F. Moghtader / Hacettepe J. Biol. & Chem., 2025, 53 (1), 97-108

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: https://hjbc.hacettepe.edu.tr/
Dergipark Akademik: https://dergipark.org.tr/en/pub/hjbc

Multifunctional Gelatin Hydrogel Microspheres Delivering Phages and Basic Fibroblast Growth Factor

Faj ve Temel Fibroblast Büyüme Faktörü Salan Çok Fonksiyonlu Jelatin Hidrojel Mikroküreler

Farzaneh Moghtader[1,2*]

[1]TiPhage ArGe Teknoloji Sanayi ve Ticaret Anonim Sirketi, Teknopark İstanbul, Pendik, İstanbul, Turkiye.
[2]NanoBMT: Nanobiyomedtek Biyomedikal ve Biyoteknoloji Sanayi ve Ticaret Limited Sirketi, Mugla, Turkiye.

ABSTRACT

The aim of this study is to prepare gelatin hydrogel microspheres (GELs) delivering phages (as antibacterial agents) and/or basic fibroblast growth factor (as a potent angiogenic factor) as multifunctional pharmaceuticals to be used in healing of infected wounds. T4 phages were propagated and tested by using E.coli as the host and the target. GELs with different crosslinking degrees were prepared by a two step process from basic and/or acidic gelatins by using different amounts of glutaraldehyde as the crosslinker. Acidic and enzymatic degradations were studies. T4 phages and/or basic fibroblast growth factor were loaded within these microspheres and release kinetics/modes were investigated/optimized.

Key Words
Gelatin hydrogel microspheres, T4 phage, basic fibroblast growth factor, controlled release.

ÖZ

Bu çalışmanın amacı, enfekte yaraların iyileşmesinde kullanılmak üzere çok fonksiyonlu farmasötikler olarak fajları (antibakteriyel ajan olarak) ve/veya temel fibroblast büyüme faktörünü (anjiyojenik faktör olarak) taşıyan – kontrollu salan jelatin hidrojel mikroküreleri (GEL'ler) hazırlamaktır. Hem konak hem de hedef olarak E.coli kullanılarak T4 fajları çoğaltılmış ve aktivite testleri yapılmıştır. Farklı çapraz bağlanma derecelerine sahip GEL'ler iki aşamalı bir proses ile bazik ve/veya asidik jelatin kullanılarak hazırlanmıştır. Jeller farklı miktarlarda glutaraldehit ile çapraz bağlanmıştır. Asidik ve enzimatik bozunmalar incelenmiştir. Bu mikrokürelerin içine T4 fajları ve/veya temel fibroblast büyüme faktörü yüklenmiş ve salım kinetikleri ve modları araştırılarak optimize edilmiştir

Anahtar Kelimeler
Jelatin hidrojel mikroküreler, T4 fajı, temel fibroblast büyüme faktörü, kontrollü salım.

Article History: Aug 13, 2024; Accepted: Nov 25, 2024; Available Online: Dec 11, 2024.
DOI: https://doi.org/10.15671/hjbc.1532795
Correspondence to: F. Moghtader, TiPhage ArGe Tecknoloji Sanayi ve Ticaret Anonim Sirketi, Teknopark İstanbul, Pendik, İstanbul, Turkiye.
E-Mail: farzaneh_moghtader@yahoo.com

INTRODUCTION

There is a great interest in developing effective therapies for the treatment of skin wounds accompanied with deep tissue losses and with severe infections. Numerous tissue engineering and regenerative therapy approaches for the restoration of the host tissue losses have been proposed and studied using several skin substitutes [1-3]. Open skin wounds are prone to bacterial invasions which may result severe/chronic infections in the wound and/or may even lead many critical subsequent systemic events accompanied with untreatable biofilm formations that may result the lost of the patient [4-7].

In general, antibiotics have been successfully applied to fight against pathogens since the discovery of penicillin by Alexander Fleming in 1928, - however - now we are faced to limitations due to the emergence of antibiotic-resistant bacteria, that is a public health challenge with extensive health, economic, and societal implications [8-10]. Bacteriophages - shortly called "phages" are the Nature's antibacterial agents - the most abundant living organism in the world - which are typical viruses. They do quite specifically infect, replicate and kill/destroy their target bacteria - however they are known as harmless for humans. Phages demonstrate several advantages over traditional antibiotics, including high specificity, self-replication, and a low likelihood of inducing bacterial resistance [11-19]. Phages have been invented and described in the first quarter of 1900, several phages mainly as cocktails have been developed, commercialized and applied clinically in the Eastern Europe (mainly Russia and Georgia) for a long-time already. During this period the Western World was using antibiotics. However as mentioned above, today there are too many bacterial strains with antibiotic resistance, and the only way to remedy this is to use phages. Today, the age of phages has become globally - much deeper look and re-evaluation of their futuristic properties - as one of the most attractive antibacterial agents - that explains the growing interest in phages.

In the context of studies presented here we have focused on bFGF in our multifunctional formulations which is a member of the FGF family of growth factors and signaling proteins - which plays several key roles in wound healing [21-26]. In 1988, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan, licensed rights for the recombinant human bFGF that was originally developed for patients with decubitus ulcers and skin ulcers. Several formulations have been used already in the last 2 decades in all levels and with very successful clinical uses mainly in Japan as nicely reviewed by Abdelhakim et al. [25].

