

PEGylation of bacteriophages increases blood circulation time and reduces T-helper type 1 immune response

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Summary

The increasing occurrence of antibiotic-resistant pathogens is of growing concern, and must be counteracted by alternative antimicrobial treatments. Bacteriophages represent the natural enemies of bacteria. However, the strong immune response following application of phages and rapid clearance from the blood stream are hurdles which need to be overcome. Towards our goal to render phages less immunogenic and prolong blood circulation time, we have chemically modified intact bacteriophages by conjugation of the non-immunogenic polymer monomethoxy-polyethylene glycol (mPEG) to virus proteins. As a proof of concept, we have used two different polyvalent and strictly virulent phages of the *Myoviridae*, representing typical candidates for therapeutical approaches: Felix-O1 (infects *Salmonella*) and A511 (infects *Listeria*). Loss of phage infectivity after PEGylation was found to be proportional to the degree of modification, and could be conveniently controlled by adjusting the PEG concentration. When injected into naïve mice, PEGylated phages showed a strong increase in circulation half-life, whereas challenge of immunized mice did not reveal a significant difference. Our results suggest that the prolonged half-life is due to decreased susceptibility to innate immunity as well as avoidance of cellular defence mechanisms. PEGylated viruses elicited significantly reduced levels of

T-helper type 1-associated cytokine release (IFN- γ and IL-6), in both naïve and immunized mice. This is the first study demonstrating that PEGylation can increase survival of infective phage by delaying immune responses, and indicates that this approach can increase efficacy of bacteriophage therapy.

Introduction

Use of bacteriophages as therapeutic agents started with their discovery by Frederick Twort and Félix d'Herelle almost a century ago. Early enthusiasm on the phage therapy, however, diminished in the Western countries due to inconsistent outcomes of the experiments, and was mostly abandoned after discovery of the antibiotics in the 1940s. However, phage therapy continued to be investigated in eastern European countries and the former Soviet Union (Sulakvelidze *et al.*, 2001). The increasingly problematic situation with respect to antibiotic-resistant pathogenic bacteria along with the lack of new antibiotics, and our better understanding of the biology of phages prompted a re-examination of phage therapy (Sulakvelidze *et al.*, 2001; Merrill *et al.*, 2003; Capparelli *et al.*, 2007; McVay *et al.*, 2007; Verbeken *et al.*, 2007). One of the lingering problems in therapeutic application is rapid clearance of administered phage particles from the circulatory system. A serial passage technique, which involved repeated animal passage of *Escherichia coli* phage lambda, was employed for selection of long-circulating phage mutants, which were shown to carry single amino acid substitutions in major structural proteins (Merrill *et al.*, 1996). However, there is a need for other or additional procedures which are more generally applicable to different phages.

The PEGylation of proteins, first described in the late 1970s (Abuchowski *et al.*, 1977a,b), relies on covalent binding (conjugation) of polyethylene glycol (PEG) molecules to primary amino groups of proteins (Katre, 1993). The PEG moieties themselves are chemically inert, non-immunogenic and biocompatible water-soluble polymers of ethylene oxide subunits. PEGylated proteins have been shown to retain their native structures and biological activities, often show improved stability, and decreased immunogenicity (Katre, 1993; Roberts *et al.*, 2002). These alterations can enhance therapeutic potency for protein drugs, such as adenosine deaminase (Levy *et al.*, 1988),

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Table 1. Relevant characteristics of phages A511 and Felix-O1.

	A511	Felix-O1	References
Morphotype and dimensions	Myovirus capsid diameter 88 nm, tail length 201 nm	Myovirus capsid diameter 72 nm, tail length 113 nm	Ackermann and Nguyen (1983); Zink and Loessner (1992)
Host	<i>Listeria</i> sp. (polyvalent)	<i>Salmonella</i> sp. (polyvalent)	Seeliger and Holl (1961); Loessner and Busse (1990)
Genome size	134.5 kb	86.2 kb	Accession No.: AF320576 (Felix-O1); DQ003638 (A511)
Number of ORFs predicted (total)	190	247	Accession No.: AF320576 (Felix-O1); DQ003638 (A511)
ORFs encoding putative structural proteins	orf81–orf106 (26 ORFs)	orf102–orf142 (26 ORFs) ^a	This work
Arginine/lysine content (average mol%) of 26 putative structural proteins	Arg: 4.07% Lys: 7.32%	Arg: 4.49% Lys: 5.95%	This work

a. In contrast to the preliminary annotation available from GenBank, only non-nested ORFs pointing into the major transcription direction were considered for annotation of the Felix-O1 genome sequence.

asparaginase (Graham, 2003) and interferon- α (Reddy *et al.*, 2002). Specificity of the reaction, e.g. PEGylation of specific amino acid residues, and the degree of PEGylation are dependent on the reaction conditions and chemistry of PEG activation (Katre, 1993). Recently, the scope of PEGylation was expanded to include large assemblies such as human and animal viruses (Croyle *et al.*, 2000) and even mammalian cells (Scott *et al.*, 1997). Other demonstrated effects of protein PEGylation include reduced humoral and cellular immune responses in innate (Croyle *et al.*, 2004; Mok *et al.*, 2004) as well as in adaptive immunity (Croyle *et al.*, 2001; 2002; 2005; Lee *et al.*, 2005), protection from neutralizing antibody *in vitro* (O’Riordan *et al.*, 1999; Croyle *et al.*, 2000), improved virus stability (Croyle *et al.*, 2000; Cheng *et al.*, 2003) and enhanced viral transduction efficiency (Inchley, 1969; Cheng *et al.*, 2003).

