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Discovery of Specific Ligands for Oral Squamous Carcinoma to Develop Anti-cancer Drug Loaded Precise Targeting Nanotherapeutics

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Abstract

Oral squamous cell carcinoma (OSCC) has a low five-year survival rate, and mostly due to late detection and a lack of effective tumor specific therapies. Using a high throughput drug discovery strategy termed one-bead one-compound (OBOC) combinatorial library, we identified six compounds with high binding affinity to different human OSCC cell lines but not to normal cells. Current work is under way to develop these ligands to OSCC specific imaging probes or therapeutic agents.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the biggest causes of morbidity worldwide with a low five-year survival rate that may be due to late detection and an absence of effective tumor specific therapies.[1,2] Therefore, the development of early-detection techniques and innovative therapies are greatly needed.

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Conflict of Interest Disclosure: **NONE**

The one-bead one-compound (OBOC) combinatorial library method has been applied to discover ligands against a number of biological targets, such as cell protein kinase substrates and inhibitors, protease substrates and inhibitors, cell surface receptor, artificial enzymes, and various ligands for the preparation of affinity column media.[3-7] In this study, the authors employed OBOC combinatorial library technology to look for the ligands which can bind OSCC cells with high-binding affinity and specificity.

Material and Methods

Cells

Normal and tumor cell lines were obtained from American Type Culture Collection, except as otherwise described. Normal human keratinocytes were gifted from Dr. Fong Tong Liu of the Department of Dermatology, University of California Davis Medical Center. The authors prepared normal peripheral white blood cells using the Ficoll-Paque gradient method from peripheral blood of a healthy donor.

Synthesis of the Initial and Focused OBOC Libraries

The authors generated the OBOC libraries on TentaGel S NH2 resin (Rapp Polymere Bmbh) using a "split-mix synthesis" approach as previously reported.[3] Standard solid-phase peptide synthesis techniques with 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and *N*-hydroxybenzotriazole (HOBt)/*N*, *N*'-diisopropylcarbodiimide (DIC) coupling were used in the construction of the OBOC libraries. The completion of each coupling step was determined with a ninhydrin test.

Generation of Rainbow Beads

Individual OBOC libraries were labeled with oil-based organic dyes that are used as the coding tags.[8] In this "rainbow beads" method, saturated solution of organic dyes, such as Sudan black B, Sudan blue 2, scarlet red, disperse yellow, and purpurin were prepared in DMF and mixed with individual libraries for one hour and washed with hexane, water, and PBS prior to screening with live OSCC cells (**Figure 1**).

Preliminary Screening With Color-Coded OBOC Libraries

OSCC3 cells were grown in DMEM media with antibiotics and 10 percent FBS. After reaching 80 percent confluence, cells were harvested by trypsin. Small samples of each color-coded OBOC libraries were then combined and screened concurrently against live OSCC cells for three hours in 5 percent CO_2 at 50 rpm. The preferred libraries with OSCC cell binding were readily identified with the color code and selected for subsequent large-scale screenings for cell surface binding ligands.

Large-Scale Cell-Based Screening with selected OBOC libraries

The six preferred OBOC libraries selected from preliminary screening were screened in a large scale (about 750,000 beads/each OBOC library) against live OSCC cells. The compound beads with a full-coating of OSCC cells were selected and treated with 8 M guanidine hydrochloride to remove the binding cells and adsorbed proteins. The OSCC cell-

binding beads were then reincubated with OSCC cells to confirm their binding ability. The OSCC binding beads were treated with 8 M guanidine hydrochloride again and screened with normal human keratinocytes (NHK) for eight hours in Epelife media at 37 degrees Celsius and 50 rpm. The OSCC binding beads that did not bind to NHK were picked up. The chemical structures and sequences of the selected compound beads were determined by an automatic micro-sequencer as previously described.[3]

Evaluation of OSCC Binding Compounds' Affinity and Specificity

The OSCC binding compounds discovered from above study were resynthesized on TentaGel beads and evaluated with five different human OSCC cell lines, including OSCC 3, OSCC 4, OSCC 10A, OSCC HOK313, and OSCC MOK 101 for one hour in Epelife media and inspected under the microscope. The compounds were also challenged with normal human keratinocytes, fibroblast cells, endothelial cells, and while blood cells for eight hours in Epelife media at 37 degrees Celsius, 50 rpm, and inspected under the microscope.