Gelatin, a natural biopolymer - a polypeptide - derived from collagen, has gained significant attention in the field of biomaterials due to its unique properties and versatile applications [26-30]. As a biomaterial, gelatin exhibits excellent properties including the followings: (i) very high biocompatibility - due to its chemical similarities to native collagen that is the main component in the extracellular matrix (ECM) in the native tissues. Gelatin matrices exert even significantly lower risk of side effects - like lower immunogenicity - compared to its raw material collagen; (ii) biodegradability is one of the essential/desirable property of gelatin materials in bioapplications. The degradation products are oligopeptides/amino acids, they are actually among the most important building blocks in our body and can trigger tissue regeneration/healing. Lower mechanical strength and high sensitivity to enzymatic degradation - means fast degradation rates *in vivo* - are considered as disadvantages in some applications like cell scaffolding - but could be significantly improved by chemical and physical modifications like simple crosslinking and/or by blending with other degradable natural and synthetic polymers; (iii) gelation properties of gelatin - mainly due to the hydrophilicity of the gelatin backbone and its high molecular weight which is about 100 kDa - means lower solubility in aqueous media - is an unique property. The gelatin gels could be formed/shaped under mild conditions (at room temperature and without the use of potentially harmful chemicals), which is particularly important for loading bioactive agents including phages and growth factors into controlled release systems without loss of their activity. These gels could be transformed into hydrogels with desired porous network structures and varying degrees of water absorption/swelling using various crosslinking methods which is an important strategy to increase their mechanical strength, lower the degradation rate and control release of the active agents that they carry; and (iv) gelatin, being a low-cost natural polymer readily available in high purity, is one of the preferred choices.

Combining tissue regeneration therapies and preventing/removal of infections in the same treatment protocol using multifunctional pharmaceutical formulations is a rather challenging strategy. In this perspective, as

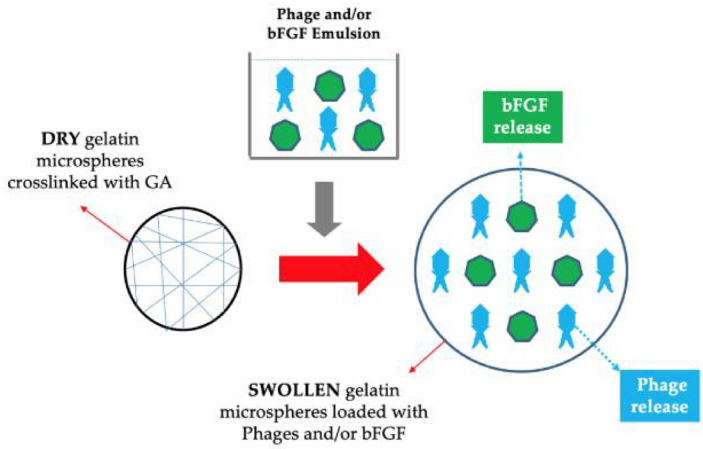

Figure 1. The Graphical abstract: Phage and bFGF carrying/controlled releasing multifunctional gelatin hydrogel microspheres cross-linked with glutaraldehyde.

described schematically in the "graphical abstract" given in Figure 1, we have attempted to develop GELs carrying/controlled releasing two active agents, namely phages as antibacterial agents against infections and/or bFGF as a potent angiogenic factor for fast and correct tissue regeneration - as multifunctional pharmaceuticals.

MATERIALS and METHODS

Materials

The host and target bacteria - *Escherichia coli* (*E. coli K12*) and its specific T4 phages were purchased from American Type Culture Collection (ATCC® 11303™ and 11303-B4 ™, respectively - Manassa, VA, USA). Native basic and acidic gelatins with different isoelectronic point (IEP) 9.0 and 5.0, respectively - with the same weight average molecular weights of 100 kDa were obtained from Nitta Gelatin Co. (Osaka, Japan). Basic fibroblast growth factor - bFGF (Sigma-Aldrich, ABD) with a molecular weight and IEP of 16 kDa and 9.6, respectively was used. ABCAM conjugation kit (Conjugation Kit - Lightning-Link®, ABCAM, ab102884, Cambridge, MA, ABD) was used for Fluorescein 5-isocynate (FITC) labelling. All other chemicals were of reagent grade, obtained also from Sigma-Aldrich (Saint Louis, USA) - and were used as received unless noted otherwise.

Propagation of the target bacteria and its specific phages

E. coli was propagated as described in our previous studies and also by others [31-33], which was briefly as follows: The host bacterial strain was cultured in the Luria-Bertani (LB) broth (25 g of LB powder in one liter) at 37°C in a rotary shaker (200 rpm) until reaching the exponential growth phase with an optical density at 600 nm (to about OD_{600}: 0.5). The medium was transferred into 15 mL sterile tubes and centrifuged at 6000 rpm for about 5 min and the pellets obtained were washed few times and re-suspended in phosphate-buffered saline (PBS, pH 7.2). The bacterial concentration - in colony-forming units (CFU/mL) in the suspension was found by a classical dilution technique [34].