The major aim of our study was to determine the effects of PEGylation on infectivity and circulation time of phages infecting *Listeria* (A511) and *Salmonella* (Felix-O1) (Table 1). These viruses were chosen because they are representative members of a group of phages which bears high potential for therapeutic applications, i.e. the broad host range, virulent Myoviruses (Seeliger and Holl, 1961; Zink and Loessner, 1992). Structural proteins of purified phage particles were chemically modified by conjugation to polyethylene glycol. We show that phage PEGylation (i) can be controlled by the amount of PEG molecules in the reaction, (ii) does not negatively affect virus particle stability or host range, (iii) increases blood circulation half-life, and (iv) represses production of T-helper type 1 (Th1)-associated immune response modulators. PEGylated phage particles elicited reduced levels of IFN- γ and IL-6, in both naïve and immunized mice. This is the first study demonstrating the benefits of chemical modification of bacteriophages, and shows that this type of surface alteration can potentially enhance therapeutic potency of phage.

Results

Clear correlation between degree of PEGylation and loss of phage infectivity

The percentage of free amino groups remaining after reaction phage proteins with monomethoxy-PEG (mPEG, molecular weight ~5000 Da) inversely correlated with the different mPEG concentrations used (Fig. 1A and B). At phage : mPEG ratios (w/w) of 1:1, 1:10 and 1:50, approximately 88% (93%), 71% (81%) and 14% (33%), of A511 (or Felix-O1) structural protein amino groups remained unmodified. The percentage of remaining free amino groups in A511 PEGylation using a 1:50 ratio was significantly lower than using Felix-O1 ($P = 0.027$). When PEGylated A511 (or Felix-O1) was tested for infectivity and compared with unmodified, nude phage [wild type (WT)], about 95% (100%), 88% (74%) and 24% (29%) of plaque-forming ability was retained for the 1:1, 1:10 and 1:50 ratios respectively (Fig. 1C and D).

PEGylation of the phage particles did not significantly alter the mass or relative proportion of structural proteins, as demonstrated by SDS-PAGE analysis (Fig. 2A). Some possible but minor changes in A511 proteins were observed after silver staining, mostly in the higher molecular mass range of the phage protein profile (Fig. 2A).

The partitioning coefficient (K) of PEGylated molecules between two different phases (Dextran 500 and PEG 8000) describes the different degree of solubility of hydrophobic molecules, and is a useful measure to estimate the relative degree of PEGylation (Delgado *et al.*, 1990). Determination of K is usually based on measuring protein distribution between the two phases. In our study, however, this was not feasible because the relatively diluted phage suspensions correspond to actually very low protein concentration (initial concentration $< 6.4 \mu\text{g ml}^{-1}$). Thus, we have modified the partitioning coefficient and the assay by measuring plaque-forming units (K_{PFU}). The K_{PFU} values for PEGylated A511 (or Felix-O1) were 0.013

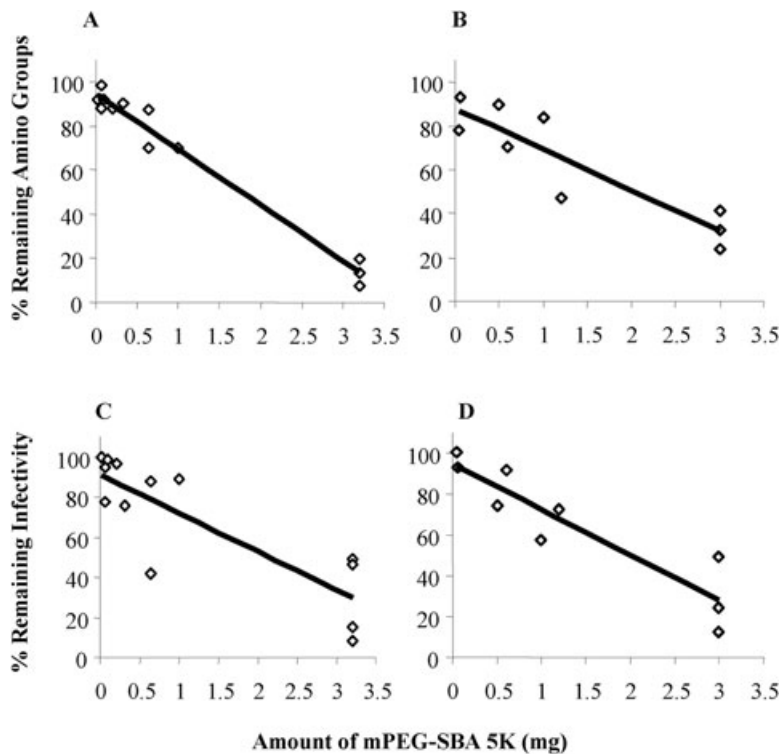


Fig. 1. Effect of PEGylation on infectivity of phage. Determination of the degree of PEGylation by measuring the percentage of remaining unmodified amino groups (A, A511; B, Felix-O1), and the percentage of infective particles after PEGylation (C, A511; D, Felix-O1). Trend lines are shown.

(0.012 for Felix-O1) (1:1 ratio of phage to mPEG), 0.029 (0.016) (1:10) and 79.90 (0.238) (1:50), compared with 0.009 [unmodified control (WT)] (Fig. 2B). After recovery of A511 particles from the two different phases and subsequent SDS-PAGE separation of the phage proteins, a gradual decrease in density of the major capsid protein recovered from the lower phase was observed, correlating with increasing mPEG concentration in the modification reaction (Fig. 2C). Modification of Felix-O1 yielded similar results (data not shown). In general, the observed mobility changes in the PEG-8000 solution (Fig. 2C) were in good agreement with previous reports (Bode, 1976). The stability of PEGylated phages in SM buffer at various temperatures (4–42°C) over a period of 3 weeks were not affected, compared with non-modified phage.

Taken together, we found that the physicochemical surface modification of A511 and Felix-O1 was possible without overly compromising viral integrity and infectivity. In all further experiments, phages were PEGylated at a 1:50 ratio.

