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# Modification of agarose with carboxylation and grafting dopamine for promotion of its cell-adhesiveness

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ABSTRACT

In order to improve bioactivity of agarose, we modified agarose by carboxylation and grafting dopamine. Under alkaline condition, carboxylated agarose was prepared using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) oxidation system by oxidizing $C_6$ hydroxyl on D-galactose ring into carboxyl group, and the maximum value of the degree of carboxylation reached 30%. With the increase of the amount of oxidant, the molecular weight of the carboxylated agarose decreased to 4 kDa by gel permeation chromatography (GPC) measure. Carboxylated agarose reacted with dopamine through EDC condensation reaction to obtain agarose grafting dopamine (Ag-g-DA), and the grafting rate of dopamine was determined to be 9.3% by UV spectroscopy at 280 nm. The structures of these modified agaroses were determined by FT-IR and $^{13}C$ NMR. Both carboxylated agarose and Ag-g-DA showed no cytotoxicity and promoted cell-adhesiveness.



## 1. Introduction

Agarose is a natural polysaccharide, consisting of 1,3-linked-β-D-galactose and 1,4-linked 3,6-anhydro α-L-galactose residue repeating units. Extracted from marine red algae, it is abundant and commercially available. Agarose forms thermoreversible gel when hot agarose solution is cooled below 40 °C. It has been extensively used in biomedical field, such as drug delivery (Mehrotra et al., 2012), cell therapy (Vinerean, Gazda, Hall, & Smith, 2011), molecular biology (Novak et al., 2011) and tissue engineering (Khanarian, Haney, Burga, & Lu, 2012) because of its special mechanical properties, biocompatibility and bioinert nature. However, agarose, like most common hydrogels, is not favorable for cell adhesiveness and growth due to its high hydrophilicity. Also, agarose does not absorb cell adhesive proteins, resulting in anti-adhesive properties. Modification is a flexible approach to promote its cell-adhesiveness. Agarose can be modified through its hydroxyl group covalently crosslinking functional groups and thus introduced cell adhesion sites to regulate cell–material interaction. Aizawa et al. created a cell-adhesive matrix of agarose modified with glycine–arginine–glycine–aspartic acid–serine (GRGDS) (Aizawa, Leipzig, Zahir, & Shoichet, 2008). Au et al. found micropatterned agarose covalently modified with collagen facilitated attachment of primary canine hepatocytes and human neoplastic hepatocellular carcinoma (HepG2) cells (Au, Hasenwinkel, & Frondoza, 2012).

Carboxyl modification can effectively improve surface properties of materials and also promote proteins absorption for favoring cell attachment, proliferation and differentiation. Park et al. found fibroblasts seeded on carboxylic acid modified PGA, PLGA, and PLLA scaffolds were spread over a larger area and had higher adhesion and proliferation rates compared to unmodified scaffolds (Park, Ju, Son, Ahn, & Han, 2007). A recent study has demonstrated that the terminal carboxylation of PIPAAm brushes gave both the acceleration of cell adhesion and rapid harvest of cell sheets (Takahashi et al., 2012). Carboxyl group is an active group that easily reacts with other groups by electrostatic adsorption or chemical reaction. Flake et al. activated the carboxyl groups of acrylic-acid-containing PEG microparticles by EDC/NHS, and scaffolds were formed by mixing with amine-containing PEG microparticles (Flake et al., 2011). TEMPO-mediated oxidation is an effective method to introduce carboxyl groups to polysaccharides such as cellulose, chitosan, paramylon and curdlan, which can be achieved in water under alkaline condition and certain amounts of TEMPO and NaBr. The reaction shows highly selective oxidation of C6 primary hydroxyl to carboxylic groups (Bordenave, Grelier, & Coma, 2008; Isogai, Saito, & Fukuzumi, 2011; Tamura, Wada, & Isogai, 2009).

