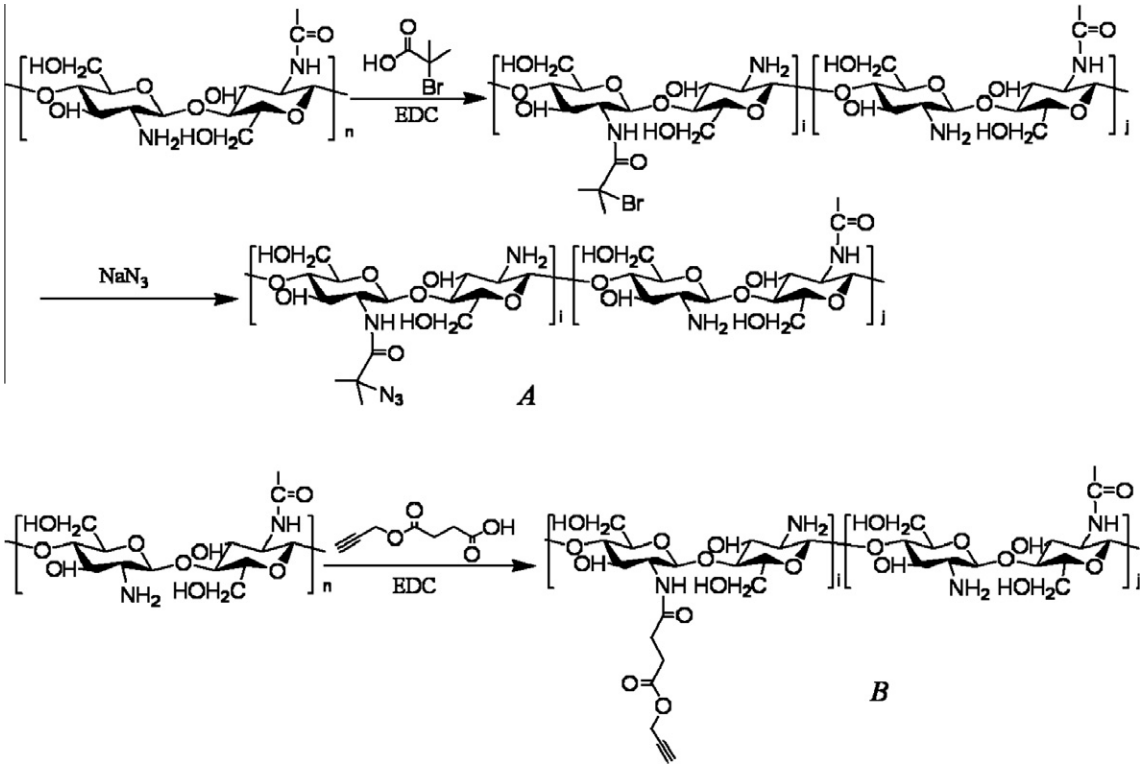
Rectangular quartz slides (15×8 mm^2) were cleaned by H_2O_2/H_2SO_4 solution (1:3 v/v) at 80 °C for 90 min and then dipped in a mixture solution containing H_2O, H_2O_2 and $NH_4{\cdot}OH$ (5:1:1 v/v/v) at 80 °C for 90 min (Caution! piranha solution is highly caustic and great care must be taken when handling.) The slides were finally washed with DI water, and dried in air to obtain a negatively charged surface. For chitosan click multilayer assembly, the negatively charged slides were sequentially immersed in CHI-Az and CHI-Alk solutions containing copper sulfate and sodium ascorbate for 15 min, accompanied by water rinsing after deposition of each layer. The dipping solutions were prepared as follows according to the literature [27]: (a) CHI-Az (1.0 mg ml^{-1}), (b) CHI-Alk (1.0 mg ml^{-1}), (c) DI water (pH 7.0), (d) copper(II) sulfate (0.4 mg ml^{-1}) and (e) sodium ascorbate (0.8 mg ml^{-1}). The pH of each solution was adjusted to 6.1 with 1 M HCl or NaOH solution. Polymer dipping solutions were made up in a constant volume ratio of 3(a or b):1(d):1(e). The washing solutions were made up in a similar ratio except using solution (c) in place of (a) or (b). Dipping solutions and washing solutions were prepared 2 min prior to use.

2.8. Characterization of multilayered films

We used UV–vis spectra obtained on a Lambda Bio40 UV/vis spectrometer (Perkin–Elmer) to monitor the LbL assembly process. The morphology of the resulting multilayer was analyzed by atomic force microscopy (AFM) using a Shimadzu SPM-9500J3 operated in tapping mode at 20 °C. The morphology of the quartz slide was also analyzed by AFM for comparison. The formation of linkages in the resulting multilayer was studied by X-ray photoelectron spectroscopy (XPS). XPS was conducted on a XSAM 800 spectrometer (Kratos, UK) with an Mg KR target. An analysis area of approximately 0.6×0.8 mm^2 was used to measure three locations per sample.

