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Direct *in vitro* selection of titanium-binding epidermal growth factor

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ABSTRACT

Epidermal growth factor (EGF) with affinity to TiO_2 surfaces was obtained by direct *in vitro* selection. A random peptide library was generated for fusion to the N-terminal of EGF, and polypeptides exhibiting affinity were selected *in vitro* by ribosome display. The best-performing polypeptide sequence was selected for synthesis using a solid-phase method and showed high affinity to TiO_2 after refolding. Molecular dynamic simulations indicated that the interaction of the selected peptide segment with the TiO_2 surface was comparable to that of a previously reported titanium-binding peptide, TBP-1. The hydroxyl groups in the selected peptide segment were found to be critical for the binding interaction. NIH3T3 cell culture for two days in the presence of the TiO_2-binding EGF showed that it was able to enhance cell proliferation as much as unmodified EGF in solution. As a result, the selected EGF construct was able to induce cell proliferation on titanium surfaces. This direct *in vitro* selection technique should extend the possibilities for the design of other surface-binding growth factors.

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1. Introduction

Metals such as titanium and titanium alloy are widely used in medicine because of their biocompatibility, nontoxicity, good mechanical properties, and excellent resistance to corrosion [1]. However, the methods for surface modification of metal with biological molecules are limited, yet the surface properties are critical for establishing the response of tissues to biomaterials and for acting as powerful signals for cell growth and differentiation. Conjugating growth factors or extracellular matrix to metal is expected to provide a source of signals that is continuous and stable, thereby efficiently stimulating cells to reconstitute damaged tissues during long-term regeneration—as has been reported for organic materials [2–16].

To biologically modify metal surfaces, the first step is typically the formation of an organic layer on the metal surface to introduce functional groups for binding to biological molecules. In addition to the well-established silane-coupling method, many other methods have recently been developed involving electrodeposition, photoimmobilization, and chemical treatment with molecules containing phosphate groups or catechol-related groups (dopamine or polydopamine) [17–25]. Preparation of biological molecules with increased binding affinity to inorganic materials has also been attempted using, for example, the recombinant insertion of a known binding motif [26,27]. However, biological molecules with natural affinity to inorganic molecules are rare. Instead, non-canonical amino acids, such as phosphoserine and 3,4-dihydxylphenylalanine, which are produced by post-translational modification, typically need to be incorporated as these show the greatest affinity.

We have developed a new, high affinity growth factor composed of canonical amino acids only, using *in vitro* selection of peptides exhibiting binding to inorganic substrates. To date, phage display has been the predominant method employed for the selection of peptides with affinity to inorganic substrates such as $BaTiO_3$ (for electronic applications), as well as SiO_2, TiO_2, aluminum, steel, semiconductor materials, platinum, silver and hydroxyapatite [8,28]. Typically, phage display technologies introduce a combinatorial library (to the order of 10^9 sequences) of 7-mer or 12-mer peptide sequences with affinity to a molecule, ligand or material. This technique has been used as a strategy to identify amino acid sequences with potential for creating biological bridges at the molecular level, between synthetic materials and biomolecules of interest.

Kashiwagi et al. fused BMP2 to an oligopeptide aptamer sequence to generate a peptide with affinity to titanium (Ti-binding

