DENDRIMER SURFACE ORIENTATION OF THE RGD PEPTIDE AFFECTS MESENCHYMAL STEM CELL ADHESION

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Mesenchymal stem cells (MSCs) are promising candidates for a range of tissue regeneration applications. Adequate scaffolds are necessary for their application in vivo, where interactions between cells and the surface material are critical. Arginine-glycine-aspartic acid tripeptides (RGD) were conjugated to polyamidoamine (PAMAM) dendrimers and used to pre-treat test surfaces. We demonstrate that pretreatment with dendrimer-presented tripeptides efficiently increases MSC adhesion to a polystyrene test surface, and that treatment effectiveness is related to how tripeptides are presented by the dendrimer to the cell. We tested both R-G-D–dendrimer and dendrimer–R-G-D arrangements and found the former to be optimal in terms of surface adhesion.

Introduction

Scaffolds are a promising approach for promoting stem-cell mediated tissue regeneration for several clinically relevant cell types, including mesenchymal stem cells (MSCs). MSCs have been the target of extensive efforts towards developing tissue engineering strategies due to their high proliferative potential, their default tissue differentiation pathway, and their key roles in the formation of specific tissues in vivo.1,2 The development of new and improved biomaterials and biomedical devices for in vitro and in vivo applications such as diagnostics, drug delivery, implants and regenerative medicine is also a major focus of investigation. Numerous studies have focused on controlling interactions between cells and surface materials, especially towards the development of surface modification protocols that enhance cell adhesion by improving interactions between an implant material and biological tissues.3,4 Coating implant surfaces with cell-adhesive molecules provides a strong mechanical contact between cells and surfaces. Additionally, it has been demonstrated that the molecular characteristics of the adhesion ligands interspersed at the cell–biomaterial interface can influence the fate of stem cells.5,6 Generally, surface modification methods involve the application of bioactive molecules to the substrate

which are then recognized by cell–surface receptors. The recognition of extracellular matrix (ECM) proteins by integrins, a family of heterodimeric transmembrane proteins, has been widely used and is of great interest for tissue engineering applications. 7,8 A variety of peptide motifs have been shown to specifically interact with integrins, many of which have also been identified within ECM proteins. The most widely studied peptides for use in biomedical devices contain the arginine-glycine-aspartic acid (RGD) sequence.9,10 RGD nanospacing has been revealed as a regulator of specific cell adhesion. The critical nanospacing is around 70 nm, and it has been known that the RGD nanospacing within a local cluster is more essential than RGD density to determine cell adhesion.11–13 A shorter nanospacing leads to a more significant cell spreading. It has been demonstrated that MSCs can be encapsulated in RGD-alginate beads while maintaining greater than 80% viability over a two weeks period.10

The modification of biomaterial surfaces with small RGD peptides offers several advantages over the immobilization of whole ECM proteins, such as easier manufacture and quality control, simpler patterning and higher density. The most widely used techniques involve self-assembled monolayers or the immobilization of RGD peptides on polymer surfaces.14 While different polymer-based coating approaches have been tested for bioapplications,15 increasing interest has focused on dendrimers, a unique category of polymeric material. The threedimensional architecture of dendrimeric systems confers them intrinsic features including structural homogeneity, integrity, controlled composition and high-density multidentate homogeneous terminal groups available for bioconjugation. These unique properties make dendrimeric systems attractive for a variety of bioapplications16 and has resulted in the growth of dendrimers as therapeutic tools in regenerative medicine over recent years.11 Polyamidoamine (PAMAM) dendrimers are biocompatible, non-immunogenic, water soluble, and have been coupled to many biological molecules such as proteins and drugs.17 The toxicity of PAMAM dendrimers increases with later generations; EC50 for G4 PAMAM dendrimers NH2 is 5–20 mM,18 and toxicity was also correlated with the zeta potential of dendrimers in mammalian cells.19