2.9. Fluoresceinamine isomer capture by ~4 μm $CaCO_3$ particles

$CaCO_3$ particles with narrow size distribution were prepared according to the literature [28,29]. In order to analyze $CaCO_3$ particles by confocal laser scanning microscopy (CLSM), we added fluoresceinamine isomer in a preparation of $CaCO_3$ to label the particles with fluorescence. Briefly, 5 ml 0.33 M K_2CO_3 solution containing 10 mg fluoresceinamine isomer was rapidly poured into 5 ml of a 0.33 M solution of $CaCl_2$ containing 10 mg PSS at room



Scheme 1. Synthesis route of azide-modified chitosan (A) and alkyne-modified chitosan (B).

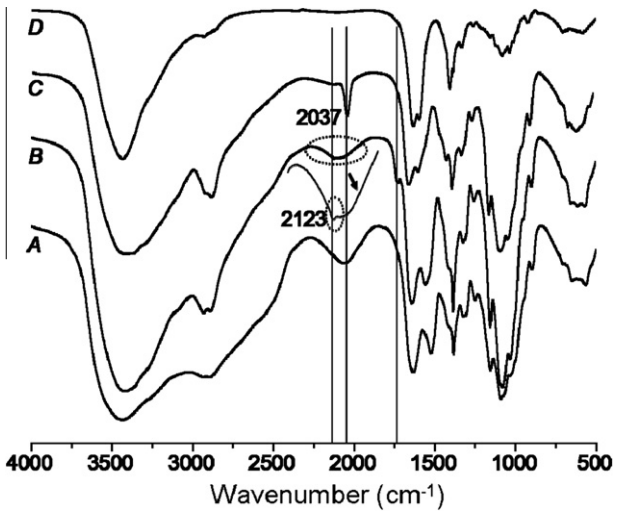


Fig. 1. FTIR spectra of chitosan (A), alkyne-modified chitosan (B), azide-modified chitosan (C) and chitosan click capsules (D). The extra curve is an expansion of the alkyne stretch shown in curve B.

temperature. After intense agitation for 30 s, the reaction mixture was left still for about 2 min. Then the precipitate was filtered off, thoroughly washed with DI water and acetone, and dried in air. The whole process was protected from light wherever possible.

2.10. Multilayer assembly on $CaCO_3$ particles

Approximately 60 mg $CaCO_3$ particles and 1.5 ml CHI-Az solution were mixed and the mixture was shaken to form a symmetrical suspension. This incubation was allowed to stand for 15 min to establish a CHI-Az layer. After adsorption, the particles were centrifuged at 10,000 rpm for 1 min, followed by washing with DI water.

The following dipping solutions were made as per those reported in Section 2.7 above. After pre-coating with CHI-Az, 1.5 ml of CHI-Alk solution (containing copper and ascorbate) were added and allowed to incubate for 15 min. Then the particles were centrifuged and washed with the washing solutions. This was followed by adsorption of CHI-Az, followed by the same washing protocol. The process was repeated until the desired number of layers was obtained. The whole process was protected from light wherever possible.

2.11. Formation of click capsules

To form the click capsules, the multilayer-coated $CaCO_3$ particles were treated with 0.2 M EDTA. Dissolution of the $CaCO_3$ core occurred after less than 1 min. The capsules were imaged by CLSM and transmission electron microscopy (TEM).

2.12. Characterization of chitosan click capsules

The core–shell and hollow particles were viewed with CLSM (Nikon C1-si, BD Laser at 488 nm). For TEM, a drop of a concentrated capsule solution was placed on a clean TEM grid and allowed to dry in air. TEM analysis was carried out with a JEM-100CX II instrument operating at an acceleration voltage of 100 kV.

2.13. Post-functionalization of (CHI-Az/CHI-Alk)-coated particles by FITC

The particles were incubated in anhydrous DMF containing FITC (10 mg ml^{-1}) for 24 h. The functionalized particles were obtained after several washing steps with anhydrous DMF and at least seven washing steps with DI water. A control sample was incubated in DMF without FITC for 24 h. The conjugation of FITC was then

analyzed by monitoring the fluorescence intensity of the resulting particles by flow cytometry.

2.14. Post-functionalization of (CHI-Az/CHI-Alk)-coated particles by lactobionic acid

Since ~4 μm $CaCO_3$ particles are too big to be internalized by HepG2 cells, we used $CaCO_3$ particles with a small size in the cell-targeting assay in order to assess the cell internalization of the capsules into tumor cells. The preparation of small $CaCO_3$ particles is as follows: 5 ml 0.33 M K_2CO_3 solution was rapidly poured into 5 ml 0.33 M $CaCl_2$ solution at room temperature. The subsequent steps are similar to those used for the preparation of ~4 μm (CHI-Az/CHI-Alk)-coated $CaCO_3$ particles. (CHI-Az/CHI-Alk)-coated $CaCO_3$ particles were then incubated in lactobionic acid solution (10 mg ml^{-1}, pH 7.0) containing EDC (2-fold molar ratio based on lactobionic acid) for 24 h. The functionalized particles were obtained after five washing steps with DI water. A control sample was incubated in solution without lactobionic acid for 24 h. The lactobionic adsorption was then analyzed using a HepG2 cell targeting experiment.