PEGylation increases phage half-life in blood and serum both in vitro and in vivo

The proportion of infective A511-wt particles in the mouse blood circulation dropped rapidly; only 3.3% of the initial PFUs remained 1.5 h after injection (Fig. 3A). The decrease continued further; we measured 0.016% residual infectivity 6 h post injection, and 0.001% infective

particles 24 h after injection. In contrast, PEGylated A511-PEG phages survived much better; 83.7% and 20.9% of the PFUs remained after 1.5 and 6 h injection respectively (Fig. 3A). The A511-PEG clearance followed an almost linear function, with 0.01% infectivity remaining after 24 h incubation (Fig. 3A). At 6 h post injection, the difference between native and PEGylated phage reached a maximum of more than 3 logs.

With respect to Felix-O1, 0.25% and 0.03% of the WT phages were found 1.5 and 6 h after injection respectively (Fig. 3B). After 3 h, this proportion was reduced to 0.07%, and to 0.005% after 24 h (Fig. 3B). Similar to A511, inactivation and/or removal of PEGylated Felix-O1 proceeded much slower, with 59.6%, 9.0%, 0.7% and 0.02% viable phage remaining after 1.5, 3.0, 6.0 and 24 h respectively (Fig. 3B).

The protective effects of PEGylation on virus neutralization was also seen when A511 was incubated in pre-serum samples taken from non-immunized mice (Fig. 3C). In fact, A511-PEG remained fully viable in pre-serum for 4 h, whereas a gradual decrease was observed for unmodified A511, with 87.4%, 73.0% and 63.3% remaining infectivity after 1, 2 and 4 h incubation respectively (Fig. 3C). In contrast to A511, PEGylated Felix-O1 was not as serum resistant and lost infectivity when exposed to pre-immunization serum, with 12.5%, 9.4% and 5.8% of infectivity after 1, 2 and 4 h incubation respectively (Fig. 3C). Results for native Felix-O1 were not significantly different; 9.5%, 7.9% and 2.7% phage

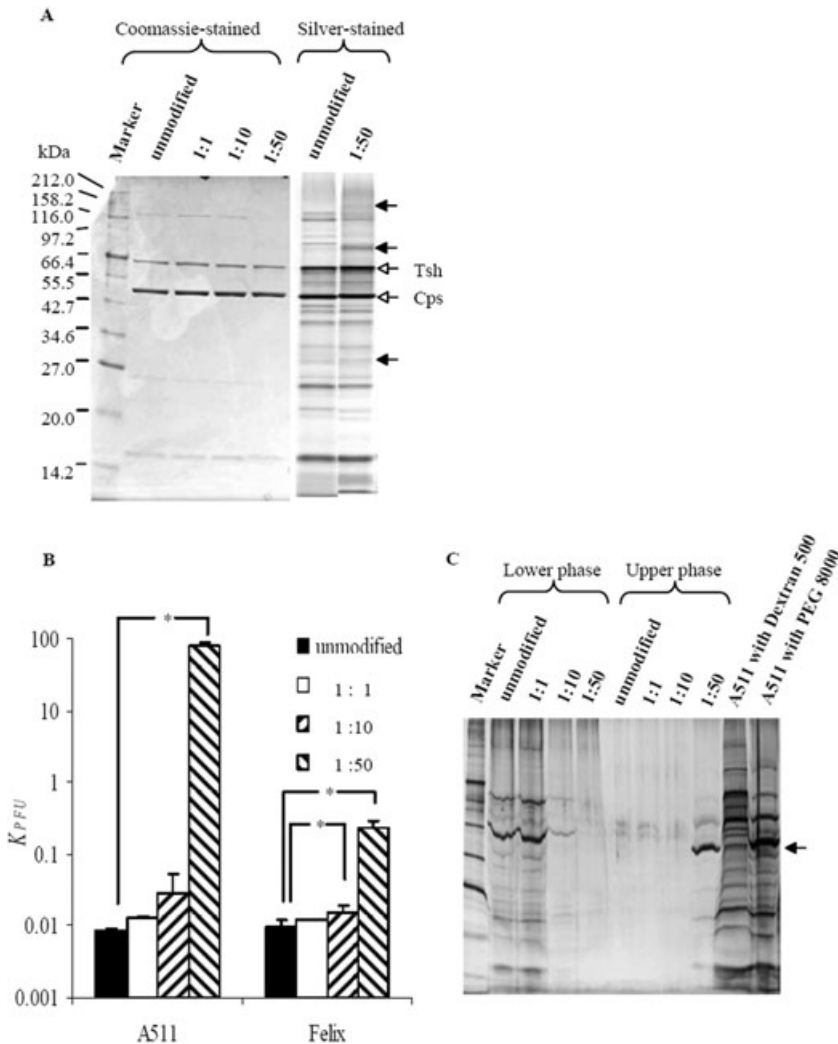


Fig. 2. A. An SDS-PAGE analysis of unmodified and PEGylated A511, stained with Coomassie or Silver. The major structural proteins (capsid Cps and tail Tsh) are marked by open arrows, and possible protein modifications/gel shifts are indicated by solid arrows.

B and C. Results of the partitioning assay (see text for details). The ratio of infective particles in the upper and lower phases is shown for both A511 and Felix-O1 (B). The asterisk (*) indicates statistical significance ($\alpha = 0.05$). (C) shows an SDS-PAGE analysis of phage A511 native and PEGylated proteins after recovery from the partitioning assay (see text). The phase transition of Cps (indicated by an arrow) is clearly evident. Samples of 30 μ l were loaded and silver-stained after electrophoresis. As controls, native A511 mixed with either Dextran 500 or PEG 8000 was also analysed.

viability was observed after 1, 2 and 4 h incubation respectively.