Additionally, the multivalent surface of dendrimers makes them ideal scaffolds for attachment via RGD-containing peptides.12,20–23 PAMAM dendrimer–RGD conjugates have been reported to enhance the targeted delivery of drugs24 or imaging agents to carcinoma cells,13,25 and have been used as delivery vectors.26,27 Large nanospacings (95–150 nm) of RGD were found to enhance differentiation of MSCs.28,29 PAMAM–RGD conjugates have also been found to mediate cellular binding and adhesion, 30–32 resulting in a unique matrix for eliciting integrinmediated cellular responses, with great potential for tissue engineering and regenerative medicine.

Results and discussion

In this paper, we aim to gain insights into the different ways in which ligands can be organized and exposed on the surface of a substrate to promote effective binding. We chose the linear RGD peptide to evaluate the effect of how the peptide is attached to the dendrimer on the recognition process. The use of dendrimers as platform for presenting the RGD tripeptide provides a unique opportunity to finely control the structural components of the cluster. This will help better understand how the spatial arrangement of RGD–dendrimer composite impacts on cellular responses and aid the design of RGD-containing molecules able to trigger more favorable cellular responses.

To examine the ability of dendritic–RGD complexes to confer cell adhesion properties, we perform in vitro cell adhesion assays using MSCs isolated from human bone marrow (BM) and using polystyrene plates as a test surface.

To decorate the peripheral dendrimer groups with biologically relevant peptide ligands, it is vital to use efficient and chemoselective conjugation chemistry to ensure complete ligand attachment to the dendrimer. Although peptide coupling reagents are often used, most chemoselective reactions use thiol–disulfide exchange to attach peptide cysteine residues to dendrimers.33,34 In this paper, we used the N-terminal cysteine residue to link peptides to end-bound maleimide dendrimers.35 Two different tetrapeptides were used, namely RGD-Cys and Cys-RGD, obtaining two different arginine-glycine-aspartic acid (RGD)-tailored dendrimers.

PAMAM–RGD peptide conjugates were synthesized as depicted in Scheme 1. Maleimido-functionalized first generation PAMAM dendrimers react with the terminal Cys residues of the tetrapeptide sequences, resulting in two D1–RGD conjugates with identical chemical composition but different amino acid arrangements, which presents the RGD fragment from the dendrimer in two different orientations for potential cell interaction. For this study the first generation (G1) PAMAM dendrimer was selected as the most suitable scaffold, as its size and number of termini available for peptide attachment should provide sufficient multivalency for effective cellular adhesion,22 and low generation PAMAM dendrimers have been shown to be potentially more biocompatible and less immunogenic.

PAMAM-G1 surface amine groups were reacted with compound 1 (Scheme 1), in order to transform the peripheral amino groups into maleimide derivatives.Maleimide-PAMAM-G1 dendrimers (2) have been used as efficient scaffolds for the covalent attachment of thiol-derivatives at the dendrimer surface.36 As outlined above, we anchored RGD peptides to the surface of compound 2 using terminal Cys-residue linkers, adapting a previously described procedure.37 The (RGD-Cys)$_8$–D1 and D1–(Cys-RGD)$_8$ compounds obtained were analyzed by liquid chromatography-mass spectrometry techniques (HPLC_MS/MS) confirming the inclusion of eight copies of the tetrapeptides at the dendrimer surface. 1HNMR spectra of both dendrimer–

RGD derivatives and the corresponding tetrapeptides are shown in Fig. 1. The spectra confirm effective binding of the cysteinemoieties to the dendrimer. Signals between 1.50 and 2.00 ppm in the 1H-NMR spectrum of both derivatives corresponds to b and g CH2 of the arginine (R) moiety. The a CH2 appears as an intense signal at 3.18 ppm. The remaining tetrapeptide signals between 2.00 and 5.00 ppmoverlap those corresponding to the dendrimer.38