Hemolytic Assay

The standard hemolytic assay was used to evaluate the potential toxicity of the compounds identified.[9] Briefly, the compounds were synthesized in solution form and purified on C18 reversed-phase high-pressure liquid chromatography . The compound structures were confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The compounds were serially diluted in sterile saline (0.9 percent) and incubated with healthy human RBC ($4x10^6$ RBC/ml) for six hours and absorbance was read by microplate reader at 550 nm. Triton X-100 1 percent was served as 100 percent hemolytic positive control. RBC without added compounds was used as the negative control.

Results

After preliminary screening 24 OBOC libraries, six libraries were selected for further studies for having positive binding beads to OSCC, including one cyclic focused X1 OBOC library, one 7-mer RGD based cyclic OBOC library and four random linear and cyclic OBOC libraries. As shown in **Figure 2**, a binding bead showed a layer of cells binding on the bead from a blue color-coded OBOC library (arrow). As a result, the blue color-coded OBOC library in this screening was selected for the large-scale screening. A total of 137 OSCC cell-binding beads were identified from X1 OBOC library (~750,000 beads). As shown in **Figure 3**, only the compound beads with a full coating of OSCC cells were picked up (arrow). The 137 OSCC cell binding beads were then screened with normal human keratinocytes. As shown in **Figure 4**, some OSCC binding beads have more keratinocyte binding while some have less NHK cell binding, and one nonbinding bead was identified (arrow). Thirteen compound beads were finally identified for their strong binding to OSCC cells but not binding to normal human keratinocytes. The chemical structures of the 13 compounds were determined by Edman chemistry. Even with the small number, a specific motif was observed. The authors have not identified a specific OSCC cell-binding compound from the other five OBOC libraries.

After resynthesis of 13 peptides on a large numbers of beads, the authors evaluated these compounds' binding property using five different human OSCC cell lines in Epelife media. **Figure 5** shows that peptide LYL13 was completely coated with a monolayer of OSCC cells after one-hour incubation. The 13 peptides were also challenged with normal human cells for eight hours. As shown in **Figure 6**, some compounds do not bind to the following normal human cells including human keratinocytes (**6a**), fibroblast cells (**6b**), endothelial cell (**6c**) and white blood cells (**6d**). The screening results of 13 compounds against OSCC, as well as normal human cells are summarized in **Table 1**. Six compounds were selected for further studies for their strong binding ability to OSCC cells but not bind to normal human cells (highlighted red). No cytotoxicity was observed to human blood cells for any of the six compounds up to the concentration of 640 ug/ml.

Discussion

Combinatorial chemistry is one of the most important approaches in medicinal chemistry to identify the specific targets on live tumor cells. Combinatorial libraries can be created using the synthetic chemistry by joining a large number of compounds ranging from natural and synthetic small molecules randomly with various building blocks. Through screening diverse initial and focused OBOC peptidomimetic libraries, the authors identified six compounds with high-affinity binding to different human OSCC cells lines but showed no binding to normal human keratinocyte, fibroblast cells, endothelial cells, and granulocytes. These six compounds were composed of natural and synthetic amino acids; therefore, they might resist proteolysis *in vivo*. A motif was observed among the six compounds. Given that these six compounds are not toxic to human red blood cells, and not binding to human white blood cells, they are safe to be as the candidates for the further study.

X1 OBOC library from which the authors discovered six OSCC binding compounds is the focused library based on a cyclic peptide motif to bind preferentially to ovarian cancer with high specificity against α3 integrin, therefore, it was expected these six OSCC binding ligands involved α3 integrin interactions.[10] However, to characterize and confirm the integrins involved, the authors needed to conduct cell adhesion-blocking assay utilizing a saturated amount of control mouse IgG or anti-human integrin adhesion-blocking monoclonal antibodies to validate the ligand and receptor interaction.