PEGylation does not increase phage circulation time in pre-immunized mice

Two weeks after the initial immunization with WT or PEGylated phages, the immunized mice received a second phage dose in order to determine the adaptive immune response to native or PEGylated phage. However, survival of A511 (PEGylated or WT) at 1 h after the second injection was only 0.0001% (Fig. 4A). Inactivation of PEGylated Felix-O1 in immunized mice also proceeded fast; from 0.003% down to 0.0002% PFUs could be recovered up to 24 h incubation. A slightly better recovery of viable phage was obtained with Felix-O1 (WT)-challenged pre-immunized mice; between 0.03% and 0.0005% of infectivity was detected after 1, 3, 6 and 24 h incubation (Fig. 4B).

In addition to the *in vivo* animal tests, we harvested mice sera 2 days before the second challenge (12 days after the first injection), and tested their potential to neutralize phage *in vitro*. Sixty minutes of incubation was sufficient to completely inactivate A511 (PEGylated or WT) (Fig. 4C). With Felix-O1, 0.2%, 0.04% and 0.004% of phage remained infective after 1, 2 and 4 h incubation respectively (Fig. 4C). PEGylation of Felix-O1 did not result in longer survival. These results indicated that PEGylation does not provide protection from adaptive immune responses.

Native and PEGylated phage both stimulate strong antigen-specific IgG response

The generation of A511-specific IgG and IgM was monitored by an ELISA assay before phage injection, and 12 days after the first immunization. In addition, serum was also collected 7 days after the second injection, from mice of the different immunization trials, receiving either native

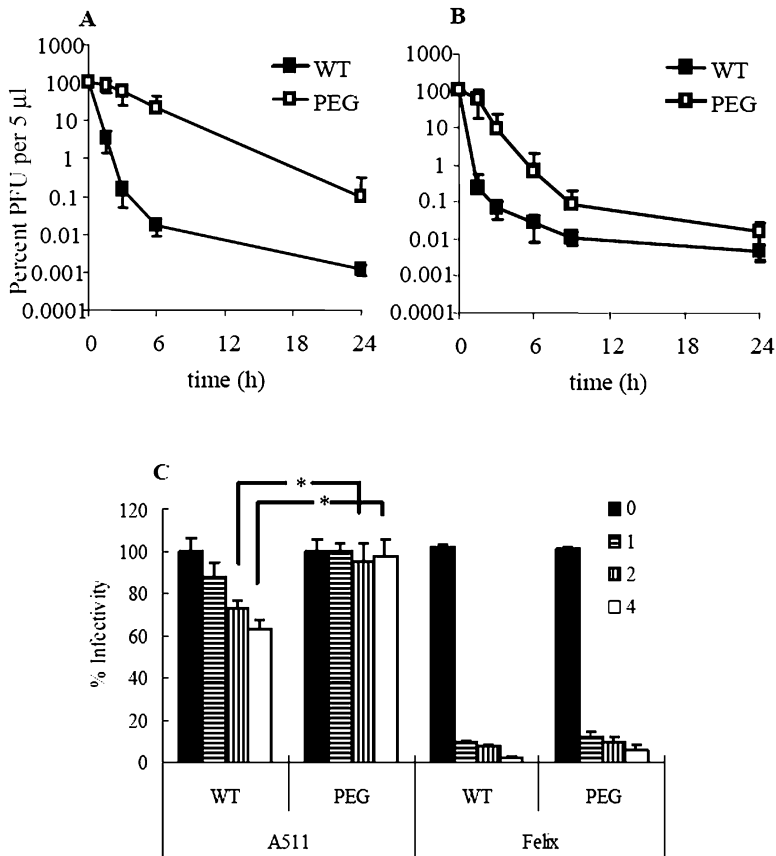


Fig. 3. Phage inactivation in the presence of innate immunity in the mouse model, both *in vivo* for A511 (A) and Felix-O1 (B), and *in vitro* (C). In (C), 0, 1, 2 and 4 indicate the incubation time in the presence of serum. In *in vivo* studies, the number of phage per 5 μl at 0 h is represented as 100%, corresponded to 1×10^6 PFU for WT A511 and 1×10^5 PFU for PEGylated A511, and 1×10^6 PFU for WT Felix-O1 and 3×10^5 PFU for PEGylated Felix-O1. The asterisk (*) denotes statistical significance ($\alpha = 0.05$).

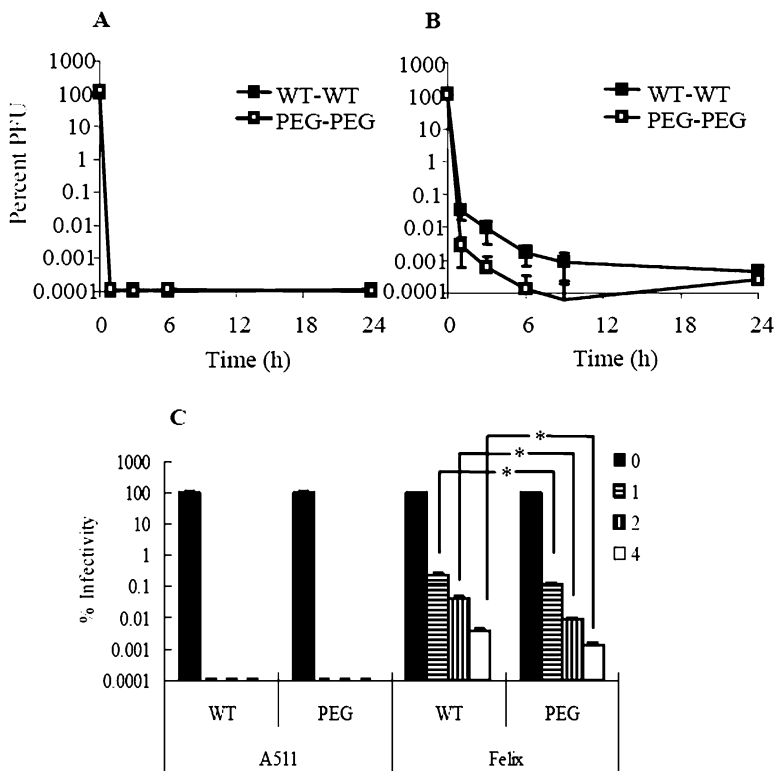


Fig. 4. Phage inactivation *in vivo* for A511 (A) and Felix-O1 (B), or *in vitro* (C) in the presence of adaptive immunity. In *in vivo* studies, the number of phage per 5 μl at 0 h is represented as 100%, corresponded to 1×10^6 PFU for WT A511 and 1×10^5 PFU for PEGylated A511, and 1×10^6 PFU for WT Felix-O1 and 3×10^5 PFU for PEGylated Felix-O1. In (C), 0, 1, 2 and 4 indicates the incubation time in the presence of serum. The asterisk (*) denotes statistical significance ($\alpha = 0.05$).

