

# Synthesis and characterization of FITC-aminated agarose as highly-fluorescent labeling agent

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## ABSTRACT

Organic fluorescence agent designed into nanoparticle can significantly promote its fluorescent efficiency. In this paper, a novel fluorescein isothiocyanate labeling aminated agarose (FITC-AA) is prepared and tested as an effective fluorescent labeling agent. IR analysis and EA proved that FITC was grafted to the aminated agarose (AA) successfully. FITC-AA spontaneously formed nanoparticle with a diameter between 159.2 nm and 1530 nm at below 37°C by gelling effect of agarose. Cell culture experiments confirmed that HL-60 cells and 3T3 fibroblast cells could be fluorescently marked by FITC-AA nanoparticles and their fluorescence sustained longer than by FITC. After deposited for half a year, FITC-AA can reversibly dissolve in hot water and form nanoparticles when cooling to 37°C, and its fluorescent properties remain. These findings demonstrate that FITC-AA is an effective labeling agent for marking cells.

**Keywords:** agarose, FITC, fluorescent labeling, nanoparticles

## 1 INTRODUCTION

New techniques for labeling molecule with fluorescent agent, in combination with nanotechnology, have been devised to directly observe molecules in living cells (Suzuki et al. 2007). Fluorescein has a very high molar absorption in the visible region, a large fluorescence quantum yield, which makes it very useful in application for a high sensitivity. Furthermore, it is commercially available (Sjoback et al. 1998, Klonis et al. 1996).

Conventional organic fluorophores, for example fluorescein isothiocyanate (FITC), have some drawbacks, such as severe background fluorescent interference, dispersivity after labeling, quenching effects of fluorescence and poor photostability, which limit their effectiveness in many applications.

Fluorescence agent designed into nanoparticle can significantly promote its fluorescent efficiency. At present study, the improvement of fluorescein dye is focused on the synthesis of organic or inorganic nanoparticles containing fluorophore. Agarose is a hydrophilic bioinlet polysaccharide, and does not specifically interact with biomolecule or cell (Bao et al. 2008), and it can spontaneously form nanoparticles at below 37°C by its gelling effect without any chemical crosslinking. On the basis of self-assembled property of agarose, it is convenient

to synthesize the agarose-based fluorescent nanoparticles. To ensure the particle size of the fluorescent agarose is small enough after agarose reacting with FITC, it can ameliorate the fluorescent effect of FITC for labeling cell. Some properties and structures of the fluorescent nanoparticles were characterized.

## 2 EXPERIMENTAL

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### 2.1 Preparation of FITC- AA

FITC-AA was synthesized as Figure 1. Agarose with different molecular weight reacted with epoxy chloropropane under alkaline condition respectively, and then excessive ethanediamine was added into the solution. The solution was stirred at 55°C water-bath for 12h. Aminated agarose (AA) was precipitated by absolute ethanol with trinal volumes of the reacting solution. The precipitation was harvested and dried with vacuum freeze drying. The borax/HCl buffer, acetone and FITC solution were heated in 55 °C constant temperature water-bath respectively. 1% AA was added into distilled water and dissolved in 55 °C constant water-bath. FITC was mixed with AA in a proportion of 1:20 in borax buffer and acetone solution, and heated at 55 °C for 12h with stirring (Huang et al. 2002, Navarrete-Casas et al. 2005). The resulting product was dialyzed in deionized water for 3 days in darkness and dried with vacuum freeze drying.

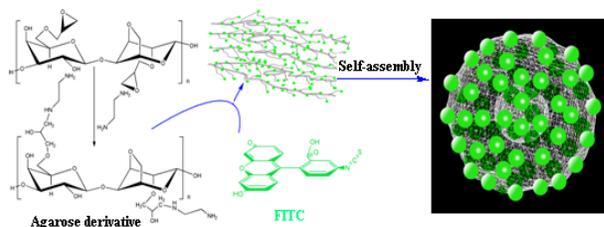


Figure 1: The chemical synthesis and self-assembly of FITC-AA nanoparticles

### 2.2 Structure analyses

FT-IR spectra of agarose and its derivatives were measured using Bruker EQUINOX-55 spectrometer as KBr pellets or thin films on KBr plates. Their elemental analyses were performed on PerkinElmer CHNS/O 2400 elementary analyzer.