L-3,4-dihydroxyphenylalanine (DOPA) is the precursor to the neurotransmitters. Five marine mussel proteins (MAPs), named Mefp-1–Mefp-5, contains DOPA (Waite, 1987). Study has demonstrated DOPA-rich polyphenolic protein secreted by the marine mussel contributes to byssal adhesion (Waite & Tanzer, 1981). DOPA functions as a cross-linking agent and mediates adhesion to the substratum, which is mainly attributed to its catechol (Wiegemann, 2005). Dopamine (DA), a major catabolite of L-dopa,

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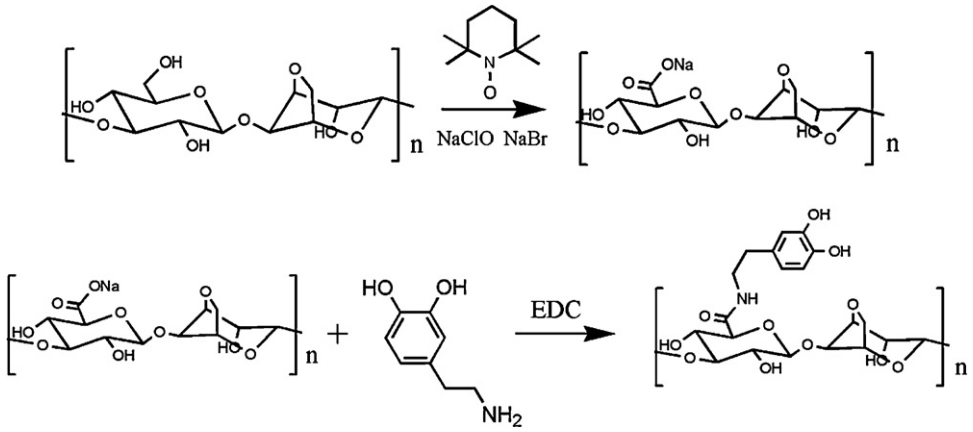


**Fig. 1.** The proposed reactions of agarose carboxylation and dopamine grafting.

coexists with amine and DOPA, which may contain both functionalities. Inspired by mussel adhesion mechanism, Ku et al. found the coated dopamine can promote cell adhesion on any type of material surfaces including the well-known anti-adhesive substrate, poly (tetrafluoroethylene) (Ku, Ryu, Hong, Lee, & Park, 2010). Lee et al. produced an adherent polydopamine multifunctional coating on a wide variety of materials. Polydopamine coatings can, in turn, serve as a versatile platform for secondary surface-mediated reactions (Lee, Dellatore, Miller, & Messersmith, 2007).

In this study, we introduced functional groups to improve the bioactivity of agarose (Fig. 1). We synthesized carboxylated agarose using TEMPO oxidation system under alkaline condition. The carboxyl content of carboxylated agarose was analyzed by titration, and GPC measured the molecular weight of agarose with different degree of carboxylation. Ag-g-DA was prepared by carboxylated agarose reacting with dopamine through EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) condensation reaction. FT-IR and $^{13}C$ NMR etc. were applied to characterize the structures of the modified agaroses. Cytotoxicity and cell adhesiveness of the modified agaroses were evaluated by in vitro cell culture experiments.

## 2. Materials and methods

### 2.1. Materials and reagents

Agarose, TEMPO (98%, MW = 156.25), sodium bromide (NaBr), sodium hypochlorite (NaClO) solution, and hydrochloride dopamine were purchased from Aladdin Chemistry Co., Ltd. EDC was obtained from Sigma.

### 2.2. Synthesis of carboxylated agarose

0.5% agarose solution was prepared by dissolving 1 g agarose in 200 ml distilled water at 85 °C, and cooled in ice water bath with stirring. TEMPO (0.02 g) and NaBr (0.4 g) were added and stirred in agarose solution until complete dissolution. 10% NaClO was added dropwise. The pH was kept at 10.5 by addition of 1 M NaOH solution. The TEMPO-oxidation reaction was performed at room temperature for 2 h. Then ethanol, amounting to 3 times volume of the reaction solution, was added in the mixture for the precipitation of the TEMPO-oxidized agarose. The TEMPO-oxidized agarose was then washed by ethanol and acetone sequentially for twice, and the solvent was removed in a rotary evaporator at 40 °C. The TEMPO-oxidized agarose was dissolved in water by heating and neutralized with 1 M HCl, and then cooled. The reacted solution was dialyzed for 3 days and freeze-dried for 24 h to obtain carboxylated agarose.

### 2.3. Synthesis of Ag-g-DA

1 g carboxylated agarose was dissolved in 100 ml PBS by heating, and then cooled. The solution was stirred for 30 min with keeping the pH at 5.5 by addition of 0.5 M dilute HCl. 0.3881 g EDC and 0.4741 g dopamine were added for 2 h grafting reaction. The mixture was purified and dialyzed for 3 d and freeze-dried for 24 h to obtain Ag-g-DA.