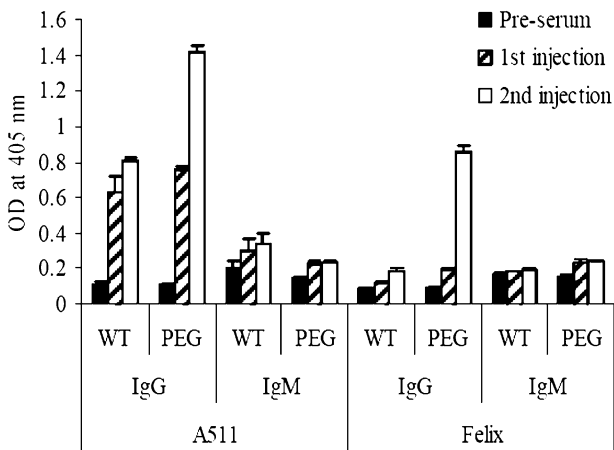


Fig. 5. Amounts of antigen-specific IgG and IgM in pre-serum, serum taken 12 days after the first injection and serum taken 7 days after the second immunization. The antigens used for ELISA plate coating and immunization were identical.

or PEGylated phages. Figure 5 shows the results obtained when the same antigen was used for coating and immunization, i.e. WT or PEGylated particles were used for coating, and 21-day sera from mice with first-WT (or PEG) and second-WT (or PEG respectively) treatment were used in the ELISA.

Phage challenge induces a T-helper type 1-associated cytokine response

When splenocytes from naïve or immunized mice were exposed to native or PEGylated phages, antigen-specific T-cell proliferation was observed 3 days after antigen treatment. The formation of blasts which accompanied the proliferation of activated T cells was monitored by light microscopy. No stimulation was observed in the control, confirming antigen-specific cell proliferation upon bacteriophage presentation. After initiation, tritium-labelled thymidine was added, and radioactivity was measured after 20 h incubation (Fig. 6). For Felix-O1, the stimulation indices in splenocytes from naïve mice were 4.4 ± 0.8 (PBS-WT) and 1.4 ± 0.3 (PBS-PEG), while in those from immunized mice, it was 7.4 ± 1.6 (WT-WT) and 2.3 ± 0.3 (PEG-PEG).

For cytokine analysis, splenocytes were treated with either the same antigen (WT_{treated} or PEG_{treated}) or the controls received PBS (PBS_{treated}). With respect to Felix-O1, the amount of IFN- γ in naïve mice was 0.40 ± 0.13 (ng ml⁻¹) for PBS_{imm}-WT_{treated}, and 0.07 ± 0.03 for PBS-PEG (Fig. 7A), while in immunized mice, they were 0.83 ± 0.18 for WT-WT and 0.39 ± 0.12 for PEG-PEG. Antigen-specific production of IL-6 was also observed (Fig. 7B), producing 19.95 ± 1.22 for PBS-WT and 4.69 ± 0.02 for PBS-PEG in naïve mice, while immu-

nized mice produced 16.73 ± 6.21 for WT-WT and 5.11 ± 0.75 for PEG-PEG (Fig. 7B). No IL-4 could be detected after phage challenge (Fig. 7C). In both naïve and immunized mice, IFN- γ and IL-6 levels stimulated by native phage were significantly higher than those seen after exposure to PEGylated phage. Similar observations were made for A511 (data not shown).

Discussion

In this work, we demonstrate that PEGylation of bacteriophages is effective to delay virus clearance and achieve longer circulation time in non-immunized animals. Our results also indicate that PEGylation can reduce cellular immune response such as antigen-specific T-cell proliferation, and decreases release of associated cytokines.

Activated polyethylene glycol was conjugated to primary amines exposed on phage structural proteins; the amide bonds formed are stable under physiological conditions. The degree of modification was controlled by mPEG concentration; phage infectivity linearly decreased with increasing PEGylation. Surprisingly, heavily PEGylated phage particles still maintained significant degree of infectivity (10–50%). Under otherwise identical reaction conditions, A511 virions were found to be PEGylated to a higher degree than Felix-O1. This may be due to the different amino acid composition of major structural proteins, the targets of the PEGylation reaction. As outlined in Table 1, both phages are morphologically similar, although A511 is slightly larger than Felix-O1. To answer the question whether a different frequency of amino-reactive Lys or Arg residues may be responsible for the different PEGylation efficiency, an *in silico* analysis was used to calculate the amino acid composition of a large

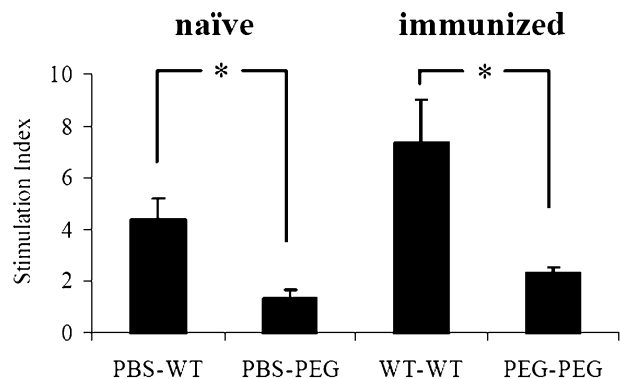


Fig. 6. Stimulation of splenocyte proliferation by bacteriophage Felix-O1. Mice splenocytes were used, which were prepared from either naïve mice (left bars) or immunized animals which have received two doses from the same antigen (right bars) either native (WT) or PEGylated Felix-O1 (PEG). Phosphate-buffered saline (PBS) was used as a control. The asterisk (*) indicates statistical significance ($\alpha = 0.05$).

