Soft Matter

Cite this: *Soft Matter*, 2011, **7**, 4876

www.rsc.org/softmatter

PAPER

Quantifying specific cell–polymer interactions using fluorescence correlation spectroscopy

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Received 29th December 2010, Accepted 10th March 2011
DOI: 10.1039/c0sm01553a

Evaluation of interactions between polymers and cells is very important in many biomedical applications, including tissue engineering, as polymer scaffolds represent the extracellular matrix in the body and provide cells with three-dimensional framework. Alginate was modified with a peptide with the sequence of arginine–glycine–aspartic acid (RGD) to prepare cell-interactive polymers, and specific interactions between mouse fibroblasts and RGD peptide-modified alginates were investigated using fluorescence correlation spectroscopy (FCS). FCS was useful for determining cell–polymer interactions in a quantitative manner at nanomolar polymer concentrations, and the results were consistent with those obtained from the fluorescence resonance energy transfer (FRET) technique. This approach of quantitatively determining interactions between cells and polymers could be useful for developing novel polymer scaffolds for tissue engineering, as well as for designing highly efficient drug delivery systems.

1. Introduction

Tissue engineering can be used as a substitute for organ transplantation, which is extremely limited by donor shortages.[1] Tissue engineering supplies patients with engineered tissues or organs that are constructed through a combination of cells and three-dimensionally structured scaffolds. In general, cells are isolated from a specific tissue of a patient and then cultured *in vitro* to attain a sufficient number of cells. The cells are then combined with polymer matrices that act as a scaffold and emulate the structure and function of the extracellular matrix (ECM) in the body, thus providing three-dimensional frameworks for tissue regeneration.[2] In addition, polymer scaffolds can deliver soluble factors such as growth factors for induction and acceleration of tissue regeneration.[3,4]

The properties of polymer scaffolds (*e.g.*, mechanical properties,[5,6] biodegradation,[7] and hydrophilicity[8,9]) have a profound effect on cellular behaviors and tissue regeneration. Since many types of cells used in tissue engineering are anchorage-dependent, it is important to investigate and evaluate the interactions between the polymers and the cells in order to effectively regenerate new tissues or organs. Adhesion is the initial cell response to matrices, and cell adhesion has a direct influence on the proliferation,[10,11] migration,[12,13] and differentiation[11,13] of the adherent cells.

In order to enhance cellular interactions with the scaffolds, cell adhesion molecules can be introduced into polymer scaffolds that have inherently poor interactions with cells. Peptides with the sequence of arginine–glycine–aspartic acid (RGD) are well-known cell adhesion molecules that have been widely used to improve the cellular adhesion of polymer scaffolds in many tissue engineering applications.[14,15] Alginate has been widely used for many biomedical applications because of its biocompatibility, low toxicity, and mild gelation conditions with divalent cations; however, alginate inherently lacks the capability of binding to cells. The RGD peptide can be chemically coupled to the alginate backbone using water-soluble carbodiimide chemistry for preparation of cell-interactive polymers. In our previous studies, we demonstrated that RGD peptide-modified alginate hydrogels have a great potential in many tissue engineering applications due to enhanced specific interactions between cells and polymers.[16]

Determination of cell–polymer interactions is critical for the design and tailoring of cell-interactive polymers for tissue engineering applications. Rheological measurement and fluorescence resonance energy transfer (FRET) method were found to be useful for evaluating these types of interactions in a qualitative and quantitative manner.[15,17] The rheological measurements of cell–polymer mixtures have confirmed the formation of cell-cross-linked hydrogels by specific interactions between cells and cell-interactive polymers.[15] The fluorescence resonance energy transfer (FRET) technique can measure interactions between fluorescent molecules (*i.e.*, donor and acceptor), in which the emission energy of an excited donor molecule transfers to an acceptor molecule. The FRET technique has been broadly applied to investigate interactions between many types of biological molecules, as well as to determine specific interactions between cells and polymers.[17,18] However, rheological measurements require high concentrations of cells and polymers in order

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to determine cell–polymer interactions, and two different fluorescent molecules need to be chemically coupled to both polymers and cells in order to monitor the FRET phenomena.