To evaluate the impact of dendrimer–RGD peptide orientation cell adhesion assays were designed to determine the ability of the two conjugates to promote human MSCs (hMSCs) adhesion on a test surfaces. Microbiology-grade polystyrene plates, 100 mm diameter, were pre-treated with either D1–(Cys-RGD)$_8$ or (RGD-Cys)$_8$–D1. Untreated plates were used as controls. Previous studies demonstrate that RGD alone was not sufficient to promote full cell spreading.39 Equal numbers of hMSCs (1.0 x 10^5 cells per mL) were applied to each plate and allowed to grow in optimal conditions for several days with one change of media at day 6. After 10 days we compared the morphology of cells on treated and untreated dishes.

MSCs cultured on untreated polystyrene plates had a rounded morphology, with little or no signs of attachment or focal adhesion (Fig. 2a). In contrast, most cells on plates treated with either D1–(Cys-RGD)$_8$ (Fig. 2b) or (RGD-Cys)$_8$–D1 (Fig. 2c) showed a spreading morphology typical of adherent cells, although the spreading morphology was significantly more pronounced on the (RGD-Cys)$_8$–D1 treated plates.

The number of cells per mL and per plate varied significantly among the conditions tested (Fig. 3). Plates treated with (RGDCys) 8–D1 contained 6.8 x 10^5 cells per mL at day 10, which is comparable to the 7.4 x 10^5 cells per mL usually obtained at that time on tissue culture grade plates and significantly more than the 4.6 x 10^5 cells per mL on plates treated with D1–(Cys-RGD)8.

An insignificant number of cells with an abnormal phenotype adhered to untreated plates (negative control). In addition, the adhered cells exhibited appreciably different resistance to trypsin treatment. Significantly, 0.50% trypsin was needed to detach MSCs from plates pre-incubated with (RGD-Cys)$_8$–D1 (Fig. 2d), rather than 0.25% trypsin needed for MSCs grown on plates pre-incubated with D1–(Cys-RGD)$_8$, in which the cells were detached quickly, leaving empty plates.

Whereas common methods for preparing artificial bioactive materials include controlling the mechanical properties of the material, incorporating bioactive signals, spatially modelling and controlling the density of bioactive signals, the way signals are exposed for molecular recognition has been less widely exploited. This study suggests that while multivalent ligand dendrimer conjugates can effectively mediate cellular adhesion, the spatial organization of ligands can have a critical effect on the ability of cells to bind RGD peptides.

Based on our recent studies,30 we have carried out experiments to test the influence of local RGD surface density in human MSCs (hMSCs) adhesion in chondrogenesis, and we analyzed the formation of focal adhesions (FAs) in cells cultured for 1 day under chondrogenic induction on the RGD–D1 nanopatterned surfaces. The preliminary data suggest that dendrimer-based nanopatterns sustained mesenchymal condensation and early chondrogenic differentiation of hMSCs, as well as cell adhesion more efficiently than the corresponding homogeneously-coated surfaces, and assisted FAs assembly and maturation at high local RGD surface densities.

Experimental

PAMAM dendrimer (generation 1, 20% w/w methanol solution) was purchased from Aldrich and used without further purifi- cation. RGD-Cys and Cys-RGD peptides were purchased from Thermo Scientific and used as received. Sephadex TM G-10 (purchased from Amersham Pharmacia Biotech AB) was used in stationary phase for size-exclusion chromatography. The synthesis of 3-maleimidopropionic acid N-hydroxysuccinimide ester 1 and the preparation of phosphate buffer saline (PBS) are described elsewhere.40,41

Preparation of compound 2

For preparation of compound 2, we adapted a previously described method for the synthesis of a maleimide-derivatized PAMAM dendrimer (generation 0).36,37,42 Briefly, after methanol removal under low pressure, 20 mg of PAMAM-G1 (0.014 mmol) was treated with 266 mg (1 mmol) of 1 in 5 mL of anhydrous dichloromethane for 48 h at room temperature. After solvent removal, the maleimido derivative 2 was obtained with a 30% yield by purification through size-exclusion chromatography.

