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Homogeneous synthesis of GRGDY grafted chitosan on hydroxyl groups by photochemical reaction for improved cell adhesion

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ABSTRACT

A novel homogeneous synthesis route was presented to produce RGD-containing peptide modified chitosan (CTS) with purpose of improving cell adhesion and growth. Bifunctional photosensitive crosslinker, Sulfo-SANPAH, was used to link cell adhesive peptide GRGDY and CTS under controlled condition. The synthesis process was proved by FTIR, MALDI-TOF MS and ^{1}H NMR analyses, and the mechanism was demonstrated clearly and completely that hydroxyl groups of CTS were prior to amino groups for nucleophilic reaction with Sulfo-SANPAH. Moreover, cell adhesion and proliferation were evaluated for GRGDY grafted CTS. The results showed that GRGDY grafted CTS formed by the novel strategy had potential application not only as drug or gene carriers but also as tissue engineered scaffolds.

1. Introduction

Chitosan (CTS), a unique cationic polysaccharide extracted from shells of crab and shrimp, has been proved an attractive biomaterial due to properties of low cost, nontoxicity, biological activity, biocompatibility, biodegradability, and potential chemical modification on reactive amino and hydroxyl groups along molecular chain (Illum, 1998; Kim et al., 2007). Therefore, it has been widely used in pharmaceutical areas for drug delivery systems (Agnihotri, Mallikarjuna, & Aminabhavi, 2004), DNA-mediated gene therapy (Borchard, 2001), and recently siRNA delivery (Liu et al., 2007). With the protonated amino groups, drug carriers based on chitosan can unspecifically associate with negatively charged cellular membrane via electrostatic interaction, which is helpful for cell absorption and subsequent release of drugs. On the contrary, without adequate amino groups, it will exert a great influence on function and strength of these carriers. In recent years, new trend has emerged to develop functionalized drug carriers with some or all following properties such as longevity, targetability, intracellular penetration, contrast loading, and stimuli-sensitivity (Torchilin, 2006).

The targeting strategies with specific ligand–receptor recognition can help drug carriers accumulate at target sites, which results in low toxic effect on healthy tissues and improved therapeutic efficiency. So far many different kinds of ligands, for example, antibody (Chiu, Ueno, & Lee, 2004), peptide (Schiffelers et al., 2004), folate (Mansouri et al., 2006), and galactose (Park et al., 2001), have been conjugated to carrier polymers including chitosan for targeting delivery to the corresponding receptors on cell membrane. Among mentioned ligands, tripeptide arginine–glycine–aspartic acid (RGD), a highly conservative amino acid sequence of adhesive proteins in extracellular matrix (ECM), has been found to play an important role in cell recognition, adhesion, migration, and proliferation. The molecular mechanism is that RGD-containing peptides can specifically recognize and bind the integrin receptors (Ruoslahti & Pierschbacher, 1987), which not only exist on the membrane of normal cells but also overexpress on the membrane of tumor cells (Su et al., 2002). Thus, the conjugation of RGD-containing peptide onto CTS can endow CTS-based drug carriers specific capacity of target delivery and association to cells and tissues, especially to tumor cells for release of anticancer or nucleic acid drugs. Many studies have attempted to modify CTS with RGD-containing peptide. However, most of them relied on the reaction on amino groups of CTS. For example, conjugation of RGD-containing peptide on CTS film and three-dimensional (3D) CTS scaffold for bone tissue engineering by EDC/NHS mediated reaction bringing imide-bond formation between amino groups in CTS and carboxyl groups in peptide (Ho et al., 2005). There were more complicated modification strategies as follows. CTS was firstly modified with succinic anhydride on amino groups, then RGD-containing peptide was introduced by EDC/NHS mediated reaction between carboxyl groups in succinic anhydride and amino groups in peptide (Li, Yun, Gong, Zhao, & Zhang,

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2006). The other example was to modify CTS with sulfhydryl groups, then to couple of RGD-containing peptide by disulfide bonds formation (Masuko et al., 2005). These kinds of RGD–CTS materials were acceptable only for the enhancement of cell attachment and subsequent cell growth and proliferation. The limitation of above method, however, appears while CTS is used to form micro- or nano-carriers for drug or gene delivery. In this situation, the protonated amino groups of CTS are usually needed to react with negatively charged polymers (e.g., alginate) to form microcarriers (Wang et al., 2006) or polyelectrolyte complex membranes (Wan et al., 2008) or to directly complex with DNA/siRNA to form nanoparticles for gene delivery (Kim et al., 2007). Therefore, it is necessary to save or only partly utilize amino groups. Under this consideration, hydroxyl groups of CTS become the alternative choice for RGD-containing peptide conjugation. In the few publications (Chung, Lu, Wang, Lin, & Chu, 2002; Chung et al., 2003), the graft of GRGD peptide on the surface of chitosan film by inducing photochemical reaction between azido groups of *N*-succinimidyl-6-[4′-azido-2′-nitrophenylamino]-hexanoate (SANPAH) and hydroxyl groups of chitosan structure was described. The photochemical reaction was heterogeneously carried out under ultraviolet (UV) irradiation, and the modified CTS film pre-neutralized by TPP or NaOH led to improved adhesion and growth of human umbilical vein endothelial cells.