After HepG2 cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin–streptomycin, 10,000 U ml^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 day in an incubator, the culture was replaced by medium containing post-functionalized (CHI-Az/CHI-Alk)-coated particles (0.5 mg ml^{-1}). The mixture was then further incubated for 4 h. Cellular adhesion or internalization was studied by CLSM after the plates had been washed six times with PBS. A control experiment was carried out, using non-functionalized (CHI-Az/CHI-Alk)-coated particles instead of post-functionalized ones.

2.15. Statistical analysis

The results are representative of replicate experiments and the results are represented by the mean and SD. The statistical analyses were performed using Student's *t*-test. Probability values less than 0.05 ($P < 0.05$) were considered to be indicative of statistical significance.

3. Results and discussion

3.1. Synthesis of chitosan with pendant groups

Alkyne and azide pendant groups were introduced onto chitosan to investigate the possibility of LbL assembly by click chemistry. The introduction of an alkyne functional moiety onto chitosan is achieved via the coupling reaction between carboxyl and amine groups in the presence of the coupling reagent EDC (Scheme 1). As for the introduction of the azide functional group, the substitution reaction between Br and NaN_3 at room temperature is widely used [30,31]. As shown in Scheme 1, the precursor Br-modified chitosan was synthesized. The azide-modified chitosan was then obtained via substitution reactions. Since LbL assembly of CHI-Az and CHI-Alk only requires a few functionalized groups, while the low degree of functionalization would retain the original biocompatibility and

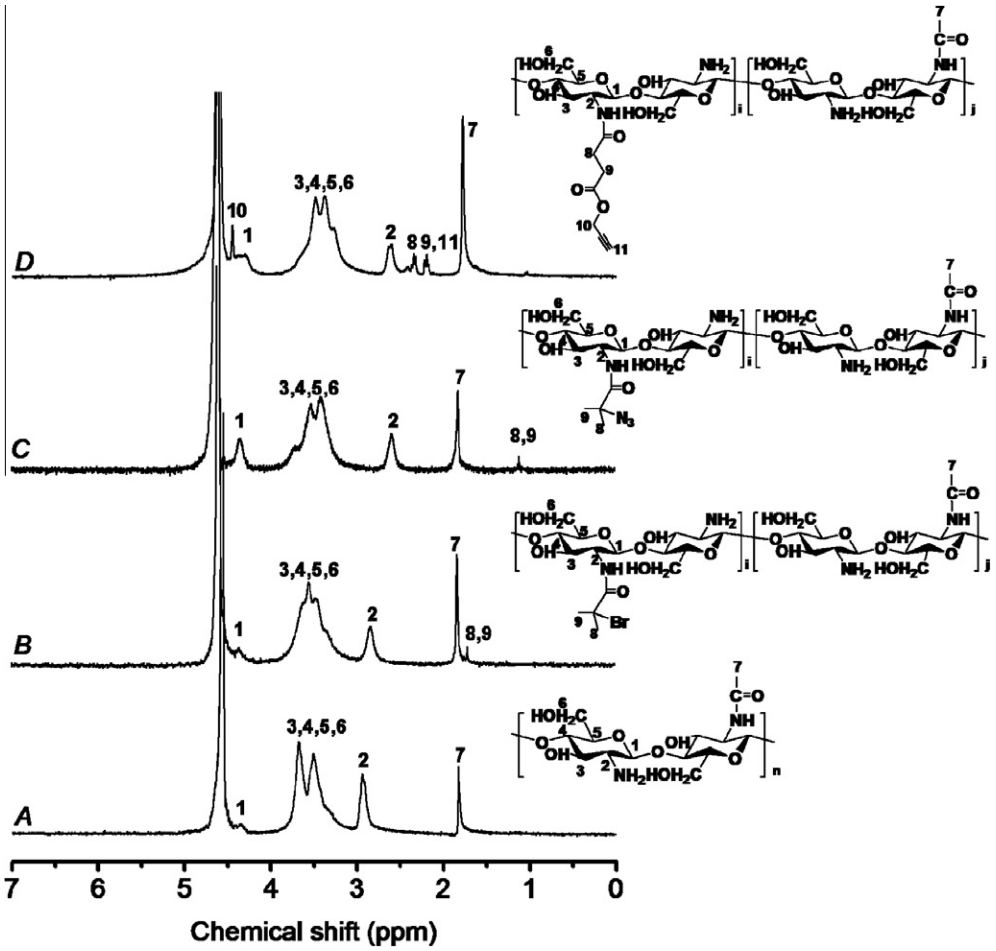


Fig. 2. ^1H NMR spectra of chitosan (A), Br-modified chitosan (B), azide-modified chitosan (C) and alkyne-modified chitosan (D).