General procedure for the preparation of dendrimer–RGD conjugates

6.5 mg (0.0024 mmol) of 2 were subsequently reacted with 10 mg (0.022 mmol) of cysteine-aspartic acid-glycine-arginine (RGD-Cys) or cysteine-arginine-glycine-aspartic acid (Cys-RGD) in 3 mL of degassed phosphate buffered saline (PBS) at 35 ºC for 48 h, under an argon atmosphere. Both D1–RGD derivatives were obtained with a 98% yield after purification by sizeexclusion chromatography. (RGD-Cys)$_8$–D1: HPLC_MS/MS: 6239.1 [M + 8]. Calcd for C238N90H384O92S8 (M+) ¼ 6230.6. D1– (Cys-RGD)8: HPLC_MS/MS: 6254.6 [M + 23]. Calcd for C238N90H384O92S8 (M+) 6230.6.

Isolation and culture of MSCs

Human BM cells were collected by aspiration from patients undergoing hip replacement surgery after informed consent and according to procedures approved by the local ethics committee. The MSC-enriched fraction was separated on Percoll (Sigma, St. Louis, MO, USA) gradient sedimentation at 20 000 g for 15 minutes and suspended in Dulbecco's modified Eagle's medium

175 (DMEM, Sigma). The BM was suspended, homogenized, and centrifuged at 400 g for 10 minutes.
176 The MSC fraction was plated at a concentration of 10^7 cells 75 cm^2 tissue culture flask and
177 maintained in DMEM containing 10% FBS, 2.5 mM L-glutamine, 100 U
178 mL
179 ———————————————————————————
180 1 penicillin, 100 mg
181 mL
182 ———————————————————————————
183 1 streptomycin and 1.25
184 mgmL
185 ———————————————————————————
186 1 fungizone. The culture medium was changed 2 times per week and the cells selected for their
187 capacity to attach to the dish surface, discarding the floating cells at the first medium change after
188 72 h. When culture flasks became near-confluent, cells were detached with 0.25% trypsin
189 containing 1 mmol L^{-1} EDTA and replated at 5 x 10^3 cells per cm^2 for experiments in 100 mm
190 polystyrene culture dishes (Corning Costar, Cambridge, MA) either of microbiologicalgrade
191 (non-surface treated) or tissue culture grade (treated to promote cell attachment). In both cases,
192 all plates were preincubated with either $(RGD\text{-}Cys)_8$–D1 or D1–$(Cys\text{-}RGD)_8$ dendrimers at 0.77
193 mg mL^{-1} for 30 min after which the dendrimer solutions were aspirated and plates air-dried under
194 sterile conditions. Cells were maintained in culture for 10 days with one change of media at day
195 six. Cells were detached from the experimental plates using a higher strength 0.50% trypsin. All
196 steps, including cell culture, were performed in a sterile laminar flow hood, and only sterile
197 materials, solutions and techniques were used.

198 **Conclusions**

199 In conclusion, we have found that the two conjugates, (RGDCys) 8–D1 and D1–$(Cys\text{-}RGD)_8$,
200 promoted MSC adhesion to the test surface, albeit with striking differences. Our data suggests
201 that the dendrimer-bound RGD peptide was recognized by cells in both cases. However, the
202 observed differences suggest that the way peptides are exposed can effectively modulate the
203 cellular response. Moreover, it shows that cell–peptide recognition is highly sensitive to R-G-D–
204 dendrimer and dendrimer– R-G-D orientations, with the R-G-D peptide sequence clearly being
205 the optimal choice for effective cell recognition. The chemical design has the potential for
206 promoting enhanced cell adhesion on solid surfaces in vivo. The fact that we observed significant
207 differences between the two peptide orientations suggests that our compound is presented to cells
208 in a stable manner rather than in a variety of different possible orientations as could occur with
209 other types of polymer attachment. Thus, when the peptide sequence is presented in an optimal
210 manner, it has the potential to establish a stronger junction between cells and the surface. These

characteristics make our conjugate strategy a promising candidate for regenerative medicine applications. The ability of hMSCs to generate distinct lineages is well-established and their tissue differentiation potential has become one of the most widely investigated topics in regenerative medicine. This new finding opens the door for further investigation of the potential of multivalent ligand– dendrimer conjugates for applications in 3D cell culture and tissue engineering.