The T4 phages were propagated by using the *E. coli* as the host bacteria prepared in the previous step - as described in the related literature [32-38], which was briefly as follows: 100 µL of *E. coli* freshly prepared with a concentration of 10^8 CFU/mL and 100 µL of T4 phage (from the stokes) with a concentration of 10^8 PFU/mL were mixed and then incubated at room temperature for 15 min and then added to the LB medium (supported with $CaCl_2$ and $MgCl_2$ - 0.001 M each). The mixture was incubated for 6 h at 37°C in a shaking incubator (200 rpm). For purification, the medium was first ultrafiltered through a sterile 0.22 µm filter and

then centrifuged at 4°C - 13,600 g for 20-30 min. The purified phages were re-suspended in sterile PBS buffer (pH:7.2) or in SM buffer (0.1% gelatin, 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5) and stored at 4°C until use. Phage concentration/titers - denoted as "plaque forming unit per millilitre" (PFU/mL) was determined by a "plaque assay" as follows [32,33,39,40]: Phage emulsions with different concentrations were prepared by dilution of the initial phage suspension, 100 µL from each one and 400 µL of *E. coli* suspension were mixed and added to LB medium (semi-liquid - agar 7.5 g/L) and incubated at 37°C for 24 h, and the lysis plaques were counted. The phage with a concentration of 10^8 PFU/mL was stored at 4°C until use.

Activities of the T4 phages propagated in the previous step were obtained in the "soft agar over layer" tests [32,33,35,41]: T4 phage nanoemulsions were dropped on the petri dishes carrying the *E. coli* overlayers on the soft agar in the culture dishes and incubated at 37°C overnight. Note that the *E. coli* lawn plates prepared were originally turbid. However, when *E. coli* were destroyed by the phages - transparent zones were formed due to lysis of the bacteria which was presented to exhibit the effectiveness of the phages.

Preparation of the GEL microspheres

A two step protocol was applied as described by Tabata and his group [42-44]: In the first step, 20 mL gelatin solution 10% w/v was heated up to 40°C and then dropped into about 600 mL of a special olive oil dispersion phase (Wako Pure Chemical Industries, Ltd., Wakayama, Japan) and then dispersed by vortex mixing to yield a water-in oil-dispersion. The dispersion was cooled down to 4°C and cold acetone was added to the dispersion medium and stirring was continued for about 1 h to reach gelation of gelatin. In order to remove the residual olive oil, the gelatin gel beads were washed three times with cold acetone by also centrifugation (at 5000 rpm at 4°C for 5 min) and air-dried and stored at 4°C. The GEL hydrogel microspheres were separated into fractions with different sizes using sieves with different apertures (Seisakusyo Ltd, Osaka, Japan) and the fraction between 32-53 µm was used in the following experiments.

In the second step, the microspheres were chemically crosslinked using glutaraldehyde (GA) as the crosslinking agent with three different GA/GEL ratios, i.e., 0.05; 0.10 and 2.0 mg/mg - to obtain gelatin hydrogel microspheres with three different crosslinking degrees [42]: The dry gelatin microspheres absorbed all of the liquid phase containing GA in the selected concentration, and they were incubated at 4˚C for 12 h. The microspheres were treated with 100 mM glycine at 37˚C in about 1 h to block the unreacted aldehyde groups of GA molecules. They were separated from some possible aggregates by filtration and washed with double distilled water few times and separated from the supernatant by centrifugation. They were then air-dried and stored at 4˚C until use. In order to calculate the average diameter of the microspheres, diameters of about 100 hydrogel microspheres within the sample were measured and the average values with standard deviations were calculated using the Image J (NIH, Bethesda, USA) computer program in the software of the microscope.

The swellabilities and water uptake of the cross-linked gelatin hydrogel beads were assessed as follows: The dried hydrogel beads were swollen in distilled water at 37 °C for 24 h to reach the swelling equilibrium. Water uptake was calculated using the weights of the swollen and dried gelatin beads, presented as a percentage. Photographs of gelatin hydrogel beads in the water-swollen state were captured using a microscope (CKX41, Olympus, Tokyo, Japan). In order to calculate the average diameter of the swollen beads, diameters of about 100 hydrogel beads within the sample were measured, and the average values with standard deviations were calculated using the Image J software (NIH, Bethesda, USA) of the microscope.

Degradation of the GEL microspheres

Both acidic hydrolysis (in HCl) and enzymatic degradation (using collagenase) of the GEL microspheres produced were investigated. The facilitated degradation/acidic hydrolysis of the microspheres within HCl was studied by the following protocol [42]: 5 mg of air-dried crosslinked microspheres were put into a 2 mL tube containing about 750 µL double-distilled water and allowed to fully swell in about 1 h at 37°C. Then, 750 µL 2M HCl was added and incubated at 37°C for different time periods to follow the degradation with time. At selected intervals, the tube was centrifuged at 5000 rpm for 5 min at 37°C, 200 µL of the supernatant was taken, and 200 µL 2M HCl was added into the tube to continue the degradation test. Absorbance of 200 µL supernatant taken from the tube was measured at 260 nm using a UV spectrometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK). By using the absorbance values, the

total mass remaining was obtained and plotted against time to demonstrate the degradation profile.