### 2.4. Characterization of the modified agarose

#### 2.4.1. FTIR spectroscopy

FTIR spectra of agarose, carboxylated agarose (—COONa), carboxylated agarose (—COOH) and Ag-g-DA were recorded using a Vertex 70 Bruker spectrometer (Bruker, Germany) in the 4000–400 $cm^{-1}$ range with a resolution of 2 $cm^{-1}$ using KBr pellets.

#### 2.4.2. NMR spectroscopy

Samples were dissolved in deuterated water ($D_2O$). $^{13}C$ NMR spectra at 35 °C were recorded using BRUKER AVANCE 500 MHz spectrometer (BRUKER, Germany).

#### 2.4.3. Degree of carboxylation analysis

732 cation resin (Guangzhou Second Chemical Reagent Factory, China) was initially soaked in saturated salt water for 10 h to make it swollen, then soaked in 4% NaOH solution to remove organic

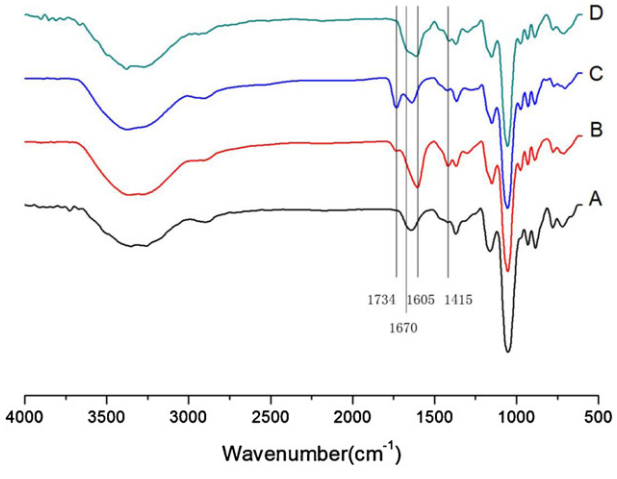


**Fig. 2.** FT-IR spectra of (A) agarose, (B) carboxylated agarose (—COONa), (C) carboxylated agarose (—COOH), and (D) Ag-g-DA.