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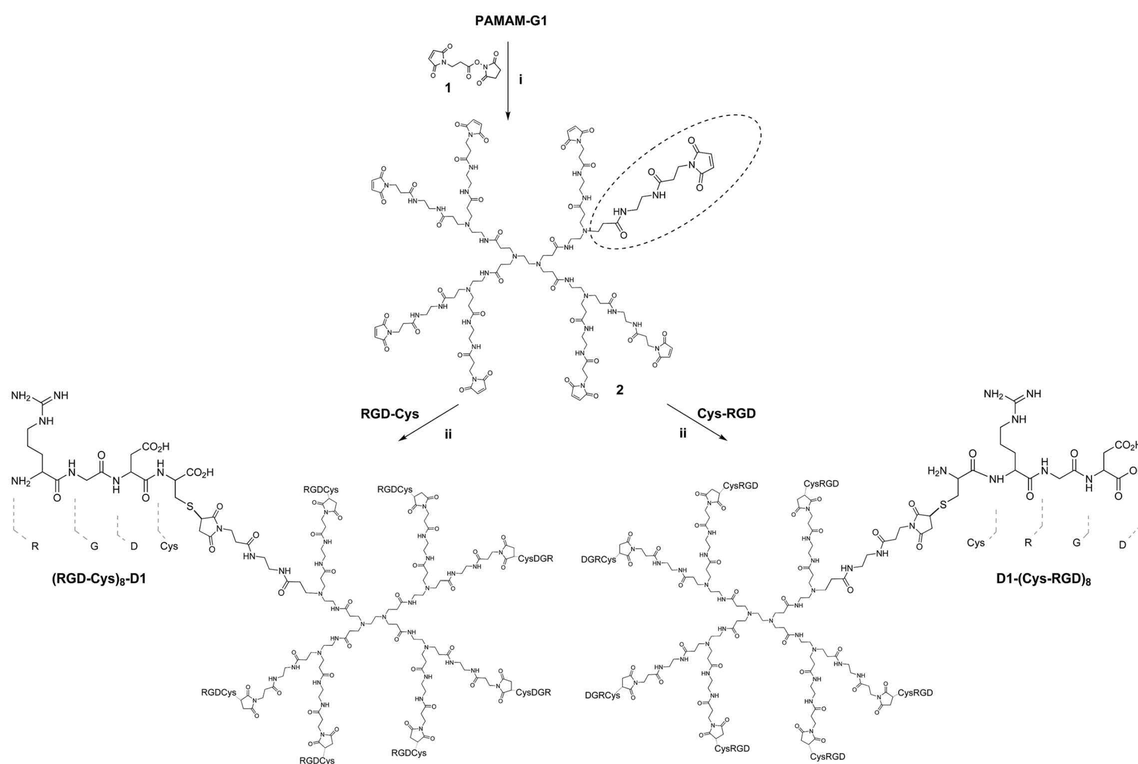

Scheme 1 Synthesis of RGD-tailored dendrimers from PAMAM-G1. (i) DCM, r.t., 48 h; (ii) PBS, 35 ºC, 48 h.