OSCC are derived from the epithelial lining of the oral mucosa. Given the fact the oral cavity is easily accessible, the disease should be detected earlier. Current diagnostic techniques involve light-based detection systems, fluorescence visualization and brush cytology.[11,12] Unfortunately, only 35 percent of cases are caught early in time. The ligands discovered from the authors' study demonstrated clearly that they bind strongly to OSCC cells but do not bind to normal keratinocytes in this *in vitro* study. Work is under the way to evaluate these ligands with pre-OSCC lesions (dysplastic) or benign lesions (ulcer). If these ligands are not specific enough, the authors will generate more focused OBOC libraries to search for the highly specific ligands for OSCC, with the OSCC cell-binding motif fixed or biased while other non-essential positions containing a large number of natural and unnatural amino acids or amino acid derivatives. The specific OSCC-binding ligands can be biotinylated and complexed with streptavidin conjugated-Q-dot (or organic fluorophor) for

cell staining and flow cytometry analysis. The most specific and high-affinity ligands might be used as the chairside primary OSCC screening tool. In addition, these florescent conjugates can be used as the probes for *in situ* detection of OSCC in the clinics as well.

The most specific and high-affinity ligands for OSCC can also be used to develop the anti-cancer drug-loaded in precise-targeting nanotherapeutics. Recently, Dr. Lam's lab developed several novel nanocarriers for the delivery of paclitaxel (PTX) or other hydrophobic anti-cancer drugs.[13-17] The PTX-loaded and targeting ligand decorated nanoparticles (PEG5k-Cys4-CA8) exhibit superior anti-tumor efficacy and lower systemic toxicity profile in nude mice bearing ovarian cancer tumor xenografts when compared with equivalent doses of nontargeted PTX nanoparticles, as well as clinical PTX formulation (Taxol®).[18] Specific OSCC-binding ligands discovered in the authors' study will be conjugated to PEG5k-Cys4-CA8 nanoparticles with loading of anti-OSCC drugs to study their precise targeting and treatment effect *in vitro* and *in vivo* studies.

This project involves the identification of OSCC-specific ligands to develop more efficacious and less toxic imaging agents and nanotherapeutics for oral squamous carcinoma's earlier diagnosis and potential treatment alternative. If proven successful in animal models, the new technology can be translated into novel and effective therapeutic agents for human oral carcinoma. As a result, we expect patients with refractory oral carcinoma will benefit from such novel nanotherapies.

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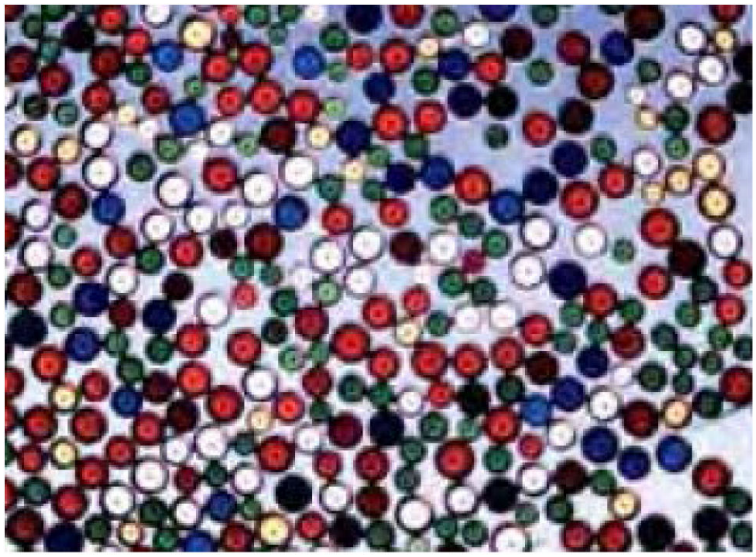


Figure 1.
Photomicrograph of rainbow beads generated using organic dyes; one color denotes one OBOC library.