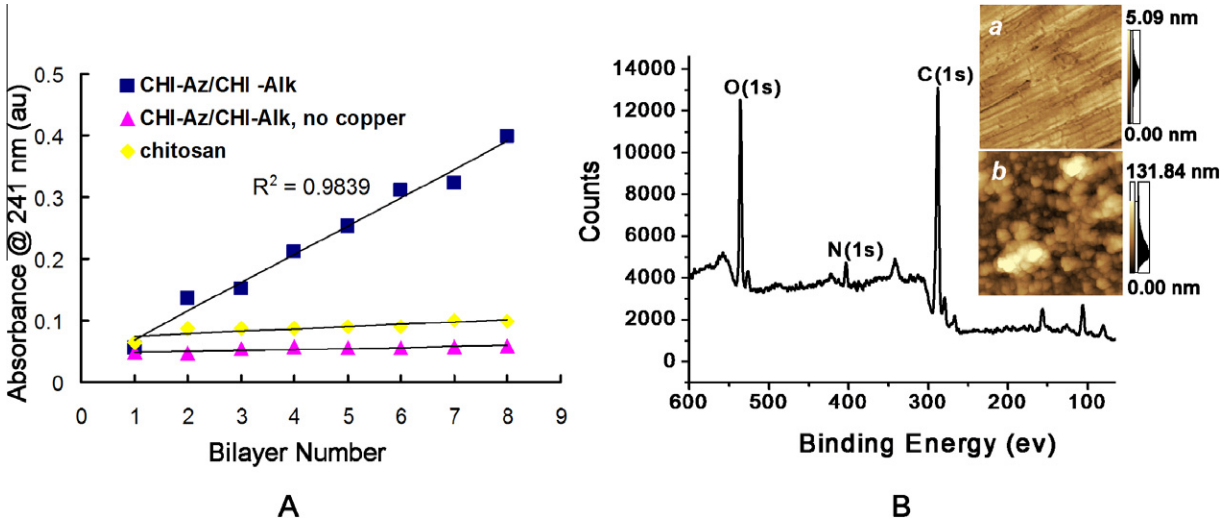
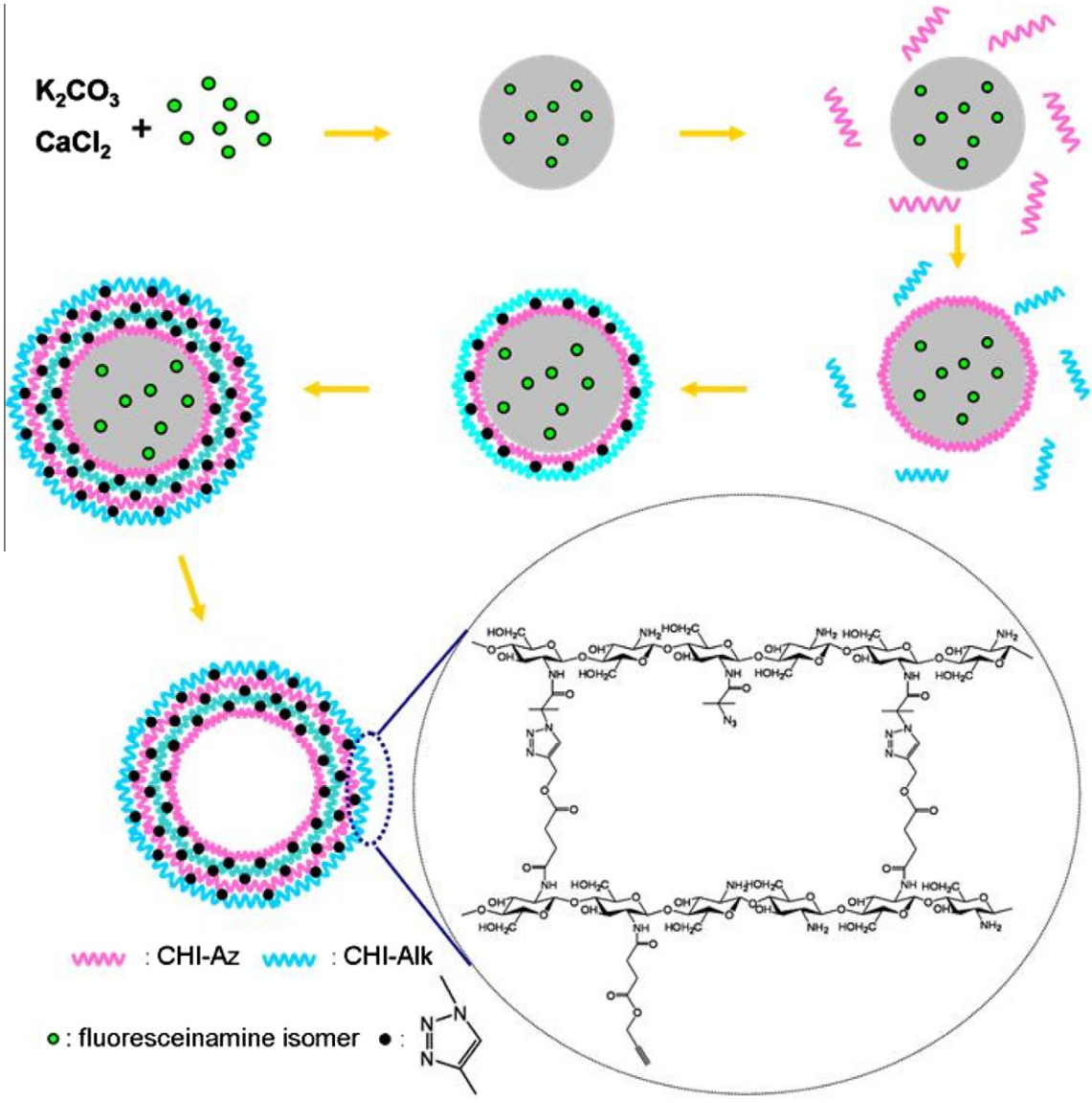