The particle size, size distribution and zeta potential of FITC-AA nanoparticles were measured using a Brookhaven Zeta Potential Analyzer. The average size of the nanoparticles was statistically analyzed as the mean value of three measurements.

FITC-AA was dispersed into distilled water. The concentration of FITC-AA in the dispersed solution was regulated to 100 µg/mL. The fluorescence spectrum of solution was examined by a Hitachi F-4500 spectrofluorimeter with a 100W xenon lamp as excitation source and Jobin-Yvon-Spex Fluorolog II spectrofluorimeter with a 450W xenon lamp as light source.

### 2.3 Fluorescent microscope observation

FITC-AA was dissolved in hot distilled water and adjusted to pH 6.8 with 0.1 M HCL. The solution was filtrated with millipore filtrator. HL-60 cells or 3T3 fibroblast cells were cultured until the confluency reached over 80%. Both of cells were plated in 24-well plates at  $1 \times 10^5$  cells/mL and cell number was adjusted with RPMI1640 or DMEM respectively. FITC-AA nanoparticles with a dosage of 100 µg/mL (weight of nanoparticles / volume of medium), and FITC with a dosage of 5 µg/mL (weight of FITC / volume of medium) were added into the culture plates respectively. We supposed the aminated agarose and the initial FITC in complete reaction and FITC was well-distributed in AA with the proportion of 1:20. In the labeling experiment, FITC-AA and FITC were incubated with the cells in the proportion of 20:1 for the same concentration of FITC in the two labeling agents.

Three observing intervals (12h, 24h, 36h) were set after labeling cells. In each observation, two cell lines were collected, and then centrifuged for 5 min at 1500 rpm and washed with PBS (pH=7.4) three times. The precipitate was immersed in 200 µL PBS and then homogenized. The images of labeling cells were observed under a Nikon TE-2000 fluorescence inversion microscope and the morphologies of cells were photographed.

### 2.4 Flow cytometry analysis

HL-60 cells were seeded in 24 well plates with a density of  $10^5$  cells/well for 24h prior to treatment. To examine the labeling efficiency of time-varying and dose-varying, observation intervals (0, 3, 5, 10, 16, 24h) with 100 µg/mL of FITC-AA nanoparticles and FITC-AA concentration gradients (0, 10, 20, 40, 60, 80, 100 µg/mL) for 12h were set to the incubation of HL-60 cells, respectively. The labeling efficiency of cells was analyzed directly by a Becton Dickinson FACSCalibur flow cytometry.

### 2.5 Properties of FITC-AA after deposit

The properties of FITC-AA were characterized with zeta potential analyzer, fluorescent microscope after half a year deposit.

### 2.6 Statistical analysis

All results were statistically evaluated by ANOVA and post hoc testing with Bonferroni's correction on SPSS statistical software. Significance was set at the 5% level ( $p < 0.05$ ).

## 3 RESULTS AND DISCUSSION

### 3.1 Characteristic of agarose and its derivatives

The FT-IR spectra of agarose and its derivatives indicate that agarose does not suffer disorganization after the activation process, and a strong, broad absorbance band of both N-H and O-H stretching vibrations of AA centered at about  $3400 \text{ cm}^{-1}$  is enlarged. After the conjugation of amine group with agarose chain, O-H stretch overlaps with N-H (Wang et al. 2007, Wang et al. 2009). Besides, the strong peak at  $1564 \text{ cm}^{-1}$  is assigned to the secondary amine (N-H). After AA grafting FITC, peaks at  $1298 \sim 1310 \text{ cm}^{-1}$  are assigned to the characteristic absorbance of N-C=S (Xu et al. 2007, Ji et al. 2007). Moreover, there are strong peaks in Curve d at about  $1650 \text{ cm}^{-1}$  and  $950 \text{ cm}^{-1}$  corresponding to phenyl groups in FITC.