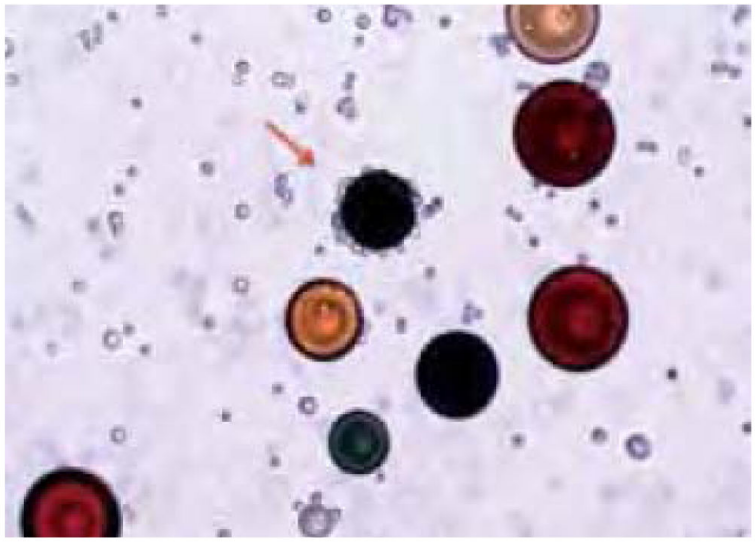


Figure 2.
Photomicrograph of screening with mixture of color-coded OBOC libraries. A binding bead was identified from the blue color-coded OBOC library (arrow).

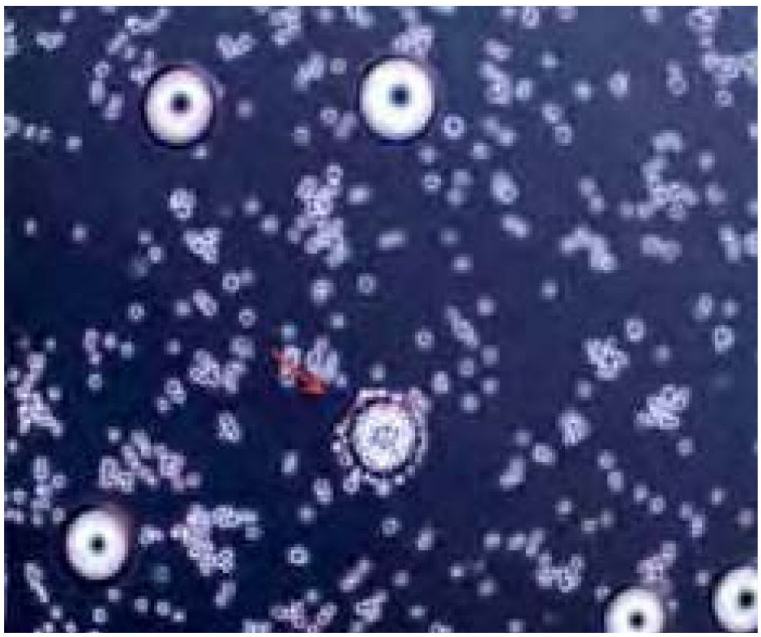


Figure 3.
Photomicrograph of the OSCC cell-binding bead identified from X1 OBOC library (arrow).

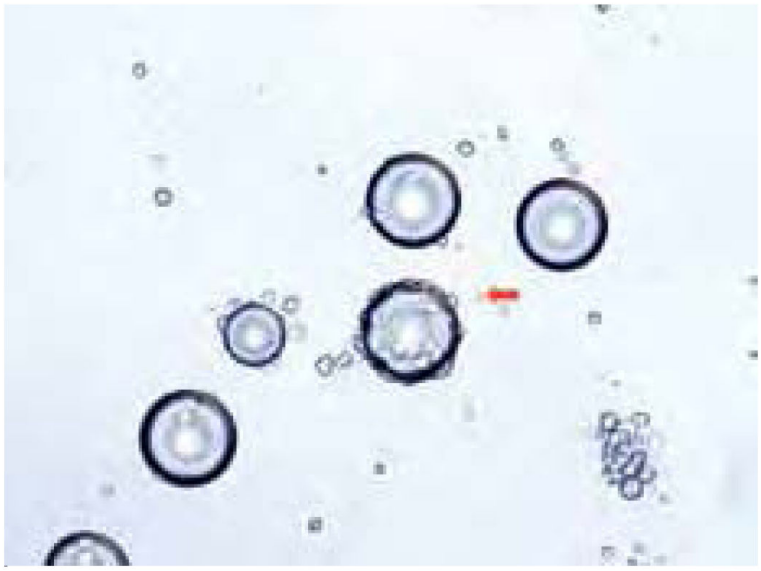


Figure 4.
Photomicrograph of a non-NKH-binding bead was identified from OSCC-binding beads (arrow).