Fig. 3. Plots of absorbance at 241 nm as a function of multilayered films assembled on quartz with increasing bilayer number by UV–vis spectroscopy (A). XPS spectra of chitosan click multilayer assembly on planar supports (B). Inset: AFM image of clean quartz slide (a) and $(CHI\text{-}Az/CHI\text{-}Alk)_8$ multilayered film assembled on quartz (b) (area = $2 \times 2\ \mu m^2$).



Scheme 2. Assembly of click chitosan multilayer (CHI-Az/CHI-Alk) on $CaCO_3$ particle and chitosan click capsule formation.

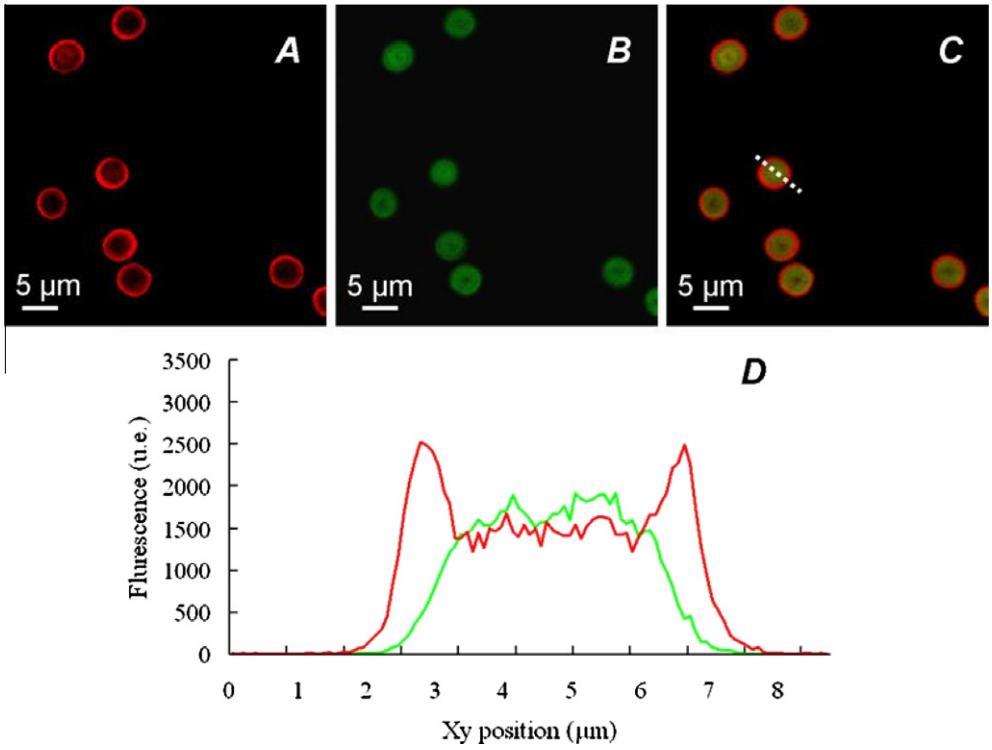


Fig. 4. CLSM images of chitosan multilayer coating on the $CaCO_3$ particles (A), $CaCO_3$ particles filled with fluoresceinamine isomer (B) and chitosan-coated $CaCO_3$ particles (C). The profile in (D) corresponds to the line in (C).

biodegradability of chitosan, the degrees of functionalization of chitosan were kept below 10%, and all of the experiments were carried out in water. The CHI-Alk was then modified with rhodamine B dye to enable the capsules to be studied by CLSM.