Enzymatic degradation of the GEL microspheres were studied by using collagenase - 1 mg of the dried hydrogel microspheres was put in 1 mL of collagenase solution containing 0.1 mg enzyme supported with magnesium/calcium ions and incubated at 37ºC [45]. At selected intervals, the tube was centrifuged at 5000 rpm for 5 min at 37ºC, 200 µL of the supernatant was taken, and 200 µL stoke enzyme solution was added into the tube to continue the degradation test. The supernatant was centrifuged at 8000 rpm for 10 min and the gelatin amount within the precipitate was determined by a ninhydrin test which is briefly as follows [46]: 100 µg precipitate was suspended in 200 µL distilled water and 100 mg $Ba(OH)_2.8H_2O$ was added and hydrolysed at 120°C in about 10 min incubation. The supernatant was separated and its pH was brought to 5.1 with 10% of trichloroacetic acid - then 1 mL 2% ninhydrin was added and the complex formation was achieved at 100°C in about 5-10 min incubation. The blue-purple colour intensity (absorbance/OD value) was measured at 570 nm. The calibration curve was prepared by using glycine solutions with different concentrations prepared from 1 mM glycine stoke solution.

Phage and bFGF loading and release within/from the GEL microspheres

For loading of phages within the gelatin microspheres, the following simple protocol was used. T4 phage nanoemulsions with a concentration of 10^8 PFU/mL. 200 µL was added into the tube containing 2 mg of crosslinked/dried GEL microspheres and incubated for 1 h at 37°C. Note that all the aqueous media was completely uptaken by the dried microspheres - because the amount of the aqueous phase much lower than the amount of water in the wholly swollen microspheres - means that loading efficiency was almost 100%.

For release of phages from the GEL microspheres - about 200 mg of the fresh microspheres carrying phages were incubated in 50 mL PBS buffer at 7.4 - by gently shaking for up to 24 h at 37°C. About 100 µL samples were withdrawn from the medium at selected time intervals (replaced with fresh medium). Then the amount of active phages release were followed by a "plague assay" as described above. The cumulative amount of phages released during incubation period was plotted against time to demonstrate the phage release kinetics/modes.

bFGF with a molecular weight and IEP of 16 kDa and 9.6, respectively was firstly labelled with FITC by using the ABCAM conjugation kit by following the protocol described in the company's website. 100 µg FITC was used for 100 µg bFGF. The FITC-labelled bFGF was stored at 4°C'de in dark until use. The GEL hydrogel microspheres were loaded with the FITC-labelled bFGF with a very similar loading protocol described above for phages loading. The FITC-labelled bFGF solution containing 100 µg of bFGF (in PBS, pH:7.4) was sucked up into 10 mg dried gelatin beads during swelling at 4°C, in dark in 1 h. The loading efficiency is 100%.

bFGF release from these GEL microspheres was studied in PBS (pH: 7.4) at 37°C'de in a shaking incubator with a quite low shaking rate for 48 h. The samples withdrawn from the release medium at selected time intervals were centrifuged, and the fluorescent intensities within the supernatant were measured with a fluorescence spectrometer (Perkin Elmer® Inc., Waltham, MA, ABD). By using the fluorescence intensity measurements, the cumulative amount of bFGF released during incubation period was plotted against time to demonstrate the phage release kinetics/modes.

RESULTS and DISCUSSION

***E. coli* and T4 phages propagated**

Escherichia coli (*E. coli*) was used both as the host for propagation of its T4 phage and also as the target bacteria in the activity/performance tests. It was obtained from ATCC and propagated within a LB medium. The optical density (OD_{600}) of 0.5 corresponds roughly a bacterial concentration of 10^8 CFU/mL - which was very efficient to propagate T4 phage nanoemulsion with high concentrations up to 10^9-10^{10} CFU/mL were prepared for the further studies.

The GEL microspheres produced

The GEL microspheres were prepared by a two step process. In the first step the uncrosslinked GEL microspheres were formed by gelation in dispersion of water-in-oil phase. In the second step, these GEL microspheres were chemically crosslinked by using GA with three different GA/GEL ratios (0.05, 0.10 and 0.20 mg/mg). These hydrogel microspheres do not dissolve in water, but do swell at different extends depending on the crosslin-

king density. Representative microscopic image of the GEL microspheres produced in this study are shown in Figure 2.

The optical image given in Figure 2 is an example of hundreds of pictures were taken - after several batch productions - it shows that the GEL microspheres are spherical in shape and had rather smooth surfaces but have always size distrubution - even after sieving as expected as a result of mechanical stirring in the first step during gelation. The average diameters of the crosslinked GEL microspheres obtained after swelling in water and percent water uptake (water content) of the microspheres (% by weight) were 47.5 ± 3.3 µm, 44.5 ± 5.0 µm and 41.2 ± 6.4 µm, and 98.5 ± 1.4, 96.8 ± 1.2, and 93.6 ± 1.4 % respectively which were produced with three different GA/GEL ratios of 0.05, 0.10 and 0.20 mg/mg, respectively.

Acidic and enzymatic degradation of the GEL microspheres

Here we have studied both hydrolytic (using HCl) and enzymatic (using collagenase) degradation of the GEL microspheres - which were prepared with three different crosslinking degrees - just to compare their degradation properties. As seen in Figure 3a, degradation of GEL microspheres in HCl was quite fast and almost 60-90% of the matrices were degraded in 2 h. All three hydrogel microspheres were degraded in about 6-10 h completely. The GEL microspheres with lower crosslinking degrees were degraded faster, as foresighted. But not that significantly different even the GA/GEL ratio range was quite broad in the crosslinking recipe as seen on the graphs - we were expecting much clear differences. The degradation medium was stongly acidic and since dried gels were allowed to swell in that medium at the beginning of the test - means that degradation was strated in whole microsphere matrix therefore very high degradations were observed in all cases.