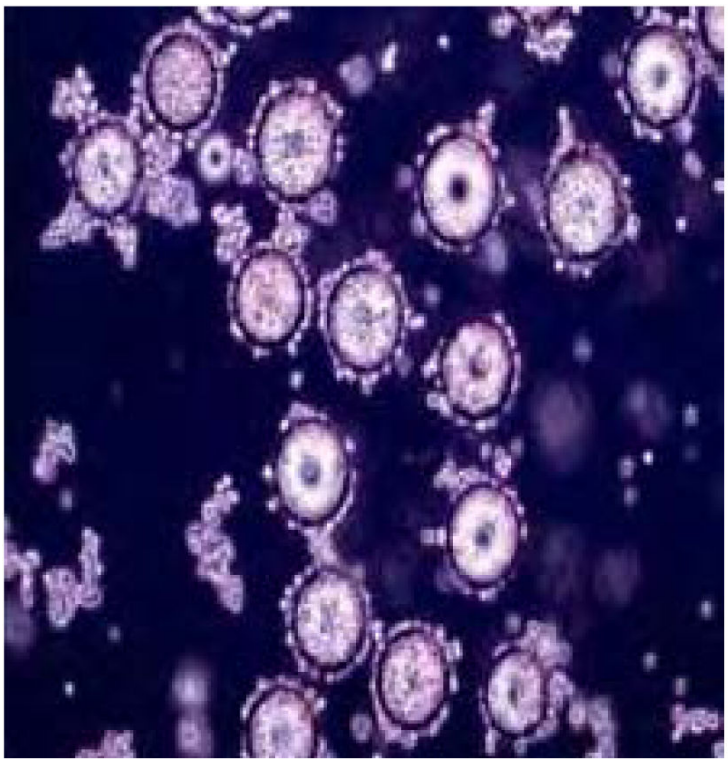
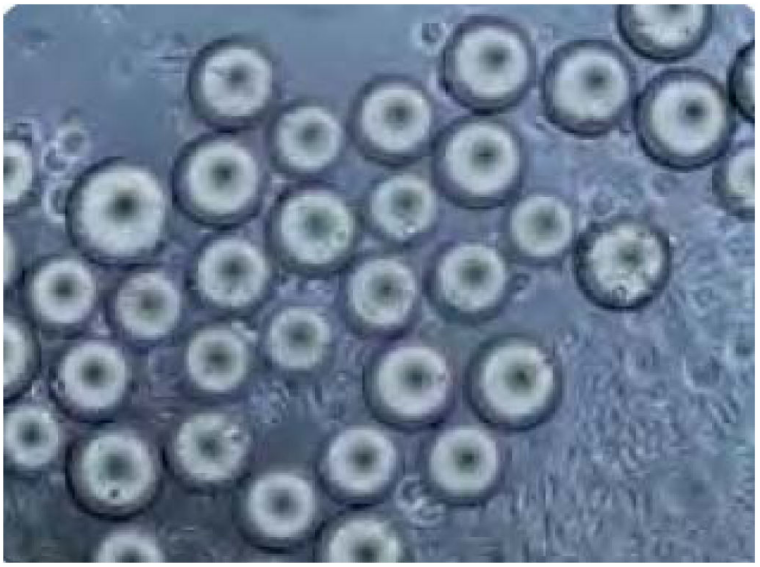
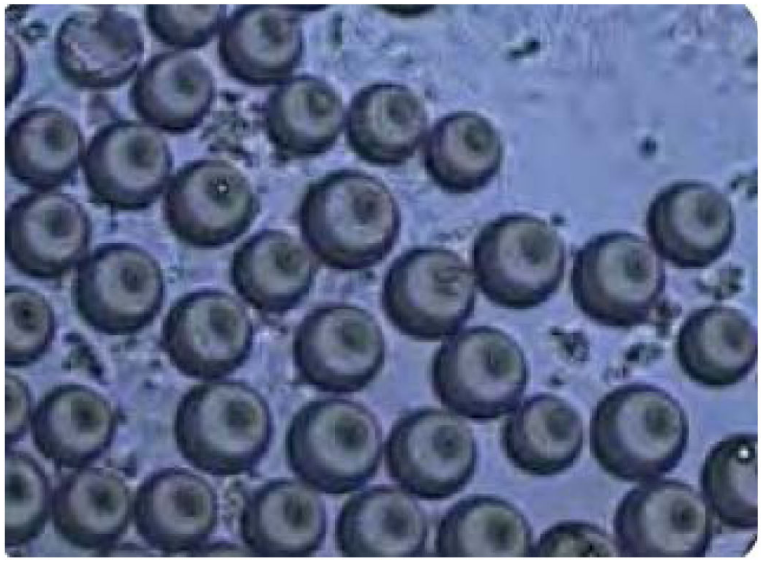