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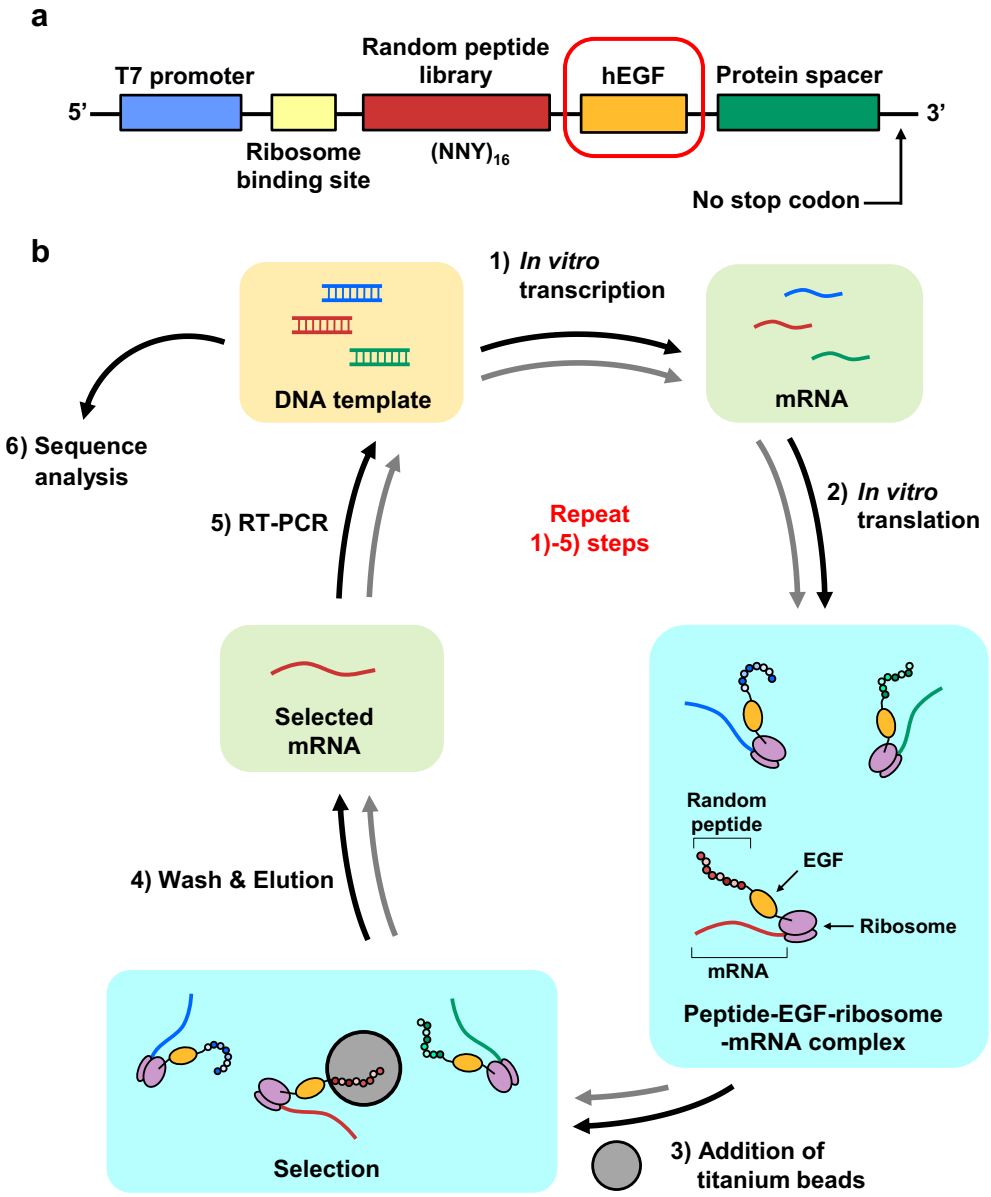

Fig. 1. Direct *in vitro* selection of titanium-binding EGF. (a) Construct. Each DNA template contained a T7 promoter, ribosome binding site, random peptide, human EGF and a protein spacer. (b) Protocol. The DNA templates were transcribed (1) and translated (2), and the resulting ribosome–polypeptide–mRNA complexes were then produced for ribosome display. TiO_2-binding sequences were selected using TiO_2-coated beads (3), bead-bound mRNA was eluted (4) and then converted into a DNA template by RT-PCR (5). This cycle was repeated five times and the final selected sequences were used for further analysis.

peptide-1, TBP-1) [29]. An optimal peptide aptamer sequence was selected using phage display [30]. The fusion of the aptamer sequence allowed the reversible binding of BMP2 to titanium, with retention of its biological activity. However, they also found that fusion of the BMP2 protein to the peptide reduced its affinity to the TiO_2 surface, which may have been the result of interference from intramolecular interactions between the binding peptide region and the BMP moiety.

To avoid this problem, we constructed a new and direct *in vitro* selection system consisting of a random peptide library fused to the epidermal growth factor (EGF) sequence, to generate a TiO_2-binding EGF. In this system, DNA sequences encoding both random sequence peptides and EGF were subjected to the selection system, rather than the peptides alone. Through this selection method, we expected to enhance affinity to TiO_2 surfaces by incorporating the effect of intramolecular interactions between the growth factor and the binding protein. In addition, we employed ribosome display for the selection [31–35] instead of phage display. Phage display has several drawbacks, such as limited sequence diversity and steric hindrance of phages. Although the T7 phage display system was developed to overcome these problems, the use of a non-biological display system provides more diversity (to the order of $10^{12–13}$ sequences) and there are no limitations resulting from toxicity. We therefore used ribosome display to take advantage of the significant increases in sequence diversity of the random peptide library that this method generates.

2. Materials and methods

2.1. Construction of DNA template for in vitro selection

For the preparation of the DNA template used in ribosome display selection, an original plasmid, 13EGFB, carrying the T7 promoter sequence, the Shine–Dalgarno sequence, *Sfi*I restriction enzyme recognition sites, a truncated human EGF sequence

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