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Peptide aptamers against titanium-based implants identified through phage display

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Abstract Commercially pure titanium (cp-Ti) is widely used in the field of long-term clinical oral implantology owing to its ability to allow close bone-implant apposition. The optimization of its function based on artificial proteins has become a key issue in the development of improved cp-Ti implants. Here, we set out to identify peptide aptamers with preferential adsorption towards titanium-based implants through the phage display methodology. Fifteen sequences were selected in the third round of biopanning. One sequence, ATWVSPY (named TBP1), had a 40% repetition rate and exhibited the strongest binding affinity to cp-Ti disks. Ten sequences were selected in the fourth round, among which the repetition rate is 80% for TBP1 and 20% for TBP2 (GVGLPHT). The peptide aptamers against cp-Ti disks can provide an alternative method of functional coating for biomaterial surfaces.

1 Introduction

Titanium is a popular biomaterial for implanted medical devices, artificial joints and cardiacstents due to its good biocompatibility, resistance to corrosion and low allergenicity. Despite these superior properties, there is still a need to improve titanium devices [1]. The biological properties of cp-Ti dental implants can be improved by surface treatments, such as plasma flame spraying, sandblasting, acid-etching, surface machining and anodic oxidation [2]. In addition to these physical and chemical methods, modification using artificial proteins which contain a set of inorganics-binding peptides has also been tried to improve the osteointegration of titanium-based implants [1, 3].

At the micro-and nanometer level, the interface between the implant and the bone tissues is characterized by the presence of an intervening extracellular matrix layer with an irregular appearance and thickness. The main components of the matrix layer include some macromolecules such as proteoglycan/glycosaminoglycans which may serve some bridging function between the implant and bone tissues [4]. Advanced strategies focus mainly on the anchorage of bone matrix components to the material surface and on the delivery of osteogenic signaling molecules to enhance periimplant bone regeneration. Biologically active components are immobilized through a variety of procedures such as adsorption, covalent coupling, electrochemical surface modifications and self organized organic layers on the implant surface [5]. The binding motifs found in crystal structures of the matrix layer around the implant have been successfully mimicked with simple peptides, i.e., RGD (present in bone sialoprotein) binds to integrin [6]; E7 (present in osteocalcin) binds to HA [7]. These biomimetic peptides, however, lack binding specificity to implants and exhibit limited biological activity [8]. In the new field of molecular biomimetics, inorganics-binding peptides and proteins are selected and designed at the molecular level through phage display methodology [9]. Using a peptide-phage system, TBP-1, a 12-amino-acid peptide aptamer (RKLPDAPGMHTW), has been isolated as a TiO_2 particle binder. Subsequent analysis has shown that its N-terminal hexapeptide, RKLPDA (minTBP-1), is sufficient for TiO_2 binding [10, 11]. Different from TiO_2 particles, cp-Ti disk has been widely used for in vitro studies because its surface characteristics are similar to

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clinical dental implants. In this study, the identification of peptide aptamers against cp-Ti disks was undertaken through a linear heptamer library to design functional peptides which preferentially adsorb to titanium-based implants.

2 Materials and methods

2.1 Isolation of cp-Ti binding peptides

Peptide sequences with preferential binding to titanium-based implants were identified by screening the Ph.D.—7^{TM} Phage Display Library (New England Biolabs, #E8100SC), consisting of $\sim 2.8 \times 10^9$ different phages with 7-mer amino acid linear peptide inserts, against cp-Ti disks. Cp-Ti circular disks with 10 mm diameter and 2 mm thickness (Baoji Nonferrous Metal Industry Co., China) were sequentially wet-polished with silicon carbide (SiC) paper to a final finish of 1000 grit, ultrasonically cleaned in ddH_2O for 15 min and oxidized with 30% HNO_3 solution for 30 min. Thereafter, disks were thoroughly degreased, washed and sonicated in a series of solvents (ethanol, acetone and chloroform for 15 min each) and dried with a stream of nitrogen gas (99.9%). All samples were sterilized under ultraviolet light for 30 min and soaked in ddH_2O overnight before use [12]. The ddH_2O was removed and replaced with blocking buffer (Tris–HCl buffer solution (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) with 0.1 or 0.5% Tween20 (TBST) and bovine serum albumin (BSA)) and incubated at 4°**C** for 1 h. The blocking buffer was discarded and the disks were washed six times with TBST (0.1%). An aliquot of the original Ph.D.—7^{TM} library containing 10^{11} pfu (plaque-forming units, input) diluted in 100 µl of TBST (0.1%) was introduced to each substrate and gently rocked for 1 h at room temperature. Non-binding phages were discarded and the disks were washed ten times with TBST (0.1%) to remove the nonspecifically or weakly bound phages. The phages remaining on the disk surfaces were then eluted with 0.2 M Glycine/HCl (pH 2.2), containing 1 mg/ml BSA for 10 min at room temperature under gentle agitation. The eluted phages were transferred to a new sterile tube and neutralized with 1 M Tris–HCl (pH 9.1). The number of eluted phages (output) was estimated by infecting *Escherichia coli* (*E. coli*) strain ER2738. Eluted phages were then re-amplified with early log phase *E. coli* ER2738. The amplified phages were isolated by PEG/NaCl (20 w/v% polyethylene glycol-8000 in 2.5 M NaCl) precipitation and used for further biopanning rounds according to the manufacturer's protocols. The similar panning procedures were repeated three more times. The third and fourth panning were different from the earlier two as 0.5% Tween20 was used. Tittering was carried out for both the eluted phages and amplified phages after each round of panning to determine the number of phages. The phages obtained from the third and fourth round were plated on LB agar media containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) and isopropyl-D-thiogalactopyranosid (IPTG) in appropriate serial dilutions to identify correct plaques. Because the ER2738 strain lacks the lacZ α gene, only the cells infected by M13mp 19 bacteriophage,which carries the lacZ α gene, can produce competent-galactosidase. Therefore, infected cells can hydrolyze X-gal and form blue phage plaques [13, 14].