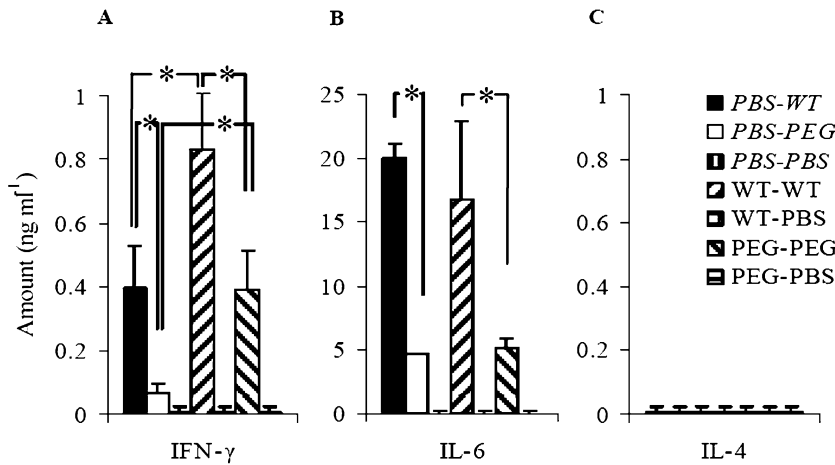


Fig. 7. Cytokine induction by unmodified and PEGylated Felix-O1 phage, in naïve (italics) and in immunized mice. Splenocytes from naïve or immunized mice which were consecutively treated with the same antigens were used. The legend indicates the immunizing antigens and the treatment antigens, either unmodified phage (WT), PEGylated phage (PEG) or buffer (PBS). (A) IFN- γ ; (B) IL-6; (C) IL-4. The asterisk (*) denotes statistically significant differences ($\alpha = 0.05$).

set of putative proteins deduced from the corresponding genes predicted to represent the major structural components (Table 1). Both phages feature a nearly identical arginine content in their structural components, whereas A511 revealed a significantly higher percentage of lysine residues (7.32%, versus 5.95% for Felix-O1). This correlates well with the different outcome of the PEGylation reaction, whose efficiency likely is influenced by the frequency of these diamino acids in the viral proteins. However, mPEG reactivity is not solely dependent on amino acid composition, as the spatial location and exposure of free amino groups must also be considered. Thus, individual optimization of PEGylation reaction conditions for each phage is required.

With respect to inactivation of phage in naïve mice, several findings must be mentioned. First, phages were inactivated quickly during circulation in the bloodstream, but with different kinetics. The different observations made for A511 (slow removal) and Felix-O1 (fast removal) may result from different levels of phage-neutralizing molecules such as circulating antibodies. This hypothesis is supported by the neutralizing effect of pre-immune serum, which affected the different phages in a similar way: A511 lost 40% activity within 4 h, whereas Felix-O1 was inactivated by more than 90%. Alternatively, the effect could also be due to a different uptake efficiency of cellular components, either non-specific and/or receptor-mediated. Obviously, these two possibilities are not unrelated. In addition, different sensitivity of A511 and Felix-O1 to endogenous proteases cannot be ruled out. Second, production of A511-specific IgG upon primary challenge was significantly stronger than the Felix-O1 specific response. This could be due to an intrinsically strong immunogenicity of A511, which might have also resulted from the longer circulation time upon antigen injection, similar to the adjuvant effect. Lastly, both A511 and Felix-O1 elicited a T1-associated

cytokine response (IFN- γ and IL-6), but no type 2 response (IL-4).

PEGylation did not negatively affect functionality and stability of the modified phage particles, and resulted in a significantly extended half-life of viral infectivity in the blood circulation of naïve mice. Several mechanisms can contribute to this desired effect. First, the modification certainly has a shielding effect against serum components, as native A511 gradually lost infectivity over time while this was not observed for PEGylated virions. This suggests an increased resistance of PEGylated phages against recognition and binding, likely through PEG-mediated epitope-masking. Second, our data show that PEGylation is effective in resistance to RES-mediated blood clearance. We found that A511 maintained only *c.* 3% of infectivity 1.5 h after injection in the blood circulation, whereas *c.* 73% remained in pre-serum after 2 h incubation. This major difference between *in vivo* blood and *in vitro* serum exposure suggests direct involvement of cellular responses in phage inactivation. Other observations also support the effect of PEGylation against inactivation by non-serum components: (i) difference in infectivity losses between PEGylated A511 and WT in pre-serum was less than 40%, whereas the loss was more than two orders of magnitude over 3 h incubation and more than 3 logs after 6 h in circulation, (ii) no significant difference in per cent infectivity loss between PEGylated and WT Felix-O1 in pre-serum, whereas a 2 log difference was found 1.5 and 3 h after injection. Moreover, in naïve mice, PEGylated Felix-O1 resulted in decreased induction of T1-associated cytokines (IFN- γ and IL-6), and lesser splenocytes proliferation.