In this study, a novel homogeneous synthesis route of conjugating CTS with RGD-containing peptide, GRGDY, was developed by bifunctional crosslinker Sulfo-SANPAH under photochemical reaction. This cross-linking agent with two dissimilar reactive ends is heterobifunctional, which is used in two-step sequential reactions to minimize undesirable intra-molecular cross reaction and polymerization. Further more, the synthesis method is indeed better than formerly reported heterogeneous way (Karakecili, Satriano, Gumusderelioglu, & Marletta, 2008), because homogeneous reaction is generally recognized easier and faster than heterogeneous reaction due to the completely mixture of reactants. Moreover, the modified CTS was characterized with FTIR, MALDI-TOF MS, and ^{1}H NMR analyses. Finally, the ability of modified CTS on cell adhesion and subsequent cell proliferation was assessed. The modified CTS can then be lyophilized with saved amino groups for long time storage. After being easily processed as any shape or structure as needed theoretically, it can be widely applied not only as drug or gene carriers but also as tissue engineered scaffolds.

2. Materials and methods

2.1. Materials

Cell adhesive peptide Gly-Arg-Gly-Asp-Tyr (GRGDY) with molecular weight (M_w) of 566.57 was purchased from Bootech Bioscience & Technology Co. Ltd. (Shanghai, China). Bifunctional photosensitive crosslinker sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino]hexanoate (Sulfo-SANPAH) with M_w of 492.40 was purchased from G-Biosciences & Geno Technology Inc. (St. Louis, MO, USA). Raw chitosan was purchased from Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang, China). Chitosan samples were obtained by heterogeneous degradation and deacetylation of raw chitosan resulting in a disperse product with M_w spanning from 10 to 100 kDa as determined by gel permeation chromatography (GPC) and DD of 67–95% determined by nuclear magnetic resonance (NMR) spectroscopy. B16–F10 cells (C57BL/6 mouse, melanoma) were obtained from KeyGen Biotech. Co. Ltd. (Nanjing, China). Trypsin, streptomycin, penicillin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium was purchased from Gibco, Invitrogen Co. (New York, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China).

2.2. Homogeneous synthesis of GRGDY–SANPAH–CTS by photochemical reaction

Firstly, Sulfo-SANPAH, bifunctional crosslinker with *N*-hydroxysuccinimide (NHS) ester ends and photosensitive azido ends, was conjugated with GRGDY peptide by NHS mediated reaction through the formation of imide linkages. Briefly, 120 mg/mL GRGDY peptide aqueous solution was added into Sulfo-SANPAH EtOH solution with the molar ratio ranging from 1:1 to 10:1. The mixed solution was then kept under darkness and stirred for 2 h at room temperature (Lin et al., 2001), which produced GRGDY–SANPAH. Secondly, 1 g of CTS was dissolved in 100 mL 1% v/v acidic solution, and then CTS solution was mixed with above GRGDY–SANPAH solution. To activate the homogeneous reaction, the above solution was put into an ultraviolet (UV) generator (Beijing, China), and was irradiated at 40 W for 10 min (Chung et al., 2003; Legrand et al., 1992). Subsequently, the reaction solution was dialyzed with double distilled water for more than 3 days until without UV absorbance at 254 nm (phenyl group of both Sulfo-SANPAH and tyrosine) and 220 nm (imide bond of GRGDY peptide) in the water outside, followed by lyophilization at −50 °C for 24 h resulting homogeneously synthesized GRGDY–SANPAH–CTS.

2.3. Fourier transforming infrared spectroscopy (FTIR) analysis

GRGDY–SANPAH–CTS samples were pressed with KBr and scanned from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. The infrared spectra were recorded using a Bruker spectrometer (Vector22, Switzerland).