Fig. 1 shows that the IR spectra of chitosan with functional groups (Fig. 1B and C) are similar to those of chitosan itself (Fig. 1A). For CHI-Alk (Fig. 1B), the typical absorption of alkyne appeared at 2123 cm^{-1}. In addition, the stretching variation absorption of C=O in alkyne pendant units existed at 1745 cm^{-1}. Based on the ^1H NMR spectra of alkyne-modified chitosan, the chemical shift at 4.6 ppm was attributed to the methylene protons of —OCH_2C≡CH. And the chemical shifts at 2.2, 2.25, 2.4 and 4.5 ppm were mainly associated with the protons of the alkyne pendant groups —C=OCH_2CH_2C=OOCH_2C≡C*H*, —C=OCH_2CH_2C=OOCH_2C≡CH, —C=OCH_2CH_2C=OOCH_2C≡CH and —C=OCH_2CH_2C=OOCH_2C≡CH, respectively. Other resonances are assigned to the backbone chain of chitosan. In the case of the azide-modified chitosan, to ensure the complete conversion from Br to azide pendant groups, a large excess of NaN_3 compared to the Br in the polymeric chain was added. In this study, the product was dialyzed against DI water for several days and the dialyzing water was changed many times to ensure the excess NaN_3 was removed completely. As shown in Fig. 1C, the peak at 2037 cm^{-1} indicated the existence of azide pendant groups. For ^1H NMR spectra, after the substitution reaction between Br and NaN_3, the original chemical shift of methyl protons (—C(CH_3)$_2$– Br) at 1.75 ppm disappeared and a new signal appeared at 1.2 ppm in the ^1H NMR spectra of the azide-modified chitosan, which was associated with the corresponding methyl protons of —C(CH_3)$_2$—N_3. This demonstrated the conversion from Br to azide pendant groups after the substitution reaction. All the other signals are related to chitosan backbone chain.

Herein, the real degree of substitution (DS) of CHI-Alk and CHI-Az were determined from their ^1H NMR spectra (Fig. 2B and C). On the basis of the ^1H NMR spectra, the DS of alkyne and azide functional groups in the polymeric chain was calculated by comparing the integration ratio of the methylene protons (2H) (—C=OCH_2CH_2C=OOCH_2C≡CH) of alkyne pendant units at 2.4 ppm and the methyl protons (6H) (—C=OC(CH_3)$_2$-N_3) at 1.2 ppm to that of the protons (5H) which positioned on C-3, C-4, C-5 and C-6 appearing at around

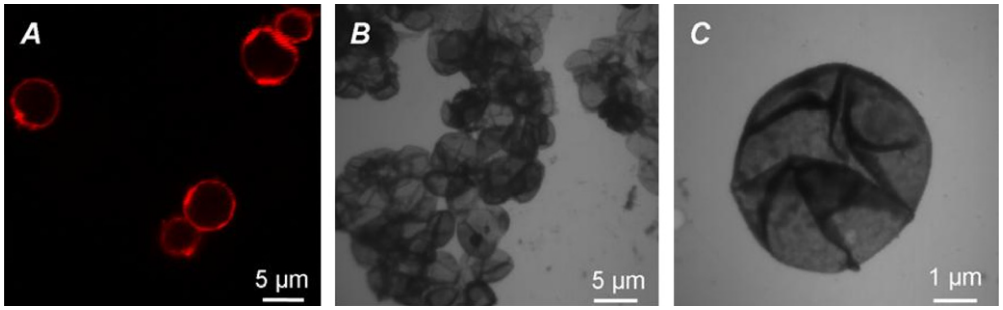


Fig. 5. CLSM (A) and TEM (B, C) images of (CHI-Az/CHI-Alk)$_8$ click capsules.

3.6 ppm respectively. ^{1}H NMR analysis confirmed that ~10% of the chitosan side chains were functionalized with alkyne moieties and ~6% were modified with azide moieties.

3.2. Multilayer assembly on planar supports

The growth of the CHI-Az/CHI-Alk click multilayer was studied via an experiment involving multilayer assembly on planar supports. The LbL assembly of CHI-Az/CHI-Alk multilayer was monitored by UV–vis spectroscopy [27,32,33] (Fig. 3A). Linear growth of the film was observed by monitoring the peak at 241 nm mentioned in a previous study [27]. A control of chitosan without click groups (control, ◇) showed a plateau in absorbance after only four layers, indicating that the click functionalities are essential for the deposition of consecutive chitosan layers and the formation of chitosan multilayer. If no copper(II) sulfate and sodium ascorbate was added (control, Δ), the absorbance remained constant low, suggesting that film did not build up and indirectly confirming that copper(I) acts as a catalyst for LbL assembly of click-modified polymers. Moreover, the absorbance of CHI-Az/CHI-Alk without copper is even lower than that of chitosan without click groups, which was attributed to the fact that the intermolecular interaction of chitosan was weakened after chitosan had been functionalized with click groups. For XPS study, a peak at 400 eV, which has been commonly assigned to triazole functionality [34], confirmed the formation of triazole linkages in the chitosan multilayer (Fig. 3B). The morphology of clean quartz slide and air-dried chitosan multilayered film was examined by AFM. The AFM images are shown in Fig. 3B as an insert picture. The *z* scale of chitosan multilayered film is ~130 nm (Fig. 3Bb) compared to ~5 nm of clean quartz slide (Fig. 3Ba). The rough surface of multilayered film proved the successful deposition of chitosan onto the quartz slide.