Similar degradation behaviour - similar to the acidic hydrolysis - was observed in the collagenase degradation tests as seen in Figure 3b. The enzymatic degradations were also quite fast even faster than acidic degradation - about 6-20% of the gelatin matrices were left in about 2 h. Degradation rate decreased when the crosslinking degree was increased. There were certainly observable differences - however all of the GEL microspheres were wholly degraded in 6 h.

Enzymatic degradation was faster then acidic degradation. In general point of view, enzymes are synthesized in the biological systems to trigger degradation rates/fates of their specific target proteins [42,47-49]. Here we have used collagenase for gelatin - it is the denaturated form of collagen. Therefore one expects this result if the gelatin is dissolve in aqueous phase not in the crosslinked microsphere form. There will be a diffusion barrier for the degrading molecules, here HCl or collagenase which will be depended on the crosslinking degree and the final network structure within the microspheres - higher crosslinking means lower diffusion rates. Therefore, one expects that degradation of the GEL microspheres should be faster in the case of HCl -

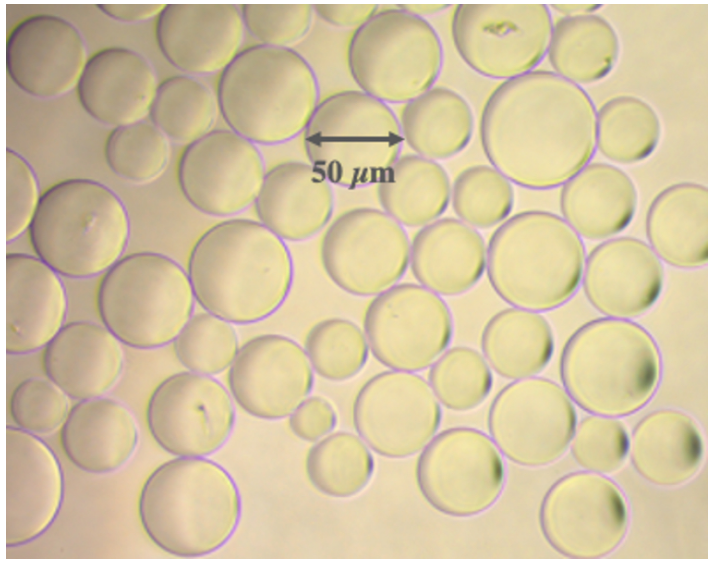

Figure 2. A repsentative image of the gelatin hydrogel microspheres prepared in this study.

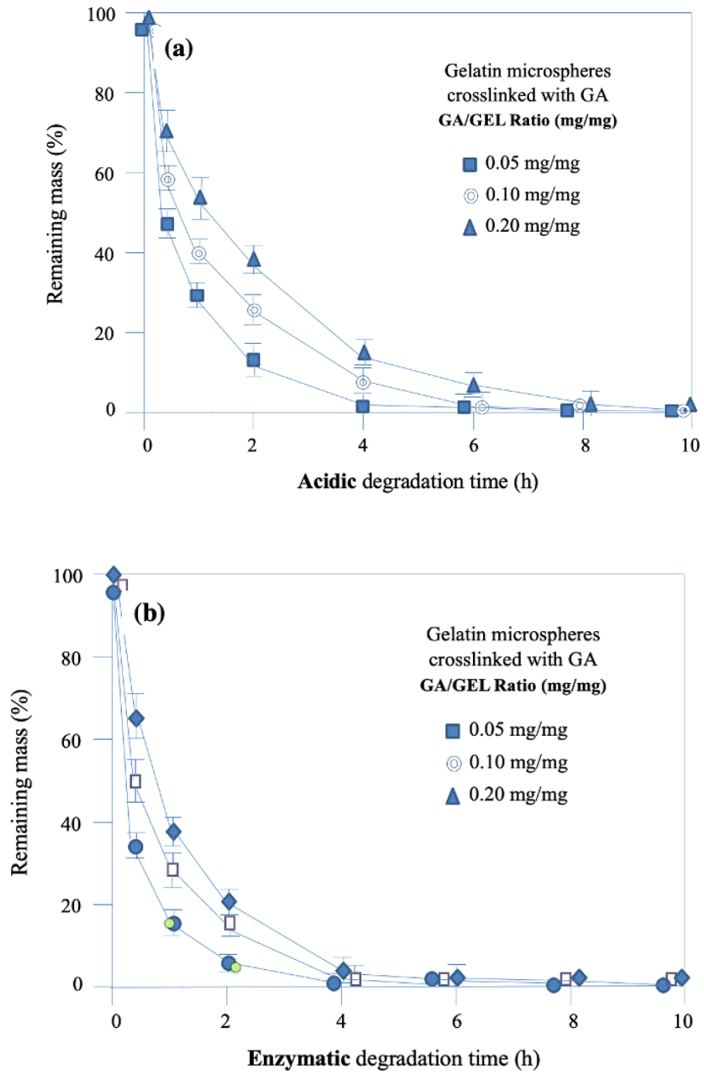

Figure 3. Degradation of the GEL microspheres crosslinked with GA with different GA/GEL ratios: (a) Acidic hydrolysis (in HCl) and (b) enzymatic degradation (using collagenase).

which is much smaller then collagenase enzyme - could much easily diffuse in the gelatin matrices. However, in order to eliminate the diffusion limitation, we have started with the dried GEL microspheres and let the microspheres do swell in the degradation media and sucks up the HCl or collaganese within the matrices. Therefore, collaganese will act quite close to the case in which gelatin solutions. Therefore, we have observed that the enzymatic degradations were faster than degradation rates in HCl - since there was no diffusion limitation.