Results of the elemental analyses showed that N-content of agarose greatly increased after amination and S-content increased after the synthesis of FITC-AA.

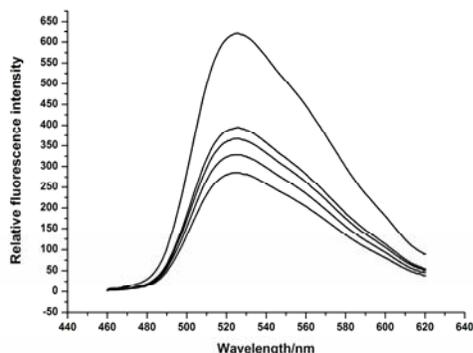
### 3.2 Size and zeta potential of FITC-AA nanoparticles

Size distribution: The molecular weight of agarose has significant effect on the particle size of agarose or its derivatives in water. The agarose with lower molecular weight forms smaller FITC-AA complex (about 104nm-410nm).

Zeta potential: Zeta potential ( $\zeta$ ) of nanoparticle is an essential parameter to evaluate the stability of the dispersed nanoparticles in solution. The surface charge of nanoparticle depends on chemical structure, diameter of particle and pH value of solution. The  $\zeta$  values of FITC-AA nanoparticles drop with the decrease of their diameters at pH 9.87. The negative  $\zeta$  values of FITC-AA nanoparticles are ascribed to the majority of the anionic form of FITC fluorescein in basic condition (Kovacevic et al. 2007, Sjöback et al. 1995). It is well-understood that the dispersed FITC-AA nanoparticles with smaller size is more stable because of larger  $|\zeta|$ .

### 3.3 Fluorescent property of FITC-AA nanoparticles

Figure 2 showed that the fluorescence intensity enhanced with the decrease of FITC-AA nanoparticle size. The mean size of the nanoparticles between 1530.8 and

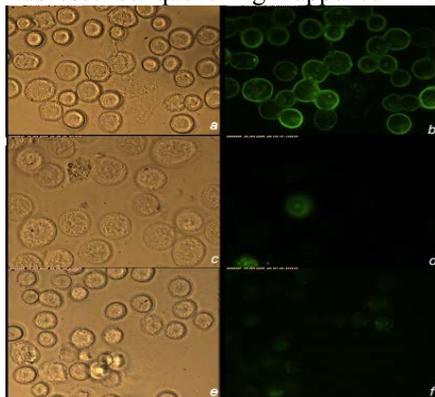


**Figure 2** Fluorescence emission spectra of FITC-AA nanoparticles with different size.

283.4 nm had no significant effect on fluorescence intensity. However, a great change appeared when the mean size of FITC-AA nanoparticles was near to 159.2 nm. In addition, the particles with larger size are more likely to pack FITC, which may result in the encumbrance of fluorescence emission and the reduction of fluorescent efficiency.

### 3.4 Fluorescent microscope examination

From fluorescence inversion microscope, the mixture of the degraded agarose and FITC, as a control group, didn't emit fluorescent light or gave off feeble and scattered light only. The result demonstrated that in the mixture, the degraded agarose couldn't react with FITC, and most of FITC was washed away. After FITC labeling cells, severe fluorescence quenching happened with time (Figure 3).