For this context, we proposed to apply fluorescence correlation spectroscopy (FCS) to quantitatively determine cell–polymer interactions at the nanomolar level of polymer concentrations without modifying cells with fluorescent molecules. FCS is a powerful tool to determine the motion of a single molecule in terms of the diffusion coefficient, concentration, aggregation and molecular interactions within living cells.[19–21] FCS is an analysis tool that measures fluctuations of fluorescent molecules in the confocal volume using a laser source at a nanoscale concentration (Fig. 1). Fluctuation of a single fluorescent molecule resulting from Brownian motion can be detected and then statistically analyzed with an autocorrelation function. The quantitative results regarding molecular motions and kinetics can be obtained from this method.[22] However, evaluation of the interactions between cells and cell-interactive polymers using FCS has not yet been reported.

In this study, we hypothesized that FCS could provide a useful means to determine the interactions between cells and cell-interactive polymers in a quantitative manner. We synthesized RGD peptide-modified alginate as a cell-interactive polymer, and the peptide was labeled with a fluorescent marker. The FRET technique was used to confirm specific interactions between the cells and the RGD peptide-modified alginate. We investigated changes in specific interactions between polymers and cells using FCS by varying the degree of substitution of adhesion ligands as well as the polymer concentration, and we also determined the relative number of polymers bound to cells.

2. Materials and methods

2.1 Synthesis of peptide-modified alginate

Sodium alginate was purchased from FMC Biopolymer and dissolved in 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma) buffer solution at room temperature (pH = 6.5, 0.3 M NaCl). A peptide with the sequence of $(glycine)_4$–arginine–glycine–aspartic acid–alanine–$(serine)_2$–lysine (G_4RGDASSK) was purchased from Anygen (Korea) and added to the alginate solution in the presence of *N*-hydroxysulfosuccinimide (sulfo-NHS, Pierce) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC, Sigma). The peptide-modified alginate was purified by extensive dialysis against deionized water for four days (molecular weight cut-off = 3500), subjected to activated charcoal treatment, and sterilization through a 0.22 μm filter. The degree of substitution (DS) of RGD peptides was defined as the number of peptides per 100 uronic acid residue in the alginate chain, and was determined using a radio-labeled RGD peptide as a tracer molecule.[17] The DS of RGD peptide-modified alginate used in this study was in the range from 0.075 to 1.5 by varying the amount of RGD peptides added to the reaction mixture. Non-peptide-modified alginate was also prepared using the same procedure, without the peptide addition.

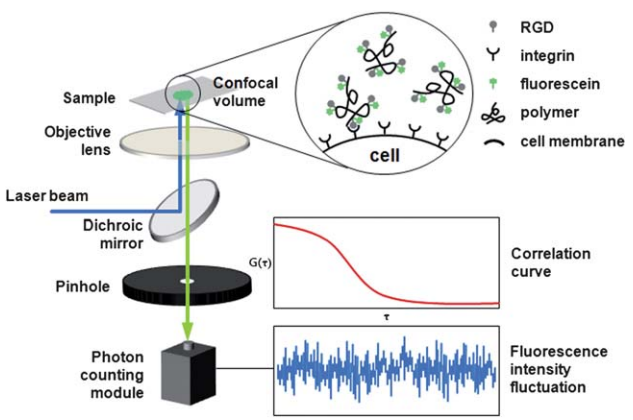

Fig. 1 A schematic representation of the FCS instrumental setup, and an illustration of ligand–receptor binding events inside the focus.

2.2 Fluorescence labeling

RGD peptide-modified alginate was labeled with Alexa Fluor® 488 carboxylic acid succinimidyl ester (Invitrogen). The peptide-modified alginate was dissolved in 0.15 M sodium bicarbonate buffer at pH 8.5 and mixed with the fluorescent dye in the presence of EDC and NHS. The molar ratio between the fluorescent dye and the peptide was kept constant at 1 : 1. Non-peptide-modified alginate was also labeled with 5-(aminomethyl) fluorescein hydrochloride (Invitrogen). Alginate was dissolved in MES buffer at pH 6.5 and mixed with 5-(aminomethyl)fluorescein hydrochloride in the presence of EDC. The same molar amount of fluorophores was added to either RGD-modified alginate or non-peptide-modified alginate solution. Both fluorescence-labeled RGD peptide-modified alginate and non-peptide-modified alginate were purified by dialysis and active-charcoal treatment, followed by sterilization through a 0.22 μm filter. Lyophilized alginate samples were dissolved in Dulbecco's modified Eagle's medium (DMEM, Gibco) without phenol red.