Although an extended circulation half-life could be demonstrated for both modified A511 and Felix-O1, the effect of PEGylation varied considerably. We found that modification-dependent survival enhancement of PEGylated Felix-O1 was less pronounced than that of A511,

both *in vitro* pre-serum and *in vivo*. Again, several explanations are possible. First, a different degree of protein PEGylation may have influenced the effects, as A511 was more heavily PEGylated than Felix-O1 at an identical of phage : mPEG ratio. Also, it is possible that initial levels of phage-neutralizing antibodies (and induced cellular uptake) were different for A511 and Felix-O1. Second, PEGylated Felix-O1 exhibited decreased splenocyte stimulation and reduced IFN- γ and IL-6 induction. Mok and colleagues (2004) also reported that PEG-modified adenoviruses reduced the innate IL-6 response, which resulted in decreased uptake by macrophages *in vitro* and Kupffer cells *in vivo*. PEGylated A511, in contrast, did not show significant difference with respect to splenocytes stimulation or cytokine production, the basis of which is not clear (data not shown). It is possible that 40 h incubation before collecting supernatants for cytokine analysis, or 3 days incubation before addition of the labelled thymidine isotope was not adequate to measure the difference. The importance of appropriate incubation time is evident from the low stimulation index observed after A511 challenge, whereas blast formation was clearly demonstrated in antigen-induced splenocyte proliferation.

After entry and distribution of phage into various organs and phage-induced immune response, clearance of viruses increases dramatically (Farber, 1969). We have also observed a strongly increased phage inactivation upon challenge of pre-immunized animals. Interestingly, A511 was inactivated much faster than Felix-O1, in contrast to the kinetics in naïve mice. This effect could be due to generation of higher anti-A511 IgG levels, which receives support from our results indicating more A511-neutralizing molecules, and a greater neutralizing capacity in serum from immunized mice. As mentioned above, stronger induction of anti-A511 IgG may also be due to longer circulation time of A511 upon primary injection. Compared with the increases seen with the PEGylated phages, the increases in antigen-specific IgG production upon the second challenge with native phage were only marginal.

In immunized animals, (i) no significant difference in *in vivo* blood half-life was observed, (ii) PEGylated Felix-O1 exhibited significantly reduced per cent infectivity in *in vitro* assays, and (iii) significantly increased PEGylated phage-specific IgG production was observed for both A511 and Felix-O1. However, it is not completely clear if the stronger ELISA signals were due to the increased IgG affinity by antibody maturation, or the absolute amount of IgG present, lastly (iv) we observed a significantly reduced cytokine production when immunized splenocytes were challenged with PEGylated phages.

Reduced splenocytes proliferation and lower levels of Th1-associated cytokines (IFN- γ and IL-6) were also observed in immunized mice challenged with PEGylated

Felix-O1, which would be expected to support longer survival of modified phage particles. However, the difference between PEGylated and native Felix-O1-specific antibody production was relatively small (compare Fig. 5, data from the first injection). Correspondingly, we found no significant difference in survival of PEGylated or native Felix-O1 in immunized mice. This finding underlines the crucial role of pre-existing neutralizing molecules such as antibodies.

With respect to future animal pathogen challenge experiments, it is apparent that longer blood circulation would require fewer injections and lower doses, to achieve the same effect as by using unmodified, nude phage. Lower doses could benefit phage therapy in several ways: (i) because phages are strongly immunogenic (Alemany *et al.*, 2000) and immune responses tend to be dose-dependent, a lower dosage could further support a lesser or delayed immune response, (ii) reduced immunity could potentiate the effect of PEGylation, requiring lower numbers of PEGylated phages at a later injection time point for the treatment. Therefore, the two parameters dosage and degree of PEGylation are clearly related, and need to be optimized in relation to each other.

The available PEGylation chemistry also offers additional approaches in order to increase the blood half-life in pre-immunized animals, such as the use of PEG molecules of different size or structure (branched or linear) (Croyle *et al.*, 2001; 2002). Moreover, besides the primary amino groups, it is also possible to use cysteine-containing sulfhydryl groups, or the hydroxyl groups in specific amino acids such as Ser or Thr as PEGylation targets, as another possibility order to further diminish or even evade the adaptive immunity response.

Experimental procedures

PEGylation of phages

Listeria phage A511 (Loessner and Busse, 1990) and *Salmonella* phage Felix-O1 (Felix and Callow, 1943) were propagated on *Listeria ivanovii* 3009 or *Salmonella enterica* serovar Typhimurium DB7155, respectively, using half-concentrated Brain Heart Infusion medium (Biolife, Milano, Italy) at 30°C (*Listeria*) or 37°C (*Salmonella*). Exponentially growing *L. ivanovii* was infected with A511 at a multiplicity of infection of 1, and further incubated for approximately 3–4 h until the culture lysed and became clear. For Felix-O1 propagation, plate lysis and phage recovery were carried out as described elsewhere (Sambrook and Russell, 2001). Purification of virions by CsCl gradient centrifugation (22 000 g, 12 h) followed by overnight dialysis (50 000 Da cut-off; Spectrum) was described previously (Adams, 1959; Zink and Loessner, 1992). Purified phage was adjusted to 10¹² PFU ml⁻¹, and stored until use in 10 mM sodium phosphate buffer (pH 7.5) at 4°C. Total phage protein was determined by a modified BCA assay as instructed by the manufacturer (Pierce).

For PEGylation, purified phages (equivalent to 2×10^{11} PFU: 64 μg of A511 or 60 μg of Felix-O1) were mixed with the *N*-hydroxysuccinimidyl ester of methoxy PEG butanoic acid (mPEG-SBA, molecular weight 5000 Da) (Nektar Therapeutics). Reactions were carried out at phage : mPEG ratios (w/w) of 1:1 to 1:50, in a total volume of 1 ml of 10 mM sodium phosphate buffer (pH 8.5), for 1 h at room temperature (24°C) with gentle agitation. Addition of a 10-fold excess of lysine (Sigma) as a blocking reagent was followed by overnight dialysis (50 000 Da pore size, Spectrum) against two changes of 10 mM sodium phosphate buffer (pH 7.3), in order to remove residual PEG and excess lysine.