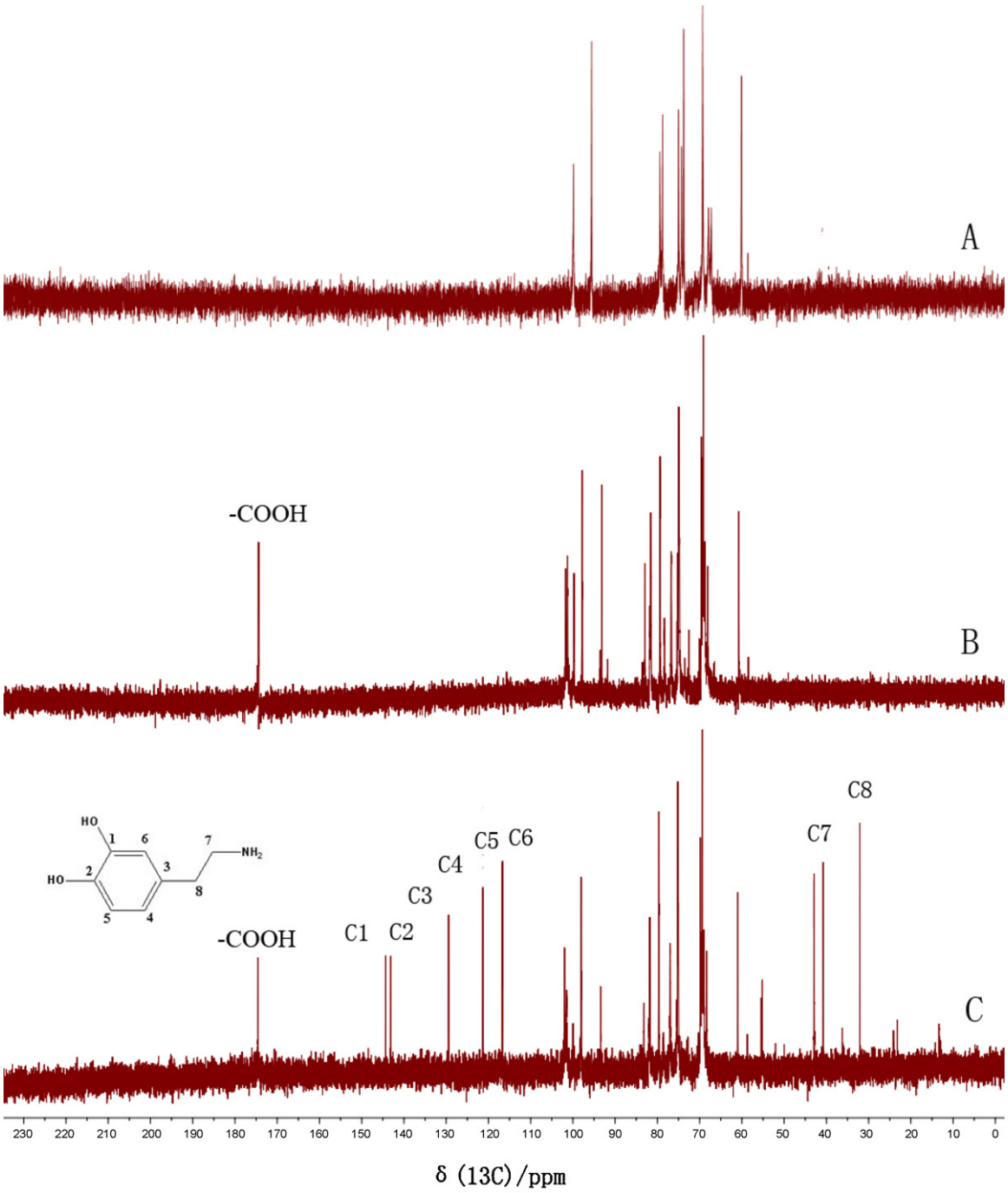


**Fig. 3.** $^{13}C$ NMR spectra of (A) agarose, (B) carboxylated agarose, and (C) Ag-g-DA.

matter. It was finally soaked in 4% HCl to remove NaOH and other compounds. The clean resin was washed by distilled water until the effluent pH was close to 7.0.

0.5 g carboxylated agarose was dissolved in 50 ml distilled water by heating and then passed through the clean 732 cation exchanger resin. The solution was collected when the effluent pH was below 7.0.

The degree of carboxylation was measured by titration with 0.1 M NaOH using phenolphthalein as an indicator ($n = 3$). The volume of titration was recorded and the degree of carboxylation was calculated as:

$$342NX + 306N(1 - X) = 0.5$$

$$NX = 0.1\,V_{NaOH}$$

$$X = \frac{30.6\,V_{NaOH}}{0.5 - 3.6\,V_{NaOH}}$$

where $N$ is the amount of substance of carboxylated agarose, and $X$ is the degree of carboxylation.

### 2.4.4. GPC analysis

The molecular weight of carboxylated agarose was characterized by GPC (Viscotek, USA). The eluent was $NaNO_3$ aqueous solution with the flow rate of 1.0 ml $min^{-1}$ at 35 °C.

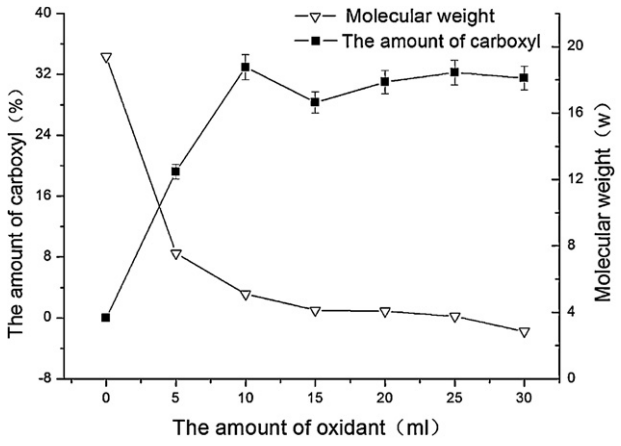


**Fig. 4.** The effect of the amount of oxidant on the content of carboxyl group and the molecule weight of carboxylated agarose.