2.4. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

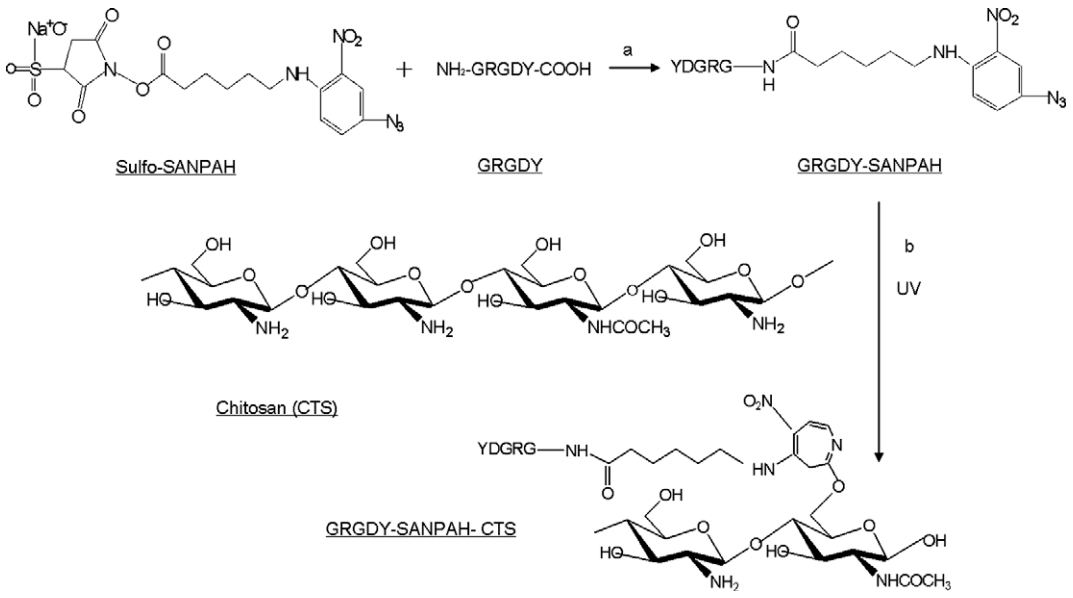
The characterization of the synthesized compound was also performed with Autoflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF MS analysis. Matrix of 2 μL α-cyano-4 hydroxycinnamic acid (15 mg/mL in 70% ACN containing 0.1% TFA) was mixed with 2 μL of sample solution, and 2 μL of mixture was deposited on the MALDI target and left in air for dryness and for further analysis by MALDI-TOF MS. For a control, 2 μL of CHCA matrix was directly deposited on MALDI target for further analysis. The analytical range of laser energy was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratio (S/N). All mass spectra shown were obtained in the positive-ion reflection mode under pressure less than 1×10^{-4} Pa with delayed time of 40 ns; each spectrum was typically added by 20 laser shots. External mass calibration was obtained by using two points that bracketed the mass range of interest.

2.5. Nuclear magnetic resonance (NMR) analysis

^{1}H NMR spectra were recorded using a ^{1}H NMR instrument (Bruker Inc., Germany) at 500.13 MHz. Lyophilizate (5 mg) was introduced into an NMR test tube (Ø 5 mm), to which 0.5 mL of 2% v/v CD_3COOD/D_2O solution was added, and finally the test tube was kept at 70 °C to dissolve the polymer in solution (Hirai, Odani, & Nakajima, 1991). Single pulse (30°) sequence was used for FID accumulation. And the pulse repetition delays were 2 s.

2.6. Fabrication of CTS or GRGDY–SANPAH–CTS (mCTS) film

To assess functional properties of grafted GRGDY peptide, the behavior of cells on CTS and GRGDY–SANPAH–CTS films were studied. First of all, CTS or mCTS film was prepared by diluting CTS or mCTS solution mentioned above to 0.1% w/v, and 10 μL of each solution was pipetted onto cover glasses (Ø 15 mm). The solution was allowed to dry about 5 min at room temperature.



Scheme 1. Illustration of homogeneous synthesis of GRGDY–SANPAH–CTS.

The formed films were immersed in 0.2 M NaOH to neutralize for 15 min, and thoroughly rinsed extensively with sterile deionized water or pH 7.4 phosphate-buffered saline (PBS) for several times, then sterilized with 75% ethanol.

2.7. Cell adhesion

B16–F10 cells (1×10^4 cells/500 μL) were seeded in 24-well plates with CTS or mCTS film. Then, cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 g/mL) at 37 °C, in a humidified atmosphere equilibrated with 5% CO_2 for 12 h. The morphology of cells was observed and recorded by phase contrast microscopy (Nikon Co., Japan) in order to compare adhesion difference of cells on CTS or mCTS film. Moreover, cells saturated by GRGDY peptide (1 mg/500 μL) prior to culture on mCTS film were also investigated.

2.8. Measurement of cell growth and proliferation

For the assessment of cell growth, B16–F10 cells (0.7×10^3 cells/500 μL) were seeded in 24-well plates with CTS or mCTS film. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 g/mL) at 37 °C, in a humidified atmosphere equilibrated with 5% CO_2. Following 5-day incubation without change of media, thiazolyl blue (MTT) assay was performed as standard protocol to assess cellular viability. After indicated incubation time, 50 μL of MTT (5 mg/mL) dye was added to each well for 1 h. Dye was solubilized with 500 μL of dimethyl sulfoxide (DMSO) solution and plates were read at 570 and 630 nm on an automated microtiter plate reader (Wellscan MK3, Labsystems Dragon, Finland). B16–F10 cells were used as control in all experiments.

2.9. Statistical analysis

Samples in triplicate were analyzed for each experiment. All quantitative results were expressed as the mean value ±SEM (standard error of measurement). Statistical analysis was performed using Student's *t*-test to compare the difference between selected groups. In all statistical evaluations, the difference was considered significant when $p < 0.05$.