2.3 Cell culture and staining

NIH3T3 mouse fibroblasts were purchased from the Korea Cell Line Bank (KCLB) and cultured in DMEM containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Gibco) at 37 °C under a 5% CO_2 atmosphere. To confirm FRET phenomena between cells and polymers, cell membranes were stained with octadecyl rhodamine B chloride (Invitrogen) for 2 h. Excess rhodamine molecules were removed by centrifugation at 1500 rpm for 3 min. The collected cells were re-suspended in DMEM without FBS and phenol red, and used for further experiments.

2.4 FRET measurement

A mixture of fluorescein-labeled alginate solution and rhodamine-stained cell suspension was placed in a 96-well tissue culture plate and incubated at 37 °C under a 5% CO_2 atmosphere for 20 min before measurements were taken. The sample was then excited at a wavelength of 488 nm, and the responding emission was collected with a spectrophotometer (SpectraMax, Molecular Devices). The excitation and emission slit width was 5 nm, and the temperature was kept constant at 37 °C. Cells treated with soluble RGD peptides for 30 min were also prepared to confirm specific interactions between integrin receptors of cells and adhesion ligands in the polymers. The degree of energy transfer

(Ψ) was determined from the emission intensity of the donor (*i.e.*, Alexa Fluor® 488) in the presence ($I_{D,+A}$), and absence ($I_{D,-A}$) of the acceptor (*i.e.*, octadecyl rhodamine B chloride).[23]

$$\Psi = 1 - \frac{I_{D,+A}}{I_{D,-A}} \quad (1)$$

2.5 Fluorescence correlation spectroscopy (FCS)

The FCS equipment (Alba, ISS) was fitted onto an inverted ECLIPSE TE2000-U microscope (Nikon) with a piezo-controlled XYZ motorized stage (Mad City Labs). The beam of a 470 nm diode laser was expanded by a beam expander. The expanded beam was reflected by two different mirrors and then entered the back port of an inverted microscope. The laser beam was reflected by a dichroic mirror (Nikon) and then focused into the sample through a 60× microscope objective lens. Emitted fluorescence photons encountered the dichroic mirror and an emission filter. The filtered fluorescence was focused by the tube lens of the microscope and exited through the side port with a focus close to the body of the microscope. The photons were focused into a pinhole, which blocked all unfocused emission photons. Signals were detected using a single photon avalanche photodiode (APD). Alignment and calibration were performed using a Alexa Fluor® 488 solution (1 nM in distilled water) with a known diffusion coefficient (430 $\mu m^2\ s^{-1}$). The fluorescein-labeled RGD peptide-modified alginate solution was mixed with cells seeded in 8-well Lab-Tek™ chamber slide systems (Nunc) and then incubated for 1 h ([cell] = 5×10^4 cells per well). The polymer solution was then removed from the well and the cells were rinsed with a PBS solution to eliminate unbound polymers. A HEPES-buffered saline solution was next added to each well (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 4.6 mM $CaCl_2$, 20 mM HEPES, and 10 mM glucose; pH 7.4). The fluctuation of photons was detected for 100 s at a sampling frequency of 100 000 Hz, and the wavelength range of the emission filter was between 510 and 560 nm. An autocorrelation curve was obtained with a 3D Gaussian function model.[24]

$$G(\tau) = \frac{\gamma}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + S^2\frac{\tau}{\tau_D}\right)^{-\frac{1}{2}} \quad (2)$$

The autocorrelation function $G(\tau)$ was defined in terms of the average time of the deviation of intensity from the average intensity. γ is a geometric scaling factor ($\gamma = 1/\sqrt{8}$ for one-photon 3D Gaussian), N is the average number of molecules in the confocal volume, and S is the ratio of the axial/radial (ω/z) dimensions of the confocal volume. τ_D is related to the diffusion coefficient by $\tau_D = \omega^2/4D$.

3. Results and discussion

Cell-interactive polymers containing adhesion ligands (*e.g.*, RGD peptide) were synthesized and tested using a FRET technique for their ability to specifically interact with cells. The specific interactions between cell receptors and adhesion ligands in polymers were first confirmed by fluorescence microscopy using fluorescein-labeled alginate and rhodamine-stained cells. The red fluorescence emission was significantly enhanced by mixing the cells with the RGD peptide-modified alginate solution (Fig. 2a), compared to those mixed with the non-peptide-modified alginate solution (Fig. 2b). It was attributed to energy transfer from the donor molecules (*i.e.*, fluorescein) to the acceptor molecules (*i.e.*, rhodamine) in the system, due to specific interactions between adhesion ligands attached to the polymer chain and cell receptors (*e.g.*, integrin) near the cell membrane. In addition, a decrease of green emission was also observed by fluorescence spectroscopy (Fig. 3a). However, when cells pre-treated with soluble RGD peptides were mixed with cell-interactive polymers, no significant change in the emission spectrum was observed (Fig. 3b). It was attributed that the soluble RGD peptides blocked the integrin receptors of cells, and these cells were thus not able to sufficiently interact with the cell-interactive polymers. This finding clearly indicates that RGD peptide-modified alginate specifically binds to integrin receptors of cells, which is consistent with previous results.[25]