*2.4.5. UV spectroscopy*

The content of the grafted dopamine ($n = 3$) was analyzed by UV–vis spectroscopy at 280 nm (UV-2550, Shimadzu, Kyoto, Japan). Various concentration of dopamine water solution was calculated to establish standard curve. The content of the grafted dopamine was converted through the standard curve. The grafting rate was calculated as

$$X + Y = 30\%$$

$$(306 \times 70\% + 320X + 492Y)m_{DA}\% = 492Y$$

where $X$ is the percentage content of carboxyl groups of $C_6$ hydroxyl groups, and $Y$ is the grafting rate. $m_{DA}$ calculated from standard curve is the percentage content of the agarose galactose unit grafting dopamine.

*2.5. Cytocompatibility test*

*2.5.1. In vitro cytotoxicity assay*

Cytotoxicity of the modified agarose was assessed by cell-counting kit-8 (CCK-8) assay. 5000 cells/well 3T3 fibroblasts were seeded in a 96-well plate and grew in DEME supplementing with 10% FBS and 1% streptomycin–penicillin. Cells were incubated overnight at 37 °C with 5% $CO_2$. Add 10 μl carboxylated agarose or Ag-g-DA with concentrations of 10, 20, 100, 200, 500 μg/ml ($n = 5$) in each well and cells were incubated for 24, 48 and 72 h. 10 μl of CCK-8 was added to each well of plate and incubated for 3 h. The absorbance at 450 nm was measured by microplate reader. Cell viabilities was calculated as:

$$\text{Cell viabilities}(\%) = \frac{OD_{450\,nm}(\text{modified agarose})}{OD_{450\,nm}(\text{control})} \times 100\%$$

where $OD_{450\,nm}$(modified agarose) is the absorbance at 450 nm of the cells cultured with carboxylated agarose and Ag-g-DA and $OD_{450\,nm}$(control) is the absorbance at 450 nm of the cells cultured without modified agarose treatment.

*2.5.2. Cell adhesiveness*

Agarose, carboxylated agarose and Ag-g-DA ($n = 5$) were coated on the glass substrate by a spin-coater (Chemat Technology Inc., Northridge, CA, USA). Before cell culture, the coated glass substrates were sterilized in 75% alcohol for 24 h and rinsed with PBS. 3T3 fibroblasts were trypsinized and resuspended in culture medium to a density of $10^6$ cells/ml. 50,000 cells were pipetted on to each glass substrate and incubated at 37 °C with 5% $CO_2$. After 4 h, 2 ml DEME supplemented with 10% FBS and 1% streptomycin–penicillin were added in each glass substrate for 12, 48 and 1 week culture, and 3T3 fibroblasts were observed under optical microscopy.

## 3. Results and discussion

*3.1. The characterization of the modified agarose*

*3.1.1. FT-IR*

FT-IR spectra of agarose and its modifications were shown in Fig. 2. Compared with agarose (Fig. 2A) and carboxylated agarose (Fig. 2B), the appearance of new peaks in the spectrum of the carboxylated agarose at 1605 $cm^{-1}$ and 1415 $cm^{-1}$ can be attributed to —$COO^-$ symmetric stretch and antisymmetric stretch. This shows that there were carboxylate groups in agarose chains after oxidization by TEMPO. An intensive peak was observed at 1734 $cm^{-1}$ (—COOH stretch) (Fig. 2C) in the spectrum of carboxylated agarose treated with cation exchange resin, which suggests that sodium carboxylate groups in carboxylated agarose were converted to free carboxyl groups. In the spectrum of carboxylated agarose grafting dopamine (Fig. 2D), the appearance of characteristic peak at 1670 $cm^{-1}$ (—CONH stretch) indicated that dopamine was grafted on the carboxylated agarose.