3. Results and discussion

3.1. Homogeneous synthesis of GRGDY–SANPAH–CTS by photochemical reaction

As described in Section 2.2, the process of homogeneous synthesis of GRGDY–SANPAH–CTS included two reaction steps (Scheme 1). Firstly, photosensitive crosslinker, Sulfo-SANPAH, was conjugated with GRGDY peptide by mixing them in aqueous/EtOH solution. The product together with both reactants was analyzed by FTIR. In comparison with FTIR spectrum of individual reactant in Fig. 1, the peak of ester band at 1739 cm^{-1} remarkably weakened in GRGDY–SANPAH spectrum accompanied by the increase of amide I peak at 1653 cm^{-1}. The reason was that *N*-hydroxysuccinimide (NHS) ester ends of Sulfo-SANPAH were unstable and easily hydrolyzed in aqueous medium, which subsequently mediated reaction with primary amino groups of peptide to form stable imide bonds. It was also noticed that ester peak at 1739 cm^{-1} decreased while amide I peak at 1653 cm^{-1} increased

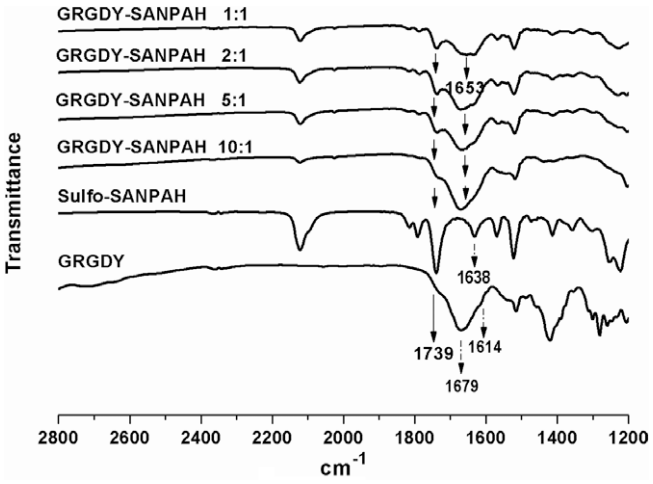


Fig. 1. FTIR spectra of GRGDY–SANPAH and individual reactant. The molar ration of GRGDY to Sulfo-SANPAH ranging from 1:1 to 10:1; Sulfo-SANPAH and GRGDY were as control.

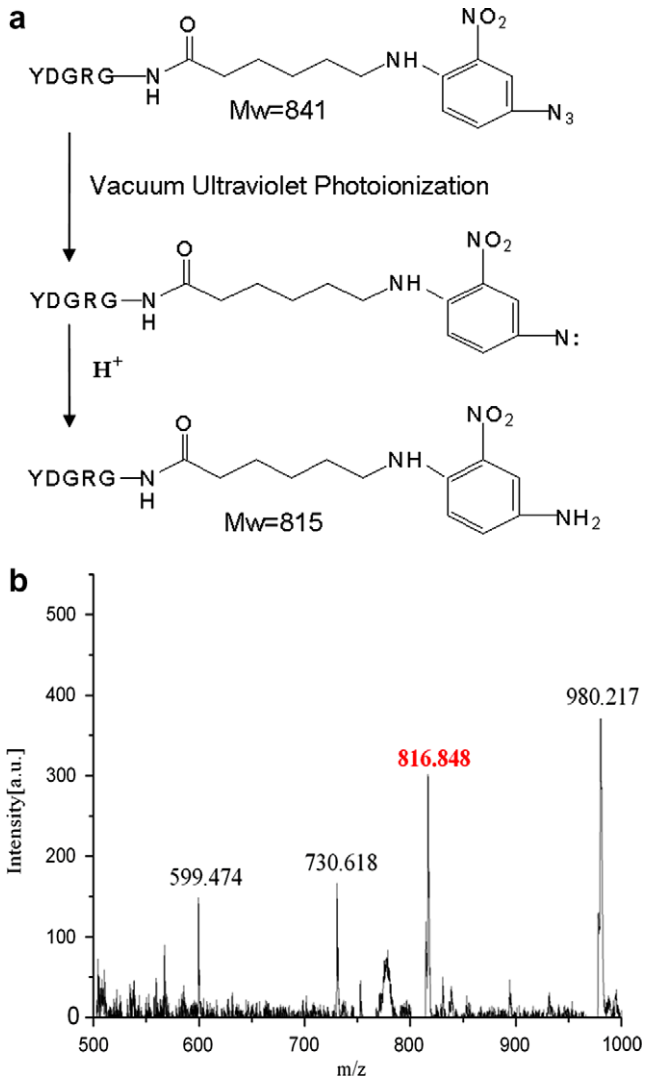


Fig. 2. Analysis principle and MALDI-TOF MS spectrum of GRGDY–SANPAH. (a) Analysis principle; (b) MALDI-TOF MS spectrum of GRGDY–SANPAH.