Table 1 The sequences of peptide aptamers against cp-Ti selected from the third and fourth round of biopanning

Round	Sample code	Sequence	Repetition rate (%)
3 ($n = 15$)	Ti-7-3-1	ATWVSPY	40
	Ti-7-3-2	AHSMGTG	6.7
	Ti-7-3-3	FSSQMRY	6.7
	Ti-7-3-4	GVGLPHT	6.7
	Ti-7-3-5	QIEPLAL	6.7
	Ti-7-3-6	RIVLPTY	6.7
	Ti-7-3-7	VQQVALL	6.7
	Ti-7-3-8	IVLPVPY	6.7
	Ti-7-3-9	GHWTRLA	6.7
	Ti-7-3-10	NLPLHST	6.7
4 ($n = 10$)	Ti-7-4-1	ATWVSPY	80
	Ti-7-4-2	GVGLPHT	20

2.2 Sequence analysis

After growing the phages on LB-agar plates, individual blue plaques containing monoclonal phage particles were picked from plates with less than ~ 100 plaques and amplified by infecting 1 ml early-log culture. The culture was centrifuged and 800 µl of the supernatant was precipitated with PEG/NaCl solution. Single stranded DNA was isolated by iodide buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 4 M NaI), cleaned in 70% ethanol, and finally resuspended in DNase/RNase free water. The purified DNA samples were sequenced by Invitrogen Biotech. Co. Ltd (Shanghai, China) with the NEB-96 gIII sequencing primer (5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′) provided in the kit. The amino acid sequence alignments were performed with Align-Version 1.02 (Scientific & Educational Software) [13] (Table 1).

2.3 Binding assay for cloned phages

The remaining 200 µl supernatant in DNA sequence was amplified in 20 ml LB Medium with vigorous shaking for

4.5 h at 37°C and then centrifuged for 10 min at 10,000 rpm at 4°C. The supernatant was transferred to a fresh tube and precipitated with PEG/NaCl solution. Thereafter, the pellet was suspended in TBS buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) and the similar procedure of PEG precipitation was repeated again. A small amount of the amplified phage eluate was titered and used as the input of binding analysis for selected clones [13]. The binding affinity of the identified phage was estimated in a way similar to the biopanning experiments, except that we used 0.5% Tween20 and an incubation time of 0.5 h. The binding efficiency was expressed as the ratio of the output phage number to the input phage number [11]. We used a cloned phage randomly selected from the original library (Ph.D.—7^{TM} Phage Display Library) as a putative negative control.

2.4 Characterization of phage binding to cp-Ti disks with laser confocal fluorescence microscopy (LCFM)

The disks were incubated overnight at 4°C with the targeted monoclonal phages at the concentration of 10^{11} pfu prepared in TBST (0.1%). A putative weak-binding phage randomly selected from the original library (named as Original) as well as 10 mM PBS buffer (pH7.4) were used as negative control and blank control, respectively. The non-binding phages were removed by washing ten times with TBST (0.1%). The phages that remained bound to the disks were labeled with mouse Anti-M13 IgG (GE Healthcare, #27-9420-01) and fluorescein-conjugated immunopure goat anti-mouse IgG (Pierce, #31569) at room temperature for 1 h. After the labeling, the substrates were washed three times with PBST (10 mM PBS (pH 7.4) with 0.05% Tween20) and mounted in 90% glycerol diluted by 10 mM PBS (pH 7.4). Following this procedure, the phages were then visualized by a TE 300L microscope (Nikon, Japan) at a 40× magnification. Approximate surface coverage of the phage particles was calculated using Image–Pro Plus 6.0 software (Media Cyberhetics Inc., USA) by comparing the approximate phage coverage in the fluorescence image to the approximate surface area of the cp-Ti disk in the dark field image. Phage clones were categorized as strong, moderate, and weak binders according to their surface coverage. The calculation was repeated twice, with the error range of ±0.05 [14].