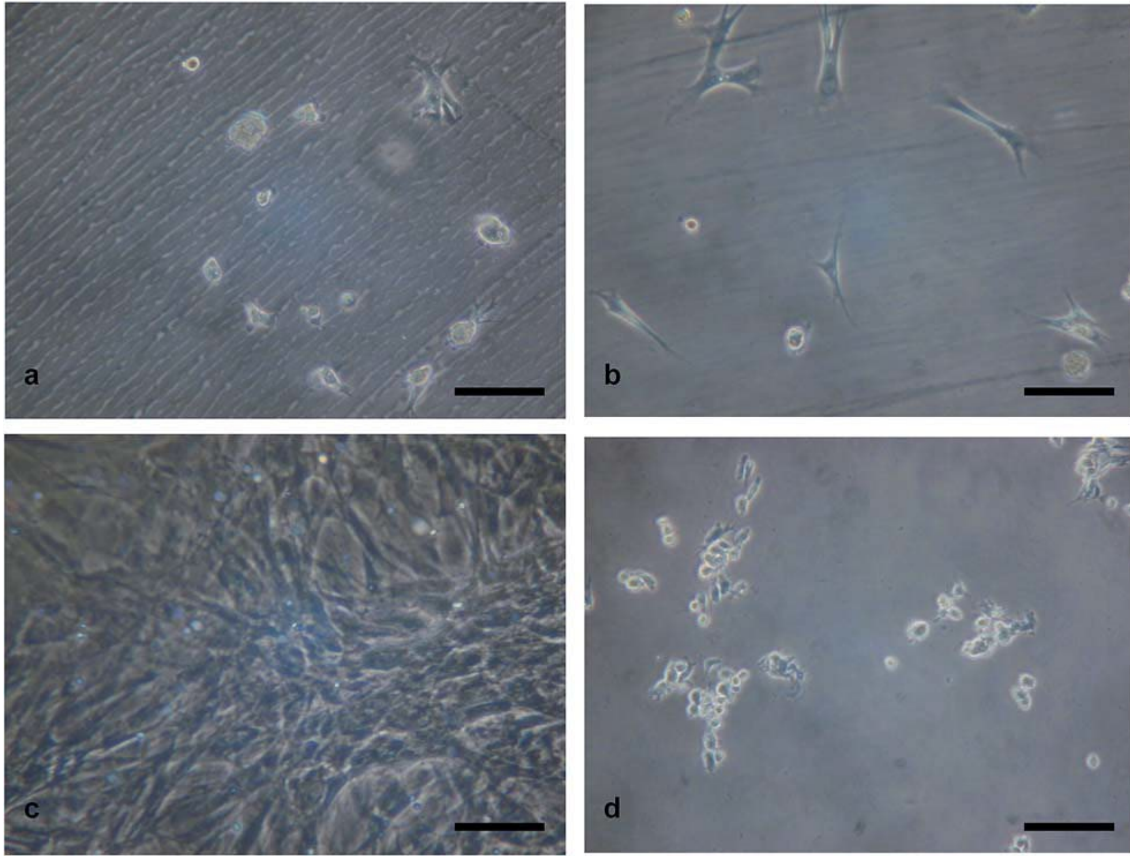

Fig. 2 hMSCs at day 10 of culture on microbiology-grade polystyrene plates, untreated (a), and treated with either D1–(Cys-RGD)$_8$ (b), or (RGD-Cys)$_8$–D1 (c). Images show different cell morphologies and number, as well as culture behavior on (c) after trypsin digestion (d). Bar, 30 microns.