3.3. Multilayer assembly on $CaCO_3$ particles

LbL assembly on colloids was performed by sequentially exposing ~4 μm $CaCO_3$ particles captured with fluoresceinamine isomer to CHI-Az and CHI-Alk solutions (1.0 mg ml^{-1}) containing copper sulfate (0.4 mg ml^{-1}) and sodium ascorbate (0.8 mg ml^{-1}) at pH 6.1. The particles were incubated for 15 min in each chitosan solution, and were then centrifuged and washed with DI water (Scheme 2). The chitosan-coated $CaCO_3$ was studied by CLSM. Fig. 4 shows CLSM images of chitosan-coated $CaCO_3$ particles. Fig. 4A and B show the localization of labeled chitosan and the $CaCO_3$ core captured with fluoresceinamine isomer, respectively. Fluorescence microscopy confirmed that the chitosan multilayer coating on the $CaCO_3$ particles was uniform. From Fig. 4B, we can see that fluoresceinamine isomer, which was added during the $CaCO_3$ particle preparation process, was successfully captured by $CaCO_3$ particles and distributed evenly. Fig. 4C represents those $CaCO_3$ particles coated with rhodamine-labeled CHI-Alk/CHI-Az shell layers. It can be seen that LbL multilayer-coated $CaCO_3$ particles have a red corona layer surrounding a green $CaCO_3$ inner core. Moreover, the diameters of the red rings are larger than those of the green ones, proving that chitosan was almost adsorbed on the surface of $CaCO_3$ particles to form a capsule wall instead of being captured into the porous $CaCO_3$. Moreover, the profile in Fig. 4D represents the fluorescence intensity of a single chitosan-coated $CaCO_3$ particle. As shown in Fig. 4D, the fluorescence intensity of labeled chitosan is higher on the edges of the particles than that in the interior, which also confirms the core–shell structure of the chitosan-coated $CaCO_3$ particles. The red fluorescence in the interior shown in Fig. 4D may due to the porous structure of $CaCO_3$ particles, leading to a small portion of labeled chitosan being captured in the interior.

3.4. Characterization of chitosan click capsules

The chitosan click multilayered core–shell particles contain $CaCO_3$ cores. Fig. 5A shows a fluorescent image of chitosan click capsules. A uniform coating with red fluorescently labeled chitosan and a regular spherical ring of the click capsules were observed. After EDTA treatment at pH 7, only a red shell layer can be seen, confirming the complete removal of the $CaCO_3$ core. At the same time, the small molecule fluoresceinamine isomer permeated out of the capsules because of the semi-permeable property of polyelectrolyte shells, which are generally known to be permeable to molecules with molecular weights below 5 kDa [35]. Moreover, it can be seen that chitosan click capsules swelled to 6.5 ± 0.5 μm compared with the 4 μm $CaCO_3$ templates. The slightly enlarged size of the hollow capsules after the $CaCO_3$ core removal may due to increased osmotic pressure in the interior void spaces filled with ionic species [36]. From the confocal images, it can be concluded that the triazole linkages between chitosan chains are readily formed under these mild conditions. To investigate the reaction of alkyne and azide functional groups in the capsule wall, the freeze-dried capsules were analyzed by FT-IR. From the IR spectra of the chitosan click capsule (Fig. 1D), we that see that the typical peaks of alkyne and azide have disappeared, suggesting that most of functional groups have been reacted. The TEM image shows that the click capsules collapsed and folded but remained intact after drying (Fig. 5B).

3.5. Post-functionalization of (CHI-Az/CHI-Alk)-coated particles by FITC

The modification of chitosan with click groups used only a minor fraction of the amine moieties on chitosan. Therefore, the remaining free amine groups in the multilayer on the surface of the particles could be used for post-functionalization with various materials, including small molecules such as drugs as well as large molecules such as peptides or biomacromolecules. The capability of the particles to be post-functionalized was demonstrated by reacting FITC with $(CHI\text{-}Az/CHI\text{-}Alk)_8$ multilayer. Chitosan multilayered click films were assembled as mentioned above with eight bilayers of (CHI-Az/CHI-Alk).

Both FITC-functionalized and non-functionalized particles were analyzed by flow cytometry. As shown in Fig. 6, non-functionalized particles exhibit ~6% fluorescence intensity, due to the fluoresceinamine isomer being captured in the $CaCO_3$ core. However, the

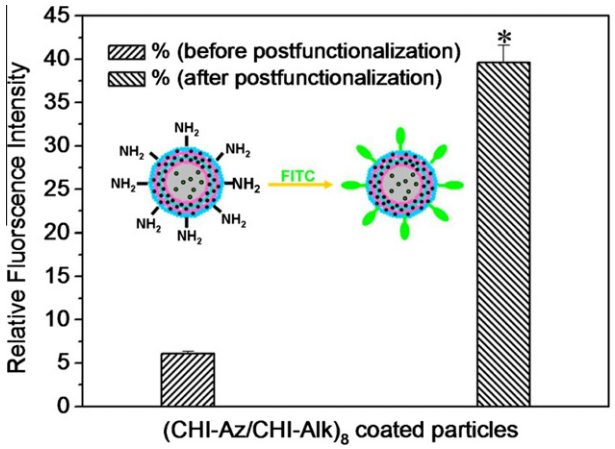


Fig. 6. Relative fluorescence intensity of $(CHI\text{-}Az/CHI\text{-}Alk)_8$-coated particles before and after post-functionalization as monitored by flow cytometry. Inset: schematic illustration of the post-functionalization process. Data are shown as mean ± SD (n = 3). ($*P < 0.05$ as compared with other samples in the same group).