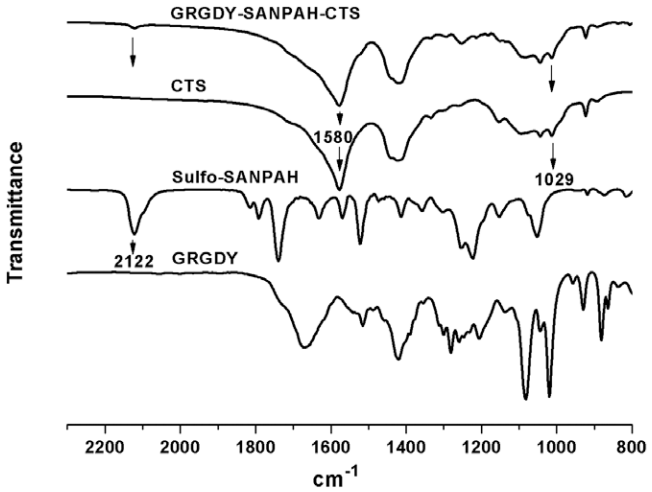


Fig. 3. FTIR spectra of GRGDY–SANPAH–CTS and individual reactant. GRGDY, Sulfo-SANPAH and CTS with UV irradiation were as control.

with the increase of molar ratio between GRGDY and Sulfo-SANPAH from 1:1 to 10:1, which demonstrated the excessive GRGDY made its amino groups react sufficiently with Sulfo-SANPAH. This is important to decrease or avoid the reaction chance between unreacted ester bonds and amino groups of chitosan in the second synthesis step. Therefore, the molar ration of 10:1 was preferred with ester bonds almost completely utilized. Meantime, MALDI-TOF MS spectrum of GRGDY–SANPAH were also recorded and shown in Fig. 2. The product of first synthesis step, GRGDY–SANPAH, should have the M_w of approximate 841 Da, but there only existed a peak at m/z 816 Da in Fig. 2b. From the analysis process shown in Fig. 2a, it was noted that the azido group of SANPAH was activated under vacuum ultraviolet irradiation releasing nitrogen molecule (M_w of 28 Da). The formed nitrene was unstable and easily associate with two hydrogen atoms, which resulted in final M_w of 815 Da. Therefore, the peak at m/z 816 Da indirectly confirmed the successful synthesis of GRGDY–SANPAH.

Secondly, following the synthesis route shown in Scheme 1, GRGDY–SANPAH–CTS was homogeneously synthesized by UV irradiation of GRGDY–SANPAH and CTS solution. The product was analyzed with FTIR and ^{1}H NMR. By comparing with the FTIR spectra of GRGDY, Sulfo-SANPAH and GRGDY–SANPAH–CTS in Fig. 3, the peak at 2122 cm^{-1} ascribing to the azido groups of Sulfo-SANPAH nearly disappeared in reaction product, which indicated that the photosensitive end of GRGDY–SANPAH was activated under UV irradiation for reaction. On the other hand, the existence of peak at 1580 cm^{-1} ascribing to N–H vibration bond, and the decrease of peaks at 1029 cm^{-1} ascribing to the O–H bond demonstrated that the amino groups at C_2 position of CTS were saved while the hydroxyl groups at C_6 position of CTS underwent a covalent modification with the $-N_3$ groups of GRGDY–SANPAH. These results were further substantiated by ^{1}H NMR spectrum of GRGDY–SANPAH–CTS (Fig. 4a). Compared with CTS (Fig. 4b), new peaks appeared at chemical shifts of 7.19, 6.93 and 1.33 ppm in GRGDY–SANPAH–CTS. The former two was known to represent the protons resonance of benzene ring, which existed in SANPAH or peptide, and the latter showed the content of $-CH_2$ residue in SANPAH. Moreover, chemical shift of 3.25 ppm representing protons resonance of C_2 of pyranose ring (Fangkangwanwong, Akashi, Kida, & Chirachanchai, 2006; Hirai et al., 1991) kept same, which suggested amino groups of CTS were unreacted and saved. While chemical shifts around 3.80–4.00 ppm representing protons resonance of C_3, C_4 and C_6 of pyranose ring showed somewhat difference, that is, decrease of middle peak at 3.90 ppm in the product spectrum. At present, it is still difficult to clearly correspond the three peaks to three kinds of protons of C_3, C_4 and C_6 position, but it is only at C_3 and C_6 positions of CTS that exist reactive hydroxyl groups, and the activity of C_3-OH is weaker than that of C_6-OH due to steric hindrance. Therefore, it was deduced that the decreased peak at 3.90 ppm can be ascribed to protons of C_6 position, which suggested C_6-OH participated in photochemical synthesis. The above results demonstrated that SANPAH and peptide were successfully introduced on CTS chain.

To our knowledge, it was said the photochemical reaction occurred between azido groups of SANPAH and hydroxyl groups of CTS or PDMS in the few reports, (Chung et al., 2002; Chung, Liu, Wang, & Wang, 2003; Li, Chen, & Wang, 2006) but almost no clear mechanism description was involved so far. Here, an analysis and explanation of possible photochemical reaction mechanism was presented as follow. The crosslinker Sulfo-SANPAH, similar to ANB-NOS, Sulfo-SAND, SANPAH, has a photosensitive phenylazide ends as shown in Scheme 1, and the possible reaction mechanism of phenylazide groups when exposed to UV light has been reported. Firstly, the phenylazide groups form short-lived intermediate nitrene groups (Murata, Tsubone, Kawai, Eguchi, & Tomioka, 2005; Wang, 2003) which subsequently follow two different reaction ways. In one way, nitrene groups can directly insert into active C–H and N–H bonds of nucleophiles or initiate addition reactions