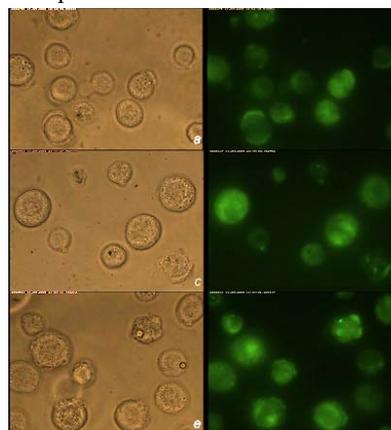


**Figure 3.** Cell morphology after FITC labeling 3T3 fibroblast cells ( $\times 1000$ ).

Especially, it was difficult to observe the fluorescence after 36h. We can find an interesting phenomenon in Figure 3(b), in which those fibroblast cells are encircled by fluorescence. It is apparent that FITC labeled the membrane of the cell more effectively. FITC-AA nanoparticles had strong specific conjugation, and the exact contours and morphology of the cells were clearly reflected in the viewing field (Figure 4). Moreover, the fluorescence maintained during the experiment. The contrast may be attributed to FITC possessing reversible dispersibility in solution, even after labeling cell, so single FITC labeling cells emits fluorescence weakly and becomes weaker with time. While the FITC-AA nanoparticle was capable to conjugate with numerous FITC molecules, after cell-uptake, the localized FITC molecules existed as an entirety and emitted strong fluorescence.

### 3.5 FCM measurement

The labeling efficiency of FITC-AA nanoparticles was quantified with flow cytometry by determining green fluorescence emitted from the labeled cells. It was found that the number of labeled cells changed with the incubation time. The labeling efficiency was relatively low in the first 5h at a concentration of 100  $\mu\text{g}/\text{mL}$ . However, there was a great increase after 10h and the rate of labeled cells amounted to 93.6%. After 16h, the labeling efficiency decreased to 56.2%, while 59.1% after 24h. With the expansion of cell number, and a continual quenching process of the fluorophore, the labeling yield of FITC-AA nanoparticles decreased after reaching to the maximum labeling yield (93.6%). Results revealed that there was an insignificant labeling effect when the concentration of FITC-AA nanoparticles was lower than 10  $\mu\text{g}/\text{mL}$ . The

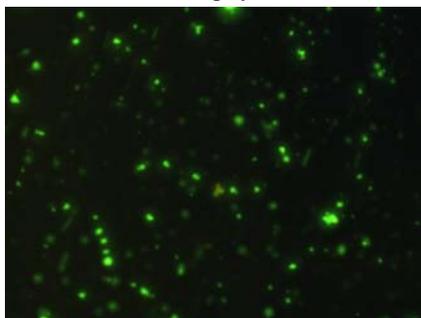


**Figure 4.** Cell morphology after FITC-AA nanoparticles labeling 3T3 fibroblast cells ( $\times 1000$ ).

tremendous change appeared at the concentration of 20  $\mu\text{g}/\text{mL}$ . With the increase of the concentration of FITC-AA nanoparticles, the number of labeled cells increased in a certain extent.

### 3.6 Properties after deposit

After deposited for half a year, FITC-AA nanoparticles enlarged its mean diameter to  $2614.0 \pm 338.1$  nm and its mean zeta potential was 1.09 mV. This meant that the mean diameter of FITC-AA nanoparticles increased significantly after deposited for a certain time. However, by the mean of solving in hot water, dispersing with ultrasonic wave and cooling to form nanoparticles again, the mean diameter of the deposited FITC-AA nanoparticles decreased to 428.4 nm. These demonstrated that FITC-AA nanoparticles can reversibly dissolve and form nanoparticles after deposit because of their physical crosslinking.



**Figure 5.** Cell morphology after incubation with half-a-year deposited FITC-AA nanoparticles for 12h in HL-60 cells ( $\times 400$ ).

Results from fluorescent microscope showed that the deposited FITC-AA nanoparticles had good labeling efficiency in HL-60 cells (Figure 5). It meant that the fluorescent properties of FITC-AA nanoparticles remained very well after deposit. Compared with quantum dots, the advantage of FITC-AA nanoparticle highlights, the storage time of quantum dots is less than three months as usual.

### 4 CONCLUSIONS

After activated with epoxy group, and then aminated, agarose can conjugate with FITC to spontaneously form nanoparticles labeling fluorescence. It was convinced that FITC-AA could be conveniently obtained by amine group on agarose chain reacting with isothiocyanate group of FITC. The molecular weight of agarose had a great effect on size and zeta potential of FITC-AA particle. The fluorescence of FITC-AA nanoparticles was dependent on the average size of nanoparticles. FITC-AA can label cells more effectively than single FITC, besides, it deposits longer than QDs. These facts demonstrate that FITC-AA could be potentially applied in labeling cell for a relatively long time.

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