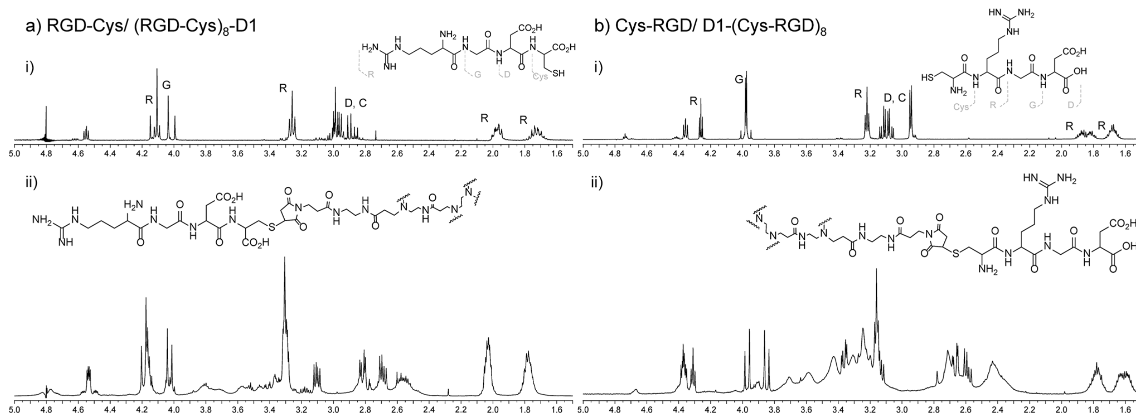

Fig. 1 1H-NMR spectra of (a) (i) RGD-Cys; (ii) $(RGD\text{-}Cys)_8$–D1 and (b) (i) Cys-RGD; (ii) D1–$(Cys\text{-}RGD)_8$–D1 in D_2Osolution. Spectra were acquired using a 600 MHz spectrometer equipped with a 5 mm TXI inverse probe.

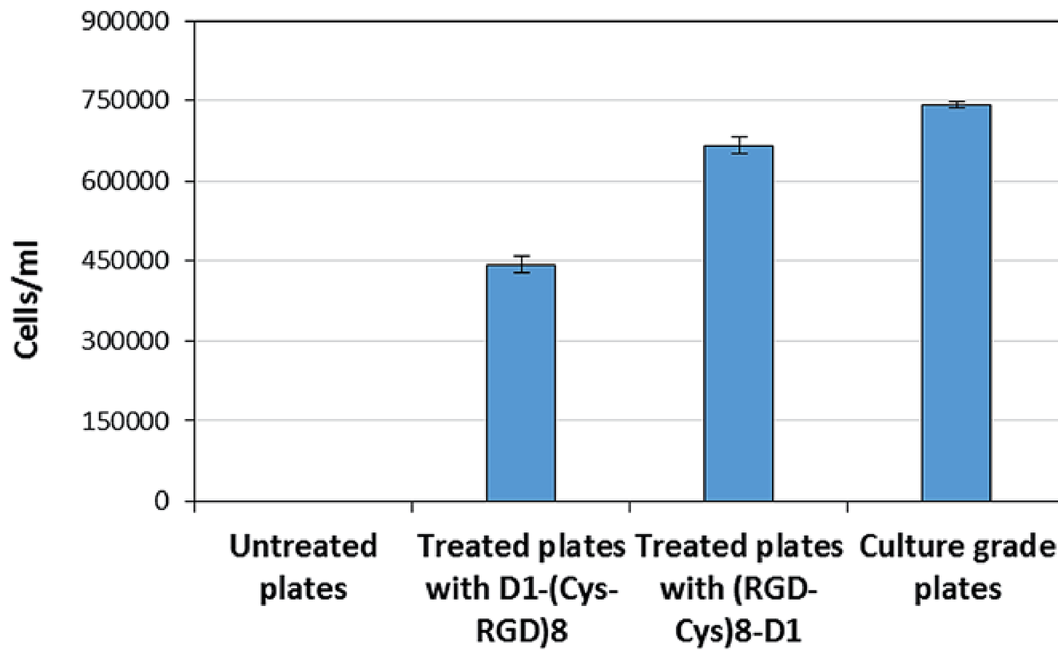

Fig. 3 The number of cells per milliliter varied significantly among the different conditions tested. (RGD-Cys)$_8$–D1 shows higher number of cells per mL due to a sensitive high recognition of cell–peptide to RG-D–dendrimer in comparison with D1–(Cys-RGD)$_8$.

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