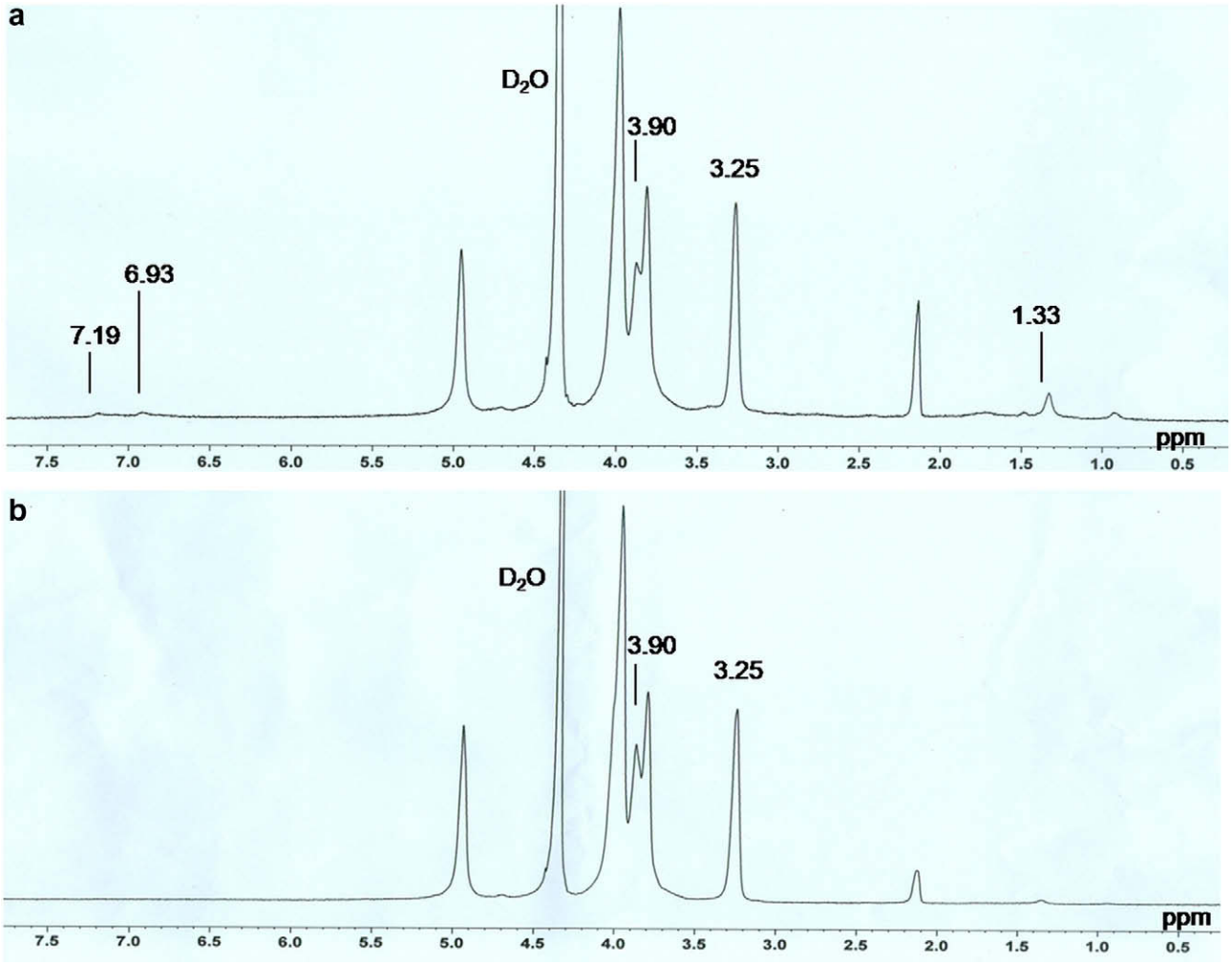


Fig. 4. ^{1}H NMR spectra of GRGDY–SANPAH–CTS and CTS. (a) GRGDY–SANPAH–CTS; (b) CTS.