Collagen/gelatin may be degraded by simple hydrolysis in the body, however, it is a quite slow process. Collagenase is a very specific enzyme that is synthesized when it is needed to trigger the biodegration rate of collagen in different/important bioprocesses like remodelling/regeneration. However, *in vivo* degradation of collagen/gelatin is quite complex - in addition to collagenase, a varity of enzymes - including gelatinases (MMP2 and MMP9), many other proteinases take also important roles - macrophages, polymorphonuclear cells, and other biological entities are involved in the degradation processes [21,42,47-49]. Therefore, it is worthy to note that: it is not possible to extrapolate the results of the degradation fade of the GEL microspheres in HCl or by collagenase given above to *in vivo* degradation. The data that are reported here could be used only to compare the degradability of the GEL microspheres with different crosslinking degrees - not extrapolation to *in vivo*.

Phage and bFGF release from the GEL microspheres

Phage release: The T4 phages propagated in this study were loaded within the GEL microspheres prepared by using native basic gelatin and with three different crosslinking degrees described above. 100% of the phage nanoemulsions (with a concentration of 10^8 PFU/mL) were loaded into the microspheres during the swelling process. The loadings were almost 100% because the aqueous phase volume used was about the volume that the dried microspheres could absorb - that were determined in the pre-tests. Then phage release from those microspheres was investigated in the release tests. Figure 4 shows the representative release behaviour. As expected, about 50-70% of the phages loaded were release in 6 h, and releases were almost completed in 24 h - actually this was the selected target of this study to have a fast respond to the infected wounds. Higher release rates were observed for the less crosslinked microspheres. There were observable differences in the release rates but not that significant in the limits of crosslinking degrees studied.

3D structure of T4 phages and various proteins located at different parts and their functional properties have been identified in detail in the related literature [50-53] - it is the most widely studied phage so far. Due to this highly complex structure and composition it is almost impossible to describe the net electrical charges, their distribution and changes with pH. Isolectric point (IEP) - pKa value of T4 phage capsid (head) has been reported in the range of 4.8-6.2, and the average pKa value of whole phage is around 4 [53-55]. However, it is widely accepted that the head of T4 phage is negatively charged while the tip of the tail is positively charged - in which phages interact with the target bacterial wall (receptors) which is negatively charged - at the physiological pH (around 7.4) with rare exceptions which stems primarly from the ionized phosphate and carboxyl grous localized on the bacterial surface. In this study, in order to reduce electrostatic interactions between gelatin matrix and T4 phages and have a rather fast release in use, only the basic gelatin (IEP: 9.0) was studied. The results demonstrated in Figure 4 approved that it was actually achieved in our studies reported here.

bFGF release: We have also studied loading and release of bFGF within the gelatin beads by applying similar methodologies for bacteriophage loading/release as decribed above. In addition to the GEL microspheres prepared by using basic gelatin (IEP: 9.0) with three different crosslinking degrees and we have also prepared hydrogel microspheres using asidic gelation (IEP: 5.0) and a mixture basic and acidic gelatins (50/50 w/w) in the initial recipe and formed the gels and crosslinked them with GA with a GA/GEL ratio of 0.20 mg/mg. All other components in the recipe and crosslinking protocol was the same. The average diameters and percent water uptake of the microspheres prepared from the recipes contaning acidic or acidic/basic gelatins were around 46 μm and 95%, respectively - quite close to the GEL microspheres prepared using only basic gelatin.

bFGF was labelled with FITC and the FITC-labelled bFGF was loaded within the GEL microspheres described above by just soaking the bFGF solution into the dried microspheres that allowed to reach almost 100% loading - very similar to the T4 phage loading. bFGF release within the PBS buffere (pH: 7.4) was followed by measuring the fluorescence intensities within the supernatant in the release tests.

Figure 5 summurizes the results of the release studies. The bFGF release from the microspheres prepared using basic gelatin with three different crosslinking densities were quite fast - similar to T4 phages as expected. Almost all of the bFGF molecules were released in about 24 h. Higher release rates were observed for the less crosslinked microspheres. There were observable differences in the release rates but not that significant. The bFGF release rates were faster than T4 phages - which may be due to higher diffusion constants for bFGF than T4 phages - because bFGF is much smaller than T4 phages. In addition, the interaction of bFGF - which is positively charged at pH 7.4 (IEP: 9.6) similar to the basic gelatin matrices (IEP: 9.0). The repulsive forces between bFGF and gelatin matrix could affect the fast release of bFGF from those positively charged gelatin microspheres.