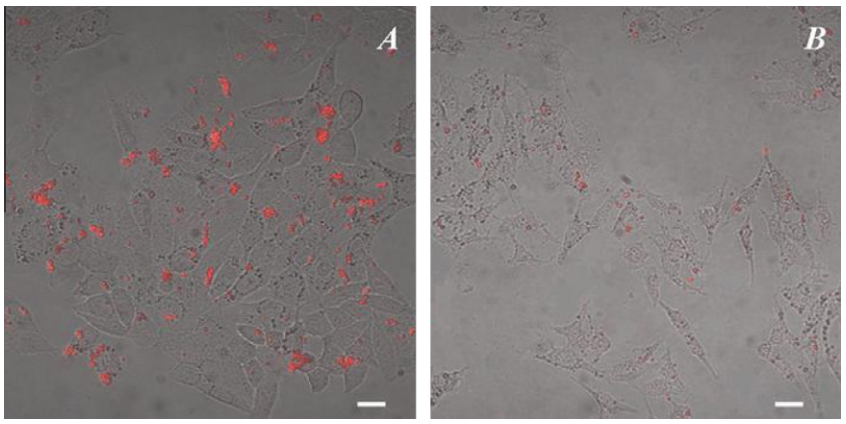


Fig. 7. CLSM images of HepG 2 cells adhered with lactobionic acid functionalized (A) and non-functionalized (B) particles under bright field with excitation at 488 nm. (Scale bar = 20 μm.).

FITC-conjugated particles exhibited a significantly improved fluorescence intensity of ~40% (Fig. 6). This proves that the particles were successfully post-functionalized by FITC and indicates that the free amine groups in the chitosan multilayer film/capsules can be used to react with other molecules to introduce other properties to the chitosan multilayer film/capsules.

3.6. Post-functionalization of (CHI-Az/CHI-Alk)-coated particles by lactobionic acid

In drug-delivery applications, therapeutic capsules are often designed with targeting moieties. To introduce targeting properties to our biodegradable multilayered click films/capsules, we post-functionalized the (CHI-Az/CHI-Alk)-coated particles with lactobionic acid. In order to make it easier for the particles to enter the cells, we used $CaCO_3$ particles of smaller size. According to the literature [37], the $CaCO_3$ microparticles are assembled from a number of tiny crystallites or nanoparticles that have previously been stabilized by the PSS molecules. Hence, here the $CaCO_3$ particles were prepared without adding PSS in order to obtain smaller sized $CaCO_3$ particles; the resulting particles were shown to have an average diameter of around 900 nm (Fig. 7). Both lactobionic acid functionalized and non-functionalized (CHI-Az/CHI-Alk)-coated particles were then used for the cell targeting assay. HepG2 cells were cultivated in the presence of the special particles to test the cell targeting behavior. As shown in Fig. 7B, non-functionalized particles showed a little non-specific binding to HepG2 cells, due to the electrostatic interaction between positively charged chitosan and negatively charged cells. However, the lactobionic acid conjugated particles exhibited significantly improved adhesion to HepG2 cells or even being internalized by the tumor cells (Fig. 7A). Since lactobionic acid was introduced to (CHI-Az/CHI-Alk)-coated particles by reacting with free amine groups of chitosan, incorporation of lactobionic acid would weaken the positive charge of the particles. Thus, for the lactobionic acid conjugated particles, the electrostatic interaction between positively charged chitosan and negatively charged cells would be weaker than that of non-functionalized particles. Therefore the difference shown in Fig. 7 indicates that the particles were post-functionalized by lactobionic acid. However, to confirm the mechanism of these targeted microcapsules for binding, further study is necessary. Further work will be focused on studying the binding mechanism of the targeted microcapsules and their applications in biomedical fields. Our approach described here, using an easy post-functionalization step to introduce targeting properties to biodegradable multilayered click films/capsules, has great potential for targeted drug delivery applications.

4. Conclusions

We have demonstrated a general click chemistry strategy to fabricate lactobionic chitosan microcapsules via LbL assembly approach. The growth behavior of the multilayer is found to be linear, and it was proven that click functionalities are essential for the deposition of consecutive chitosan layers and the formation of chitosan multilayer. Chitosan click capsules were formed by removal of $CaCO_3$ cores. Finally, in order to target the liver, lactobionic acid was conjugated onto the (CHI-Az/CHI-Alk)-coated particles. The lactobionic acid functionalized particles exhibited specific binding to HepG2 cells. After removal of the cores, we were able to obtain chitosan microcapsules that targeted the liver. This provides a versatile platform for designing novel vehicles for use in targeted drug delivery systems.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 3–7 and Scheme 2, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.11.042.

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