with double bonds; in the other way, nitrene groups experience ring expansion (Pedone & Brocchini, 2006) of singlet phenylnitrene ^{1}1a to ketenimine 3a (Hideo, Naoki, & Kazunori, 1993; Jin, Jacek, & Matthew, 2007; William & Weston, 1997) to produce a dehydroazepine intermediates, which then react with nucleophiles (Fig. 5) (Hermanson, 1996). In view of CTS structure, there are three functional groups with different reactivity, that are, primary amino group (C_2-NH_2), primary hydroxyl group (C_6-OH) and second-degree hydroxyl group (C_3-OH). Theoretically, all three active groups are nucleophilic under some condition. The C_3-OH, C_6-OH and C_2-NH_2 lead to electronegative oxygen and nitrogen due to polarization of electron. However, C_3-OH reactivity is lower than that of C_6-OH due to significant steric limitation (Yang, Liang, Dou, Shen, & Tan, 2005). On the other hand, the electronegativity of oxygen atom is larger compared with nitrogen atom (Wiberg & Rablen, 1995), and the steric hindrance of C_6-OH is smaller than C_2-NH_2. More importantly in this study, the photochemical reaction was purposely controlled to undergo in acidic condition, which meant C_2-NH_2 was protonated to form C_2-NH_3^+ that obviously resulted in weakened nucleophilicity of C_2-NH_2. All these reasons confer C_6-OH dominant nucleophilicity. Therefore, it is clear that nitrene groups react with the CTS surface by extracting hydrogen atoms from the polymer backbone under photochemical reaction, and forming links between the phenylazide groups of SANPAH molecule and hydroxyl groups of CTS. This is also a useful method when reaction priority on hydroxyl groups is needed because it can occur regioselectively and avoid complicated protection steps for C_2-NH_2.

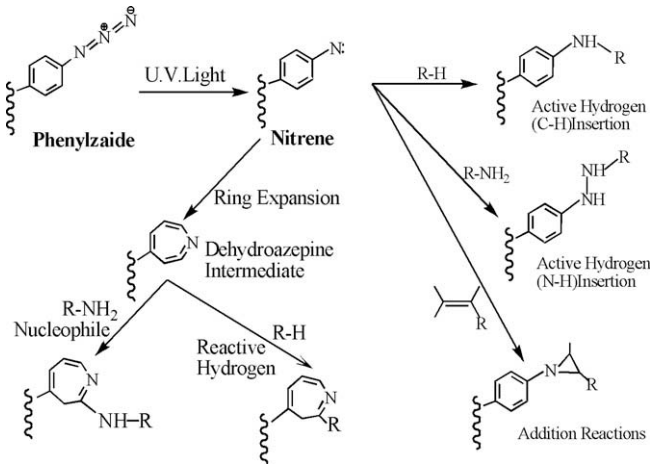


Fig. 5. The possible reaction mechanism of phenylazide group when exposed to UV light (cited from Hermanson, 1996).

3.2. Cell adhesion enhanced on GRGDY–SANPAH–CTS (mCTS) film

It is well-known that RGD-containing peptide can specifically recognize and bind the integrin receptors not only on normal cells

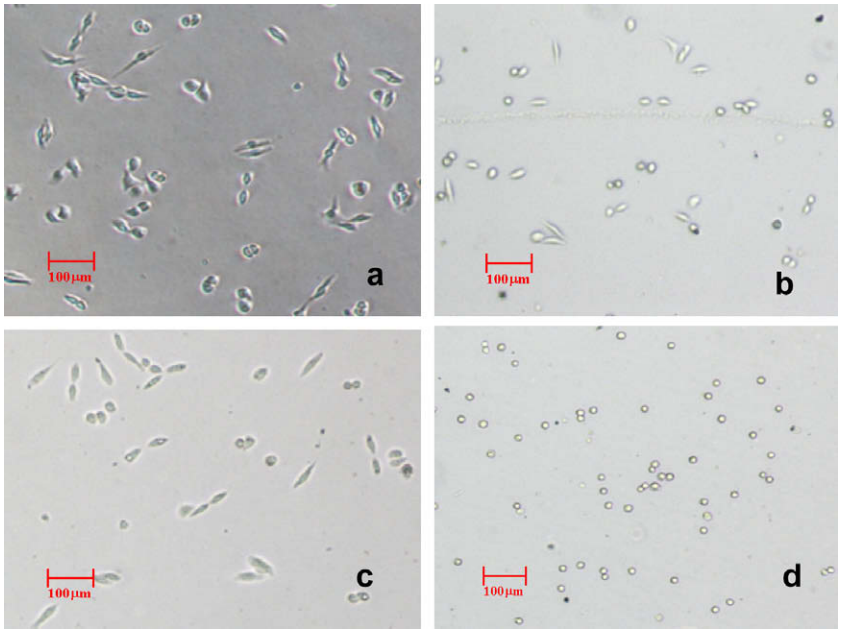


Fig. 6. Cell adhesion to different materials. (a) Cell morphology on tissue culture plate cultured for 12 h; (b) cell morphology on CTS film cultured for 12 h; (c) cell morphology on GRGDY–CTS film cultured for 12 h; (d) cell morphology on GRGDY–CTS film cultured for 12 h which cells already saturated with GRGDY peptide.

but also on tumor cells. For example, the expression of integrin αvβ3 is now detected on malignant melanoma, and correlates strongly with invasive and metastatic melanoma with a poor clinical outcome (Schadendorf et al., 1993). When RGD-containing peptide is conjugated to polymers such as CTS, the potential application as drug carriers targeted to tumor cells might be easily realized for release of anticancer or nucleic acid drugs. Therefore, the adhesion and proliferation behavior of melanoma cells (B16–F10) were evaluated on CTS or GRGDY–SANPAH–CTS film in this study.