Opposite to T4 phages - that are the antibacterial agents loaded in the gelatin microspheres - should be released in the earlier phase when they applied for the treatment of infected wounds - we were aiming sustained release of bFGF from the gelatin microspheres that we have prepared in this study. As mentioned before bFGF is avery important growth factor take important role in many bioprocesses (like angiogenesis) the tissue regeneration [25]. However, it may looses its activity in a very short time when it is used in free form in vivo. Its effectivity should be extended in longer period in the in

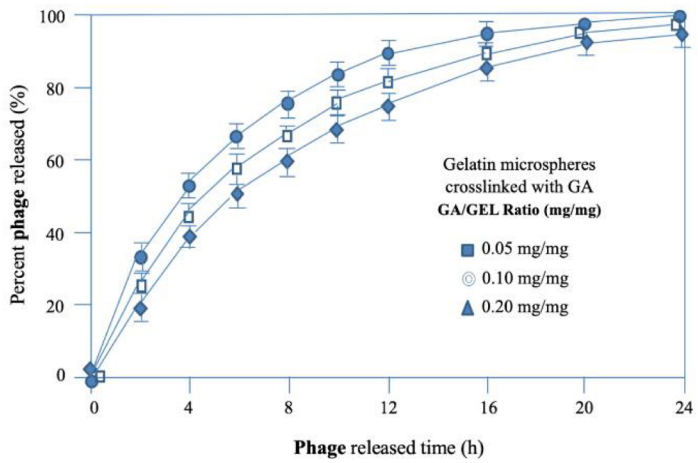

Figure 4. Phage release from of basic gelatin microspheres and crosslinked with GA with different GA/GEL ratios.

vivo regeneration process therefore the most suitable strategy is its sustained release. Therefore, in this part of our studies reported here - we repeated the related studies of Tabata's group and looked at the release behaviour of bFGF also from asidic gelatin (IEP) which is negatively charged at the pH of 7.4. A representative release curve is given in Figure 5.

As seen here, there was a rather fast release rate at the intial period, however after 6 h is slowed down and reached almost a plato. Almost 45% of the bFGF was left on the gelatin matrix even after 48 h. It seems that the rest (shown as plato in the release curves) could be released when the gelatin microspheres are degraded - which could be only in vivo - which could not be extrapolated by just using these in vitro data. Therefore, we should conclude here without any further discussions.

As an original approach, we have applied a new very simple strategy in this study, in which we have prepared the GEL microspheres by using a mixture of acidic/basic gelatins (50/50) - which made an important effect on bFGF release as seen in Figure 6. The release was also sustained but faster than comparing to the ones prepared by using only acidic gelatin. The plato reached was about 65% which was the target of that part of our studies discussed here that should be considered as a simple but important contribution of this study presented here.

CONCLUSION

The main objective of this study is to prepare multifunctional pharmaceuticals to be used in healing of infected wounds which are gelatin based hydrogel microspheres carrying and controlled releasing phages (as antibacterial agents) and/or basic fibroblast growth factor (as a potent angiogenic factor). The GEL microspheres were parepared from both basic and acidic gelatins with different isoelectric points i.e., 9.0 and 5.0, means they are positively and negatively charged at the physiological pH (7.4.), respectively. This was the intiative of presented study, based on the fact on that they do interact with different proteins and other biomolecules/entities having different electrostatic charges at the physiological pH and environmental conditions - that allow us developing controlled - sustained delivery matrices with different release rates/modes for divers bio-applications.

The GEL microspheres with different crosslinking degrees were prepared by using GA as the crosslinking agent. T4 phages and basic fibroblast growth factor were loaded into those microspheres and release kinetics and modes were investigated. The target was release of phages rather fast to have immediate effect on infections on the wound while sustained release of bFGF to reach desired tissue regeneration-healing in time. The phage from the GEL microspheres prepared with basic gelatin were quite sucessful - all the phage content were completely release in about 24 h. Using acidic gelatin in preparation of the GEL microspheres for the

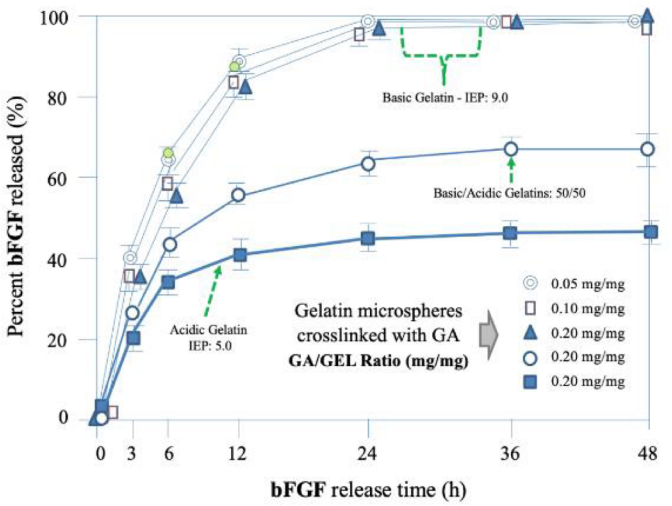

Figure 5. bFGF release from of the hyrdogel microspheres prepared from basic gelatin and crosslinked with GA with dfferent GA/GEL ratios and the ones prepared from acidic gelatin and crosslinked with GA with a GA/GEL ratio of 0.20 mg/mg.

sustained release of bFGF was sucessful that has been already presented in the previous similar literature studies. Using basic and acidic gelatin mixtures to prepare GEL microspheres was new and further contribute the sustained/controlled release of bFGF as demonstrated. This is rather important clue to prepare sustained release formulations with different rates and modes not only for the controlled release of bFGF but other potential candidates e.g., proteins and biomolecules by just simply using basic and acidic gelatins together with different ratios in the initial recipe which is under investigation in our ongoing studies.