Estimation of the degree of phage PEGylation

PEGylation efficiency was determined as described for adenovirus (Mok *et al.*, 2004), with some modifications. For the fluorescamine assay, 50 μl of fluorescamine solution (0.3 mg ml⁻¹ in acetone; Sigma) was added to serially diluted PEGylated or native (WT) phages (150 μl volume). The reaction was incubated in the dark at room temperature for 15 min, followed by fluorescence measurement at 355 nm excitation and 465 nm emission wavelengths (Victor Multilabel Counter; Perkin Elmer). The amount of fluorescence was plotted against the protein content. Percent remaining (unmodified) amino groups were calculated as $100 \times (1 - \text{Slope}_{\text{PEGylated}} / \text{Slope}_{\text{WT}}^{-1})$.

Partitioning assay and SDS-PAGE

Determination of the partition coefficient (*K*) was previously described (Croyle *et al.*, 2005) by using Dextran 500 and PEG 8000, and adopted for the determination of bacteriophages as PFUs. The K_{PFU} value represents the ratio between PFUs found in the upper and lower phases.

For SDS-PAGE, sample buffer was mixed with native or PEGylated phage particles (see figure 2 legend for amounts) and boiled for 10 min. Proteins were separated on 12% Tris-Bis gels (Criterion, Bio-Rad), and visualized by Coomassie Blue or Silver staining methods, as described elsewhere (Sambrook and Russell, 2001).

Mouse immunization

Five- to six-week-old male BALB/c mice (Charles River) were maintained in a state-of-the-art animal facility at the Department of Dentistry, Chonbuk National University, Korea, with *ad libitum* supply of food and water. One week after arrival (day 1), animals were injected via the tail vein with phage particles (0.64 μg of A511 or 0.6 μg of Felix-O1, 20 mM sodium phosphate buffer, pH 7.4; equivalent to approximately 2×10^9 PFUs in native bacteriophages). Four different treatment groups ($n = 8$) received either PEGylated or WT A511 or Felix-O1 particles respectively. Two weeks after primary injection (day 14), boosting was performed by injecting the same amount of modified or native phages.

In vivo blood clearance of phages in naïve and immunized mice

Naïve ($n = 8$ for each group) and immunized ($n = 5$) mice were designated to be animals either 1 week after arrival or 2

weeks after the primary injection respectively. Phages were injected via the tail vein, in amounts of 0.64 μg (A511) or 0.6 μg (Felix-O1), in 100 μl of 20 mM sodium phosphate buffer. Five microlitres of blood was collected by axillary bleeding at different time points (see Figs 3 and 4), and used for PFU determination. The PFU count at time '0' was obtained using blood which was taken right after antigen injection, and calculated by assuming 2 ml of total blood per mouse (35 g on average).

Phage neutralization in serum prepared from naïve or immunized mice

Mice sera were obtained from non-immunized (pre-serum) or immunized mice (12 days after the first injection). Serum was diluted 1:1 (PBS, pH 7.2) (90 μl), mixed with phages (0.64 μg for A511 and 0.6 μg for Felix-O1) (10 μl), and incubated at 37°C. Neutralization of phage was monitored by determination of PFUs after 0, 1, 2 and 4 h.

Antibody titration

Mice sera from the individual treatment groups were combined for antibody titration from pre-serum, 12 days after primary injection and 7 days after second injection (21 days after the first injection). The used ELISA protocol was reported elsewhere (Coligan *et al.*, 2001). In brief, a 96-well ELISA plate (Nalge-Nunc) was coated with phages (100 μl , 0.64 μg for A511 and 0.6 μg for Felix-O1). Serum (1:400 dilution in PBS) and AP-conjugated rabbit anti-mouse IgG (Sigma) (1:5000 dilution in PBS) were used as primary and secondary antibodies respectively. A colorimetric AP substrate (Sigma) containing 100 mM di-ethanolamine and 1.1 mM MgCl₂ (pH 9.8) was added according to the manufacturer's protocol. Colour development was stopped after 20 min by adding 100 μl of 0.3 N NaOH, and absorbance was measured at 405 nm.

Phage-induced T-cell proliferation

Mice splenocytes were prepared as described elsewhere (Coligan *et al.*, 2001) and seeded into 96-well flat-bottomed tissue culture plates (4×10^5 cells well⁻¹). They were then challenged with bacteriophages (0.128 μg of A511 and 0.12 μg of Felix-O1, equivalent to 4×10^9 PFU well⁻¹). After incubation (3 days, 37°C, 5% CO₂), 0.5 μCi of ³H-labelled thymidine was added, followed by further incubation for 20 h. Counts per minute (CPM) were determined after harvesting cells from each well (96-well cell harvester, Molecular Devices) using a liquid scintillation counter (Wallac 1409 DSA, PerkinElmer). The stimulation index was calculated as $(\text{CPM}_{\text{antigen-treated}} / \text{CPM}_{\text{PBS-treated}})^{-1}$.

In vitro cytokine production

Mice splenocytes prepared as above were re-suspended in 1 ml of complete RPMI-10 with antibiotics (RPMI with 10% FBS, 100 U penicillin, 100 U streptomycin) (Hyclone). Splenocytes were seeded onto 24-well flat-bottomed tissue

culture plates (0.5 to 1×10^7 cells well⁻¹), and phages ($0.64 \mu\text{g}$ of A511 or $0.6 \mu\text{g}$ of Felix-O1) were added, followed by incubation for 40 h in a cell culture incubator (Forma Scientific) with 5% CO₂ at 37°C. The supernatant was collected and used for analysis of cytokines (IFN- γ , IL-4 and IL-6) at the Bank for Cytokine Research (Jeonju, Korea).

Statistical analysis

If applicable, data were analysed by Student's *t*-test at an $\alpha = 0.05$ (statistical significance level).

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