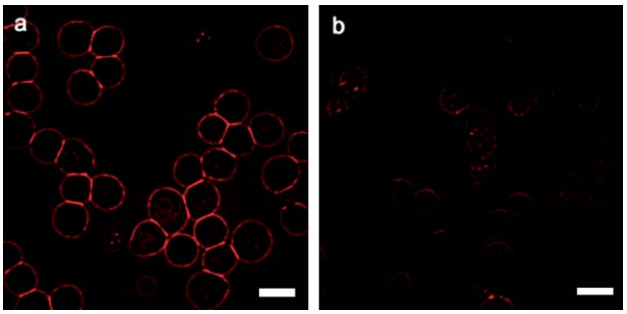

Fig. 2 Fluorescence microscopic images of cells mixed with (a) fluorescein-labeled RGD peptide-modified alginate and (b) fluorescein-labeled non-peptide-modified alginate. Samples were incubated for 20 min after mixing, and images were taken with excitation between 450 and 490 nm ([cell] = 5×10^6 cells per ml; [polymer] = 0.015 wt%; DS = 0.15; scale bars, 10 μm).

The degree of energy transfer (Ψ) was determined from fluorescence spectra and used to quantify the interactions between cells and cell-interactive polymers. The Ψ value changed depending on the concentrations of polymers and cells in the systems. The Ψ values increased when the polymer concentration increased from 0.005 to 0.1 wt% ([cell] = 5×10^6 cells per ml) (Fig. 4a). As the polymer concentration increased, the number of polymers bound to cells increased and the emission energy transferred from polymers to bound cells also increased. An increase in the cell concentration from 2.5×10^5 to 5.0×10^6 cells per ml also influenced the Ψ values ([polymer] = 0.015 wt%, Fig. 4b). We also found that a certain number of cell-interactive polymers and cells are required for FRET, and this FRET phenomenon is strongly dependent on the concentrations of cells and polymers in the system.

Interactions between polymers and cells were next investigated by FCS. The fluctuation of emission energy from fluorescein-labeled alginate was detected in the presence of cells and transferred to an autocorrelation function. We found that specific interactions between RGD peptide-modified polymers and integrin receptors of cells significantly altered the autocorrelation curves (Fig. 5). We obtained two distinct autocorrelation curves when the RGD peptide-modified alginate solution was added to cells (Fig. 5a and b). The autocorrelation curve of RGD peptide-modified alginate bound to integrin receptors of cells shifted to the right (Fig. 5a), compared with that of the unbound, free RGD peptide-modified alginate in the system (Fig. 5b). The diffusion