In the film preparation process, it was noticed that the synthesized red GRGDY–SANPAH–CTS can directly dissolved in 0.2 M NaAc–HAc buffer (pH 5.6) with concentration of 0.1% w/v, while CTS can hardly dissolved in the same buffer and had to be dissolved at lower pH (~4.5). This improved solubility of mCTS was thought to be from the hydrophilicity of grafted GRGDY peptide.

When B16–F10 cells were seeded and cultured for 12 h, it was found cells spread well on the control surface without film (culture plate well) demonstrating good cell adhesion (Fig. 6a). On CTS film, only a few cells in the visual field of microscope can spread while most were in the round shape (Fig. 6b), which suggested a little cell adhesion. On the contrary, most spreading cells instead of round ones on mCTS film were shown in Fig. 6c, which also suggested good cell adhesion. According to Section 2.4, both CTS film and mCTS film were immersed in NaOH solution before cell culture. It meant that the protonated amino groups had been neutralized resulting in decreased positive density on both films. Therefore, cells with negative charges on membrane should be difficult to adhere on both films only depending on electrostatic interaction. However, cells really exhibit good adhesion on mCTS film, which can undoubtedly be thought the GRGDY peptide grafted on CTS contributed to the enhanced cell adhesion. Furthermore, cells pre-treated in GRGDY peptide solution were cultured on mCTS film. As seen in Fig. 6d, almost all cells were round shape and suspended, which meant that cells cannot spread for cell adhesion. It is simply because integrin receptor (e.g., αvβ3, α5β1, αIIbβ3) on cell surface can interact strongly with RGD-containing peptide (Ruoslahti & Pierschbacher, 1986). The pre-treatment of cells with short, linear RGD-containing peptide saturated integrin on cell surface, which can competitively inhibit the recognition and binding of CTS grafted GRGDY. The phenomenon demonstrated RGD-integrin interaction between cells and mCTS really played an important role on cell adhesion.

3.3. Cell proliferation improved on GRGDY–SANPAH–CTS (mCTS) film

For the assessment of cell growth and proliferation on CTS and mCTS films, MTT assay was carried out to detect cell viability during cell culture. The relative cell viability with remarkable contrast on different culture surfaces were shown in Fig. 7. Basically, cells cultured on control surface (culture plate well) displayed rapid initial proliferation, then grew and proliferated well through the culture period compared with that on CTS and mCTS films, which was in accordance with the good cell adhesion in Fig. 6a. This maybe because most normal cells do not divide in the unanchored state (Vasil'ev and Gelfand, 1981), while even cells seeded on CTS surface do not start the cell cycle and are inhibited in the G0/G1 phase in cell cycle analysis (Mao et al., 2004). However, it can be seen

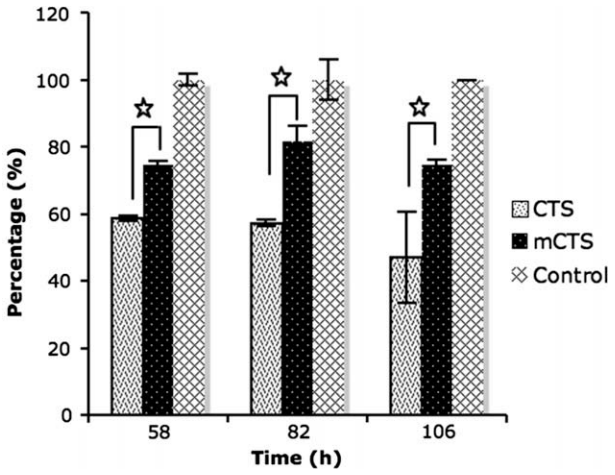


Fig. 7. Cell growth and proliferation on GRGDY–CTS film and CTS film ($n = 3$; tissue culture plate used as control; GRGDY–CTS film $^{*}p < 0.05$; compared to CTS film).

that cell growth and proliferation on CTS film modified with cell adhesive peptide GRGDY showed improvement by approximate 20% higher than that on CTS film, and kept stable through the culture period in comparison with the increased cell growth and proliferation on neutralized CTS film.

Therefore, MTT assay also confirmed that RGD-integrin interaction is critical to cellular response to biomaterial surface, and GRGDY peptide grafted CTS was beneficial to cell spread and adhesion by interacting with integrins of cells, which subsequently did promote cell growth and proliferation.

4. Conclusion

Our study presented a novel homogeneous strategy of RGD-chitosan synthesis by photochemically selective reaction under controlled condition. The use of bifunctional photosensitive cross-linker, Sulfo-SANPAH, in modification of CTS by homogeneous way displayed advantages including easy, fast, convenient, efficient and free of cytotoxic organic contamination and reagents for the protection/deprotection of amines. FTIR, MALDI-TOF MS and ^{1}H NMR analyses demonstrated the successful synthesis of GRGDY–SANPAH–CTS. The mechanism of photochemical reaction was clearly and completely described to occur between azido groups of SANPAH and hydroxyl groups of CTS. Finally, cell culture studies showed GRGDY peptide grafted CTS was beneficial to cell spread and adhesion by interacting with integrin of cells, which subsequently did promote cell growth and proliferation.

Acknowledgements

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