2.5 HNO_3 treatment

The isolated monoclonal phage that had the strongest affinity to cp-Ti disks was used in this experiment. Cp-Ti disks without any treatment, incubated in 30% HNO_3 solution for 30 min, or 65% HNO_3 for 1 h at room temperature received phage binding tests, respectively. The binding affinity was assessed by output/input efficiency.

3 Results and discussion

Research on the binding of polypeptides to inorganic materials has drawn increasing attentions in recent years. The phage-peptide library used in this study was based on random heptapeptides fused to the minor coat protein (pIII) of the M13mp19 bacteriophage. We named the peptide sequence Ti-7-3-1 and the cloned phage displaying it ¢Ti-7-3-1. After three rounds of panning, the output/input ratio (6×10^{-6}) was high enough for us to clone and sequence 15 phages, among which six displayed the same peptide sequence as Ti-7-3-1 (ATWVSPY, named TBP1). The output/input (5.6×10^{-8}) ratio decreased dramatically in the forth panning (Fig. 1). Ten clones were sequenced, among which eight displayed the same peptide sequence as Ti-7-3-1 and the rest two displayed the same as Ti-7-3-4 (GVGLPHT, named TBP2). According to the binding analysis based on output/input efficiency, ¢Ti-7-3-1 showed the strongest binding force at a density of 7.2×10^6 pfu/ml, whereas the phage in the control group at a density of 3×10^5 pfu/ml (Fig. 2). The strongest binding clone (¢Ti-7-3-1), moderate binding clone (¢Ti-7-3-4), putative weak-binding clone (Original) and 10 mM PBS (pH7.4) containing no clones (blank control) were used for subsequent LCFM analysis. From the fluorescence images, a large number of phages both in TBP1 and TBP2 groups bound on cp-Ti disks. But there was nearly no phages in the PBS control group which just had some background staining. From the semi-quantitative measurement based on surface coverage, ¢Ti-7-3-1 was characterized as the strongest binder with a surface coverage of 46.04%, ¢Ti-7-3-4 was the moderate binder with 27.56% coverage, the Original was the weak binder with a coverage lower than 1% (Fig. 3). These results further verified the binding analysis results based on output/input efficiency.

Phage display is an approach commonly utilized in isolating peptide aptamers against solid inorganic materials. Using this technology, short amino acid sequences (typically 7–15) have been selected and isolated that are specific to various inorganic materials including noble

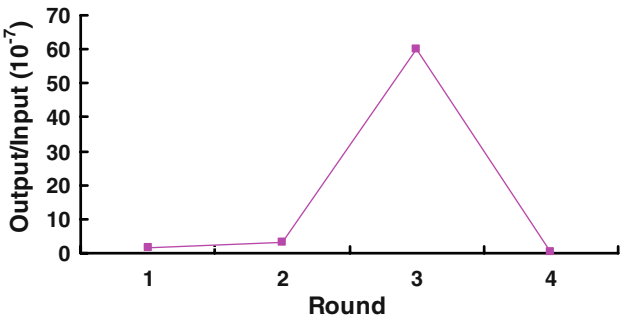

Fig. 1 The output/input ratio in four rounds of biopanning

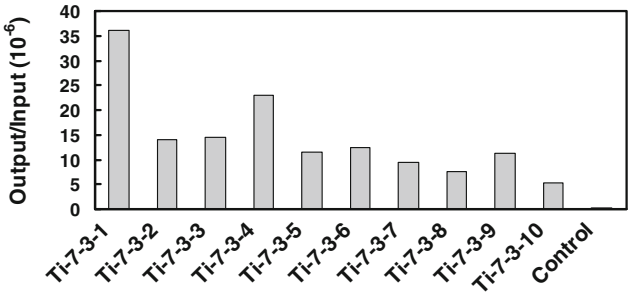

Fig. 2 Binding affinity of cloned phages to cp-Ti disks assessed by output/input efficiency