*3.1.2. $^{13}C$ NMR*

$^{13}C$ NMR spectra of agarose, carboxylated agarose and Ag-g-DA were showed in Fig. 3. Chemical shift at $\delta = 174.6$ ppm was visible (Usov, Ivanova, & Shashkov, 2009), which suggests that $C_6$ primary hydroxyl of agarose chain unit converted to $C_6$ primary carboxyl. In the spectrum of Ag-g-DA, strong absorptions at 144.3, 143.1, 129.4, 121.3, 116.7, 116.7, 40.7 and 32.1 ppm were attributed to $C_1$, $C_2$, $C_3$, $C_4$, $C_5$, $C_6$, $C_7$ and $C_8$. In addition, the peaks at 174.5 and

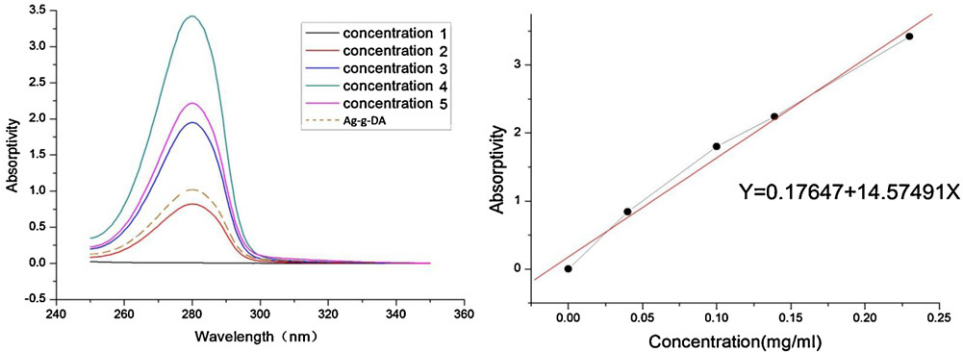


**Fig. 5.** UV spectra of dopamine solution, standard curve and Ag-g-DA.

174.6 ppm overlapped which were corresponded to —CONH and —COOH, which suggested that dopamine was partially grafted on the carboxylated agarose. All of results suggested that Ag-g-DA was successfully synthesized.

### 3.1.3. The carboxyl content and molecular weight of carboxylated agarose

Fig. 4 shows the effect of the amount of oxidant on the content of carboxyl group and the molecule weight of carboxylated agarose. During the reaction, the amount of oxidant influenced the degree of carboxylation of agarose. The carboxyl content continued to increase with the oxidant when the amount of oxidant is lower than 10 ml, and kept equilibrium value at about 30% when the amount of oxidant was between 10 ml and 30 ml. The possible mechanism is that one of the four hydroxyl groups, the C6, in agarose unit is mainly oxidized, and few of other hydroxyl groups are oxidized. During oxidization process, the molecular weight was dramatically decreased at first, then kept equilibrium at 4 kDa when the amount of oxidant was over 10 ml. Depolymerization of some polysaccharides was inevitable in TEMPO oxidation system under alkaline condition (Fan, Saito, & Isogai, 2007; Isogai & Kato, 1998; Kato et al., 2002). Obviously it is easier to oxidize the low molecular weight agarose and thus raise the degree of carboxylation of the agarose. But as biomaterials, it is undesirable for them to be processed with low molecular weight. Therefore, the amount of NaClO used was 10 ml in order to produce modified agarose with a high degree of carboxylation and high molecular weight.

### 3.1.4. Grafting rate analysis

Ag-g-DA was obtained from grafting to agarose with 30% degree of carboxylation and used to measure the grafting rate (Fig. 5). Quantitative determination of the concentration of dopamine was accomplished in comparison with standard curve. The calculated grafting rate was 9.3%. This meant 31% carboxyl groups were grafted with dopamine.

## 3.2. Cytocompatibility test

### 3.2.1. Cytotoxicity

The cytotoxicity of the modified agarose was compared with control, and concentrations range from 10 to 500 μg/ml. Results showed that carboxylated agarose and Ag-g-DA were non cytotoxicity compared to agarose (Fig. 6). With five different concentrations, cell survival rates of both modified agaroses were approximately 100% in the 24 h, 48 h and 72 h. Thus, the result also suggested that the cytotoxicities of modified agaroses were dose-independent as well as time-independent. Non-cytotoxicity of the modified agaroses meets essential requirement of biomaterials for biomedical applications.

### 3.2.2. Cell adhesiveness

Morphologies of the 3T3 fibroblasts cultured on glass substrates coated with different agaroses were showed in Fig. 7. No normal 3T3 fibroblast (spindle-shaped) was observed in glass substrate coated with agarose, and just dead cells formed agglomerates. 3T3 fibroblasts showed monolayer formation with a well-ordered arrangement in the glass substrates coated with carboxylated agarose and Ag-g-DA. The results showed that cell attachment was enhanced by using the modified agarose. After 12 h, 48 h and 1 week cell culture, the density of fibroblasts was 181%, 162% and 158% larger in glass substrate coated with Ag-g-DA than with carboxylated agarose by counting cells in the same area on the glass substrate, which suggested cell adhesive promotion of Ag-g-DA was more effective than carboxylated agarose.