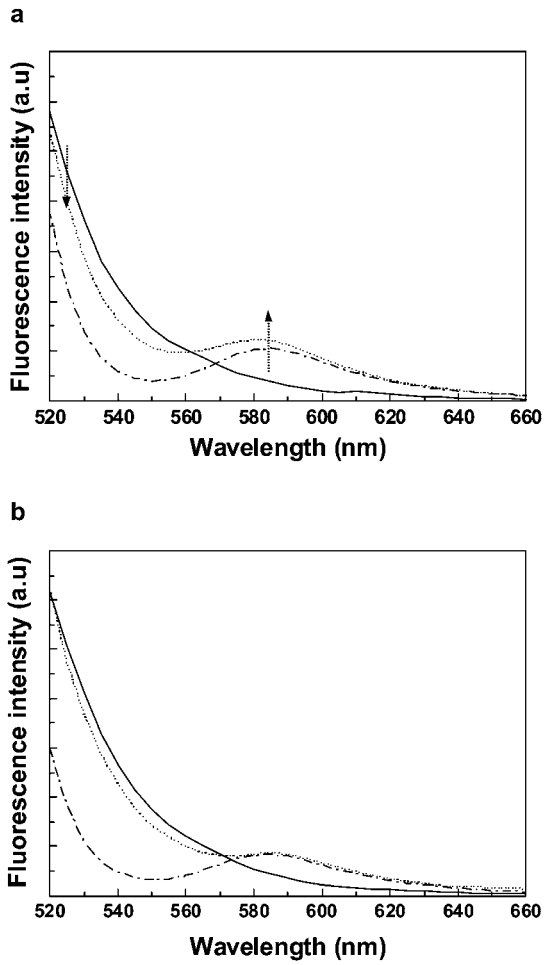

Fig. 3 Changes in fluorescence intensity when an RGD peptide-modified alginate solution is mixed with (a) cells and (b) soluble RGD-pretreated cells ([cell] = 5×10^6 cells per ml; [polymer] = 0.015 wt%; DS = 0.15; λ_{ex} = 488 nm). RGD peptide-modified alginate was labeled with fluorescein and cells were stained with octadecyl rhodamine B chloride. Fluorescence spectra of polymer only (solid line), cell only (dash-dotted line), and cell/polymer mixture (dotted line).

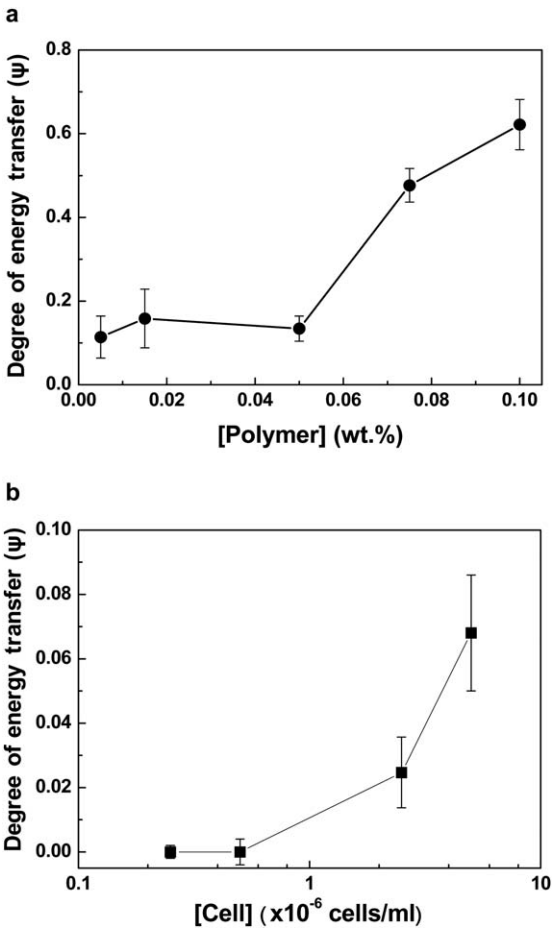

Fig. 4 Changes in the degree of energy transfer (Ψ) as a function of (a) polymer concentration ([cell] = 5×10^6 cells per ml) and (b) cell concentration ([polymer] = 0.015 wt%; DS = 0.15).

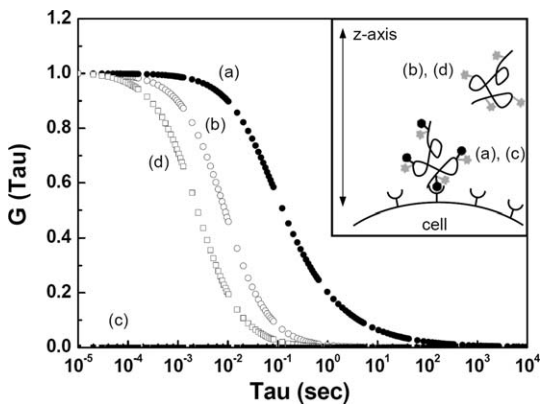

Fig. 5 Autocorrelation curves of (a and b) fluorescein-labeled RGD peptide-modified alginate and (c and d) non-peptide-modified alginate based on measurements either on the cell surface (a and c) or above the cell surface (b and d) (DS = 1.5; [polymer] = 3 nM; [cell] = 5.0×10^4 cells per well).

coefficients for free and bound RGD peptide-modified alginate were 15.8 ± 4.7 and 0.5 ± 0.4 $\mu m^2 s^{-1}$, respectively. However, an autocorrelation curve for non-peptide-modified alginate bound to cells was not obtained (Fig. 5c), unlike those above the cell surface (Fig. 5d), as all the alginate molecules were removed from the well during the rinse process due to negligible interactions with cells. This finding clearly indicates specific interactions of RGD peptide-modified alginate with cells and FCS measurements can be used to investigate this type of interactions. Autofluorescence of cells used in this study did not significantly influence our FCS measurements.