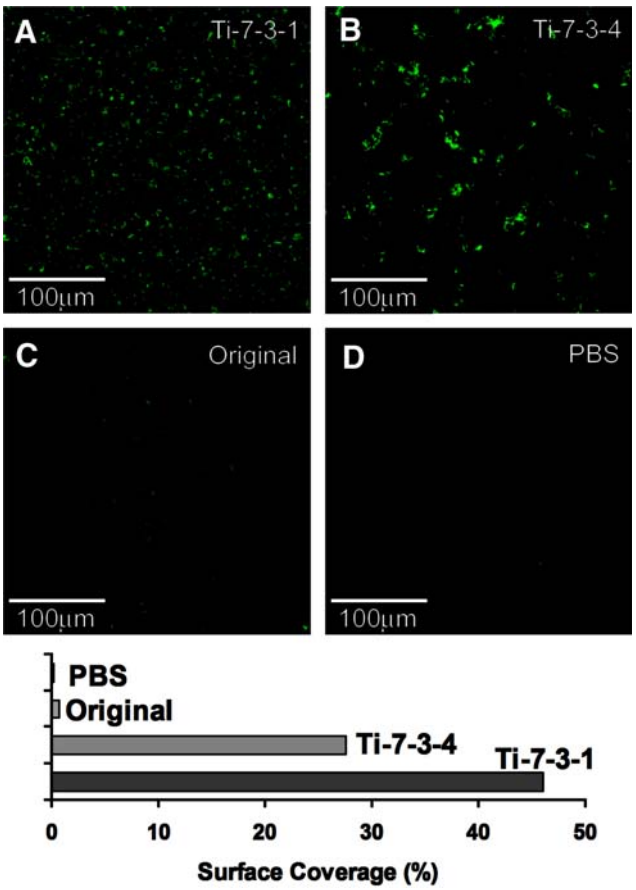

Fig. 3 Immunofluorescent images (*upper*) and surface coverage percentages (*lower*) of phage clone of ¢Ti-7-3-1, ¢Ti-7-3-4 on surfaces of cp-Ti disks. Monoclonal phage from the original library (Original) and PBS were served as negative control and blank control, respectively

metals, semiconductors, minerals and oxides including TiO_2 [15–18]. A peptide binder against TiO_2 particles, RKLPDA (MinTBP-1) had been isolated from Ph.D.-12 phage display peptide library in previous studies [11]. Different from previous studies, we selected peptide binders from cp-Ti disks directly, which have similar surface characteristics as actual dental implants. A heptapeptide motif, ATWVSPY, was successfully screened against

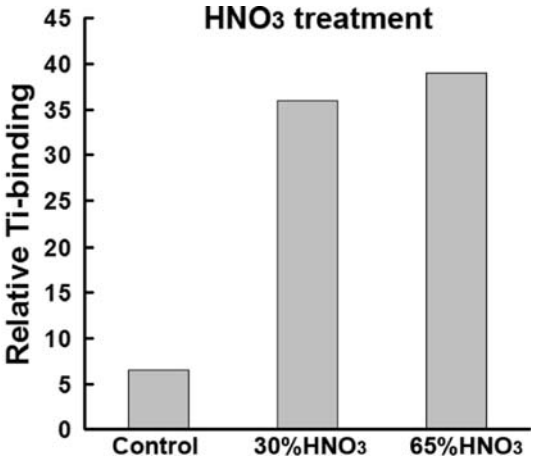

Fig. 4 Effect of HNO_3 treatment on phage-Ti interaction. The binding affinity was determined by output/input efficiency

cp-Ti disks and showed the strongest binding affinity. It might provide a new approach for surface molecular modification for cp-Ti implants.

As for the effect of HNO_3 treatment on phage binding, it was found that the binding affinity increased obviously after treating cp-Ti disks with 30% HNO_3 for 30 min in contrast to the non-oxidized cp-Ti, which is similar to the results of Sano [11]. While there was no significant difference between the 30% HNO_3 group and the 65% HNO_3 group (Fig. 4). It is known that a native thin oxide film about 5–10 nm thick, composed of amorphous and non-stoichiometric titanium dioxide (TiO_2) would form on the polished surfaces of cp-Ti disks [18, 19]. The good biocompatibility and osteointegrability of titanium may be associated with the native oxide layer which may favor the positive response of the tissue to titanium implants under certain conditions [20]. Nitric acid (HNO_3) is a strong oxidizer, which is commonly used in passivating titanium implants. When titanium is immersed in 20–40% HNO_3 for at least 30 min at room temperature, the oxide film thickness in the surface of titanium increased [21]. Our data imply that the oxidized titanium with a proper thickness of oxide film would contribute to the binding of phages on cp-Ti disks [22, 23].

4 Conclusions

We have successfully isolated peptide aptamers against cp-Ti disks through phage display methodology and proved that the peptide aptamers exhibit different binding affinities to cp-Ti disks. The peptide, ATWVSPY, was found to have the strongest binding affinity to cp-Ti disks based on output/input efficiency and LCFM. Results of the effect of HNO_3 treatment on phage affinity suggest that phages are coupled to the titanium oxide surface and a proper oxide

film thickness would contribute maximally to the binding of phages to cp-Ti disks. The peptide aptamers isolated from cp-Ti disks directly, whose surfaces are wholly modified according to the principle of clinical implant utilization, might be used to explore artificial proteins that bring biological active functions to the surfaces of titanium-based implants in future investigation.

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