Figure 5.
Photomicrograph of live OSCC cells bind to LYL 13 compound beads after one-hour incubation.





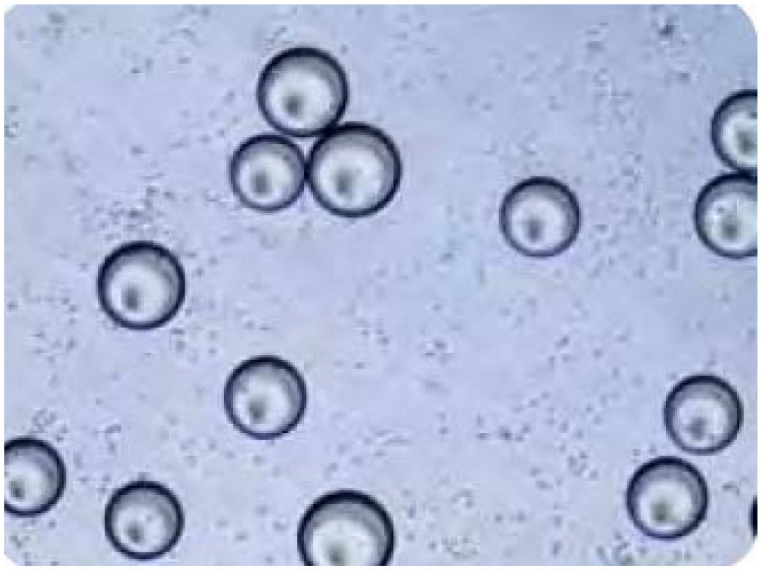


Figure 6.

Photomicrograph of OSCC-binding compound beads challenged with normal human cells. After incubation of OSCC-binding beads with human normal cells of NHK (6a), fibroblast cell (6b), endothelial cells (6c), and WBC (6d) for eight hours, six of the OSCC-binding compounds were identified for not binding to these normal human cells. Some of cells attached to the surfaces of a Petri dish with time (6a, 6b, and 6c).

Table 1

Screening results of 13 compounds (LYL1-LYL13) with OSCC cell lines and normal human cells

Cell name	LYL1	LYL2	LYL3	LYL4	LYL5	LYL6	LYL7	LYL8	LYL9	LYL10	LYL11	LYL12	LYL13
OSCC3	4+	4+	1+	4+	4+	−	4+	4+	4+	+	1+	4+	4+
OSCC4	4+	4+	3+	4+	4+	−	4+	4+	3+	−	4+	3+	3+
OSCC10A	4+	3+	1+	4+	3+	−	2+	4+	3+	−	1+	3+	4+
OSCCHok313	4+	1+	1+	−	4+	2+	2+	2+	2+	−	2+	4+	4+
OSCCMok101	2+	±	−	+	±	−	±	+	2+	−	±	±	2+
NHK	−	±	−	+	−	+	+	−	2+	−	−	−	±
HUVEC	−	−	−	−	−	−	−	−	−	−	−	−	−
NUFF	−	−	−	−	−	−	−	−	+	−	−	−	+
WBC	−	−	−	−	−	−	−	−	−	−	−	−	−

* OSCC: oral squamous carcinoma cell line; NHK: human Karatinocyte; HUVEK: human endothelial cell; NUFF: human fibroblast cells; WBC: human granulocytes.

* LYL1 to LYL13 are the names of OSCC binding compounds.

* 4+: bead full coated with cells; 3+: bead 3/4 coated with cells; 2+: bead 2/4 coated with cells; 1+: bead 1/4 coated with cells; and ±: only fewer beads have cell binding.