We further investigated the effect of polymer concentrations on the number of polymers bound to cells (Fig. 6), while maintaining the same cell density ([cell] = 5.0×10^4 cells per well). The number of bound polymers was calculated from photon counting histogram. When the RGD peptide-modified alginate solution was tested, the number of bound polymers increased as the polymer concentration increased. The relative number of bound RGD peptide-modified alginate, determined by normalization with the number of bound non-peptide-modified alginate at the same polymer concentration, was varied from 2.1 ± 0.2 to 6.1 ± 0.2. This finding was consistent with the results obtained from FRET experiments (Fig. 4a). No significant change in the number of

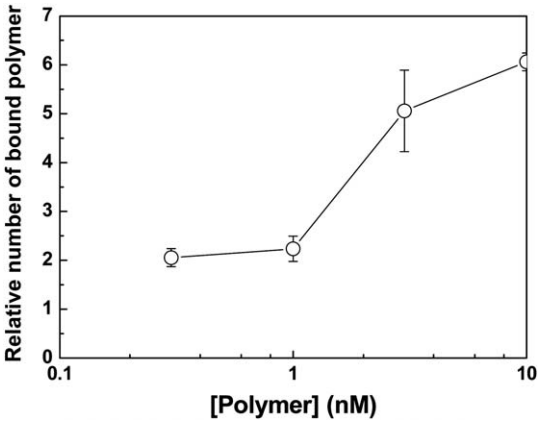

Fig. 6 Relative number of bound RGD peptide-modified alginate depending on the polymer concentration (DS = 1.5; [cell] = 5.0×10^4 cells per well).

bound polymers was found when the non-peptide-modified alginate solution was used at various concentrations due to the lack of specific interactions between cells and polymers.

We next tested whether the degree of substitution (DS) of RGD peptides to the alginate backbone could influence the number of polymers bound to cells. The DS was varied from 0.075 to 1.5 (Fig. 7). When RGD peptide-modified alginate with the DS of 0.075 was used, the relative number of polymers bound to cells was 1.9 ± 0.1. This number increased 3-fold for RGD peptide-modified alginate with the DS of 0.15 and no further significant increase was observed for those with higher DS used in this study. This finding might be attributed to the inherent stiffness of an alginate backbone, which may restrict the access of RGD peptides to cells. Alginate is a linear copolymer containing blocks of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues, and the repeated structure of α-L-guluronate residues mainly contributes to its chain stiffness.[26] Alginate used in this study originally contains high contents of G residues, and a use of alginate with different *M*/*G* ratios or a control of the backbone rigidity (*e.g.*, oxidation[7]) may change binding behavior of the RGD peptide-modified alginate.

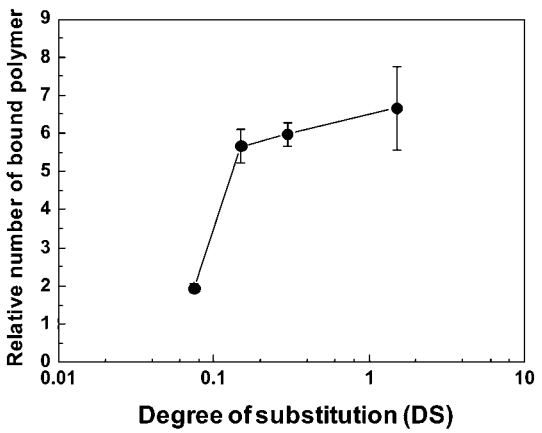

Fig. 7 Relative number of bound RGD peptide-modified alginate depending on the DS of the RGD peptide ([polymer] = 3 nM; [cell] = 5.0×10^4 cells per well).

4. Conclusion

Specific interactions between RGD peptide-modified alginate and cells were quantified using fluorescence correlation spectroscopy. FCS was useful for investigating specific interactions between cells and adhesion ligand-modified polymers at nanomolar concentrations in a quantitative manner. The number of cell-interactive polymers bound to cells was dependent on the concentration of polymers as well as on the number of adhesion peptides coupled to the polymer backbone in the system. In addition, a certain number of cells and polymers were required to induce specific interactions between cells and polymers, and FCS was useful to determine these values. This approach for quantitatively evaluating cell–polymer interactions could be critical in the design and tailoring of novel polymers for tissue engineering applications, as well as for many drug delivery systems such as polymer prodrugs.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2010-0016065, 2010-0012608).

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