Surface characteristics, such as chemical composition, charge, roughness and hydrophilicity, influence cell adhesion, though its mechanism has not been fully understood. Thus, chemical modification and surface morphology change could induce cell attachment. Agarose is known to be non-adhesive for cells, but we introduced functional groups to improve cell adhesion. Both carboxylated agarose and Ag-g-DA can enhance cell adhesiveness. Carboxylated agarose contains carboxyl groups which may

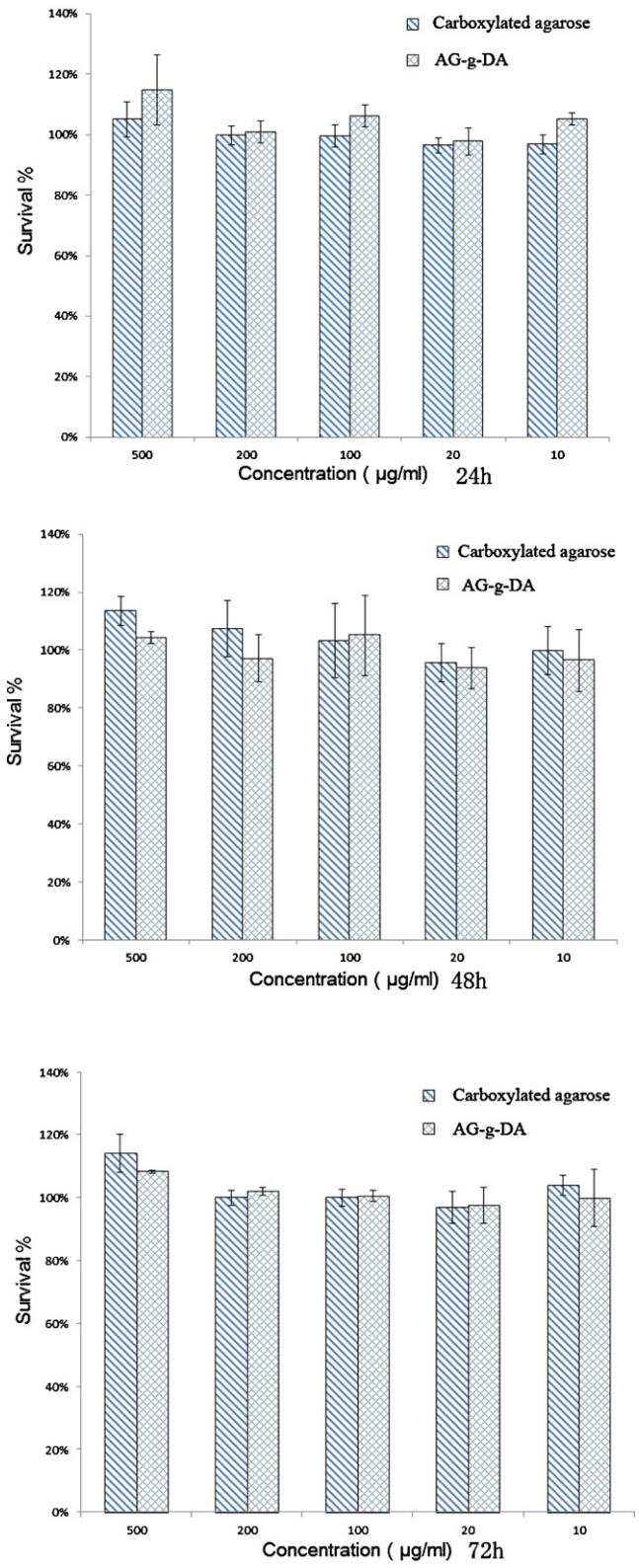


**Fig. 6.** Cell survival rates of carboxylated agarose and Ag-g-DA.

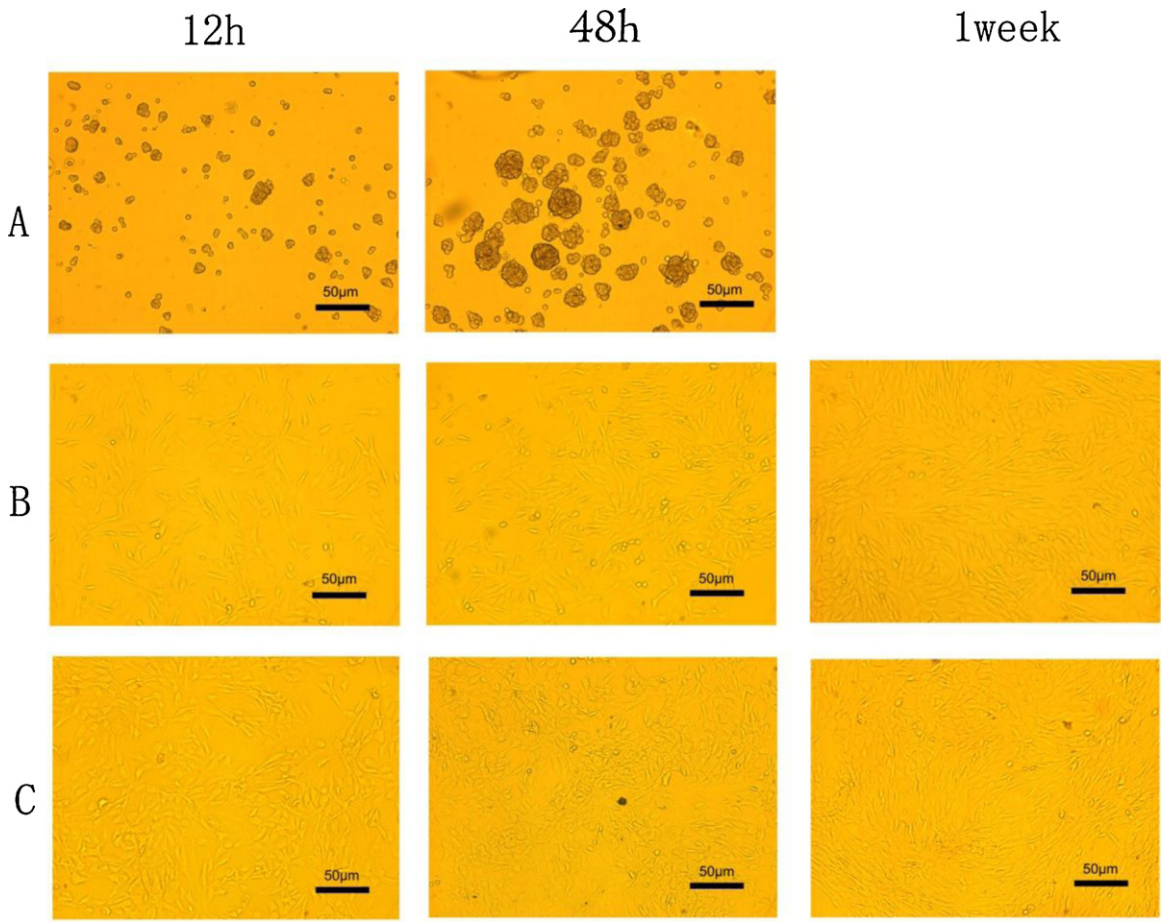


**Fig. 7.** Morphologies of the 3T3 fibroblasts cultured on glass substrates coated with of (A) agarose, (B) carboxylate agarose, and (C) Ag-g-DA.

play roles for ligand immobilization with cell membrane surface. Dopamine has inherently cell-adhesion sites for cell–material interactions, and provides an amino to covalently link with carboxylated agarose. Cell adhesion belongs to the first phase of cell–material interaction and the quality of this first phase will influence the cell's capacity to proliferate and to differentiate itself on contact with the implant (Anselme, 2000). Therefore, modified agaroses with capacity of cell adhesion is expected to extend its applications in biomedical engineering.

## 4. Conclusions

In this study, the $C_6$ primary hydroxyl of agarose was expected to be oxidized to $C_6$ carboxylate group by TEMPO oxidation system at pH 10.5, and dopamine was grafted on carboxylated agarose. Carboxylated agarose and its grafting dopamine showed no cytotoxicity and could enhance cell adhesion. This work suggests that agarose modified with functional groups is a promising route for improving its bioactivity and merits further study.

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