Acknowledgments

All kind of supports of TiPhage and NanoBMT are kindly acknowledged. This study was partially supported in the context of TÜBİTAK-TAGEM 1003 project (No: 123O633). Offering high quality of gelatin samples by Nitta Gelatin Inc. (Osaka, Japan) is highly appreciated.

References

1. D.K. Ozhathil, M.W. Tay, S.E. Wolf, L.K. Branski, A narrative review of the history of skin grafting in burn care, Medicina (Kaunas), 57 (2021) 380.
2. L.A. Elfawy, C.Y. Ng, I.N. Amirrah, Z. Mazlan, A.P.Y. Wen, N.I. Md. Fadilah, M. Maarof, Y. Lokanathan, Mh. B. Fauzi, Sustainable approach of functional biomaterials - tissue engineering for skin burn treatment: A comprehensive review, Pharmaceuticals (Basel), 16 (2023) 701.
3. A.M. Jorgensen, N. Mahajan, A. Atala, S.V. Murphy, Advances in skin tissue engineering and regenerative medicine, J. Burn Care Res., 44 (2023) 33-41.
4. J. Hurlow, K. Couch, K. Laforet, L. Bolton, D. Metcalf, P. Bowler, Clinical biofilms: A challenging frontier in wound care, Adv. Wound Care, 4 (2015) 295-301.
5. H.C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice, S. Kjelleberg, Biofilms: An emergent form of bacterial life, Nat. Rev. Microbiol., 14 (2016) 563-575.
6. M. Malone, T. Bjarnsholt, A.J. McBain, G.A. James, P. Stoodly, D. Leaper, M. Tachi, G. Schultz, T. Swanson, R.D. Wolcott, The prevalence of biofilms in chronic wounds: A systematic review and meta-analysis of published data, J. Wound Care, 26 (2017) 20-25.
7. S. Li, P. Renick, J. Senkowsky, A. Nair, L. Tang, Diagnostics for wound infections, Adv. Wound Care (New Rochelle), 10 (2021) 317-327.
8. S. Dhingra, N.A.A. Rahman, E. Peile, M. Rahman, M. Sartelli, M.A. Hassali, T. Islam, S. Islam, M. Haque, Microbial resistance movements: An overview of global public health threats posed by antimicrobial resistance, and how best to counter, Front. Public Health, 8 (2020) 535668.
9. World Health Organization, Global antimicrobial resistance and use surveillance system (GLASS) report 2021, Geneva.
10. C.J.L. Murray, K.S. Ikuta, F. Sharara, L. Swetschinski, G.R. Aguilar, et al., Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis, Lancet, 399 (2019) 629-655.
11. A. Pirisi, Phage therapy - Advantages over antibiotics, Lancet, 356 (2000) 1418.
12. S. Matsuzaki, J. Uchiyama, I. Takemura-Uchiyama, M. Daibata, Perspective: The age of the phage, Nature, 509 (2014) S9.
13. S. Hesse, S. Adhya, Phage therapy in the twenty-first century: Facing the decline of the antibiotic era; is the time for the age of the phage?, Annu. Rev. Microbiol., 73 (2019) 155-174.
14. T. Luong, A.C. Salabarria, R.A. Edwards, D.R. Roach, Standardized bacteriophage purification for personalized phage therapy, Nat. Protoc., 15 (2020) 2867-2890.
15. J.-P. Pirnay, Phage therapy in the year 2035, Front. Microbiol., 3 (2020) 11.

16. S. Royer, A.P. Morais, D.W. da Fonseca Batistão, Phage therapy as strategy to face post-antibiotic era: A guide to beginners and experts, Arch. Microbiol., 203 (2021) 1271-1279.
17. G.F. Hatfull, R.M. Dedrick, R.T. Schooley, Phage therapy for antibiotic-resistant bacterial infections, Annu. Rev. Med., 73 (2022) 197-211.
18. N.J. Anyaegbunam, C.C. Anekpo, Z.K.G. Anyaegbunam, Y. Doowuese, Ch.B. Chinaka, O.J. Odo, H.C. Sharndama, O.Ph. Okeke, I.E. Mba, The resurgence of phage-based therapy in the era of increasing antibiotic resistance: From research progress to challenges and prospects, Microbiol. Res., 64 (2022) 127155.
19. P. Ioannou, S. Baliou, G. Samonis, Bacteriophages in infectious diseases and beyond - A narrative review, Antibiotics (Basel), 12 (2023) 1012.
20. D. Gospodarowicz, N. Ferrara, L. Schweigerer, G. Neufeld, Structural characterization and biological functions of fibroblast growth factor, Endocr. Rev., 8 (1987) 95-114.
21. Y. Tabata, A. Nagano, Y. Ikada, Biodegradation of hydrogel carrier incorporating fibroblast growth factor, Tissue Eng., 5 (1999) 127-138.
22. Q.M. Nunes, Y. Li, C. Sun, T.K. Kinnunen, D.G. Fernig, Fibroblast growth factors as tissue repair and regeneration, PeerJ, 12 (2016) 1535.
23. J.W. Park, S.R. Hwang, I.S. Yoon, Advanced growth factor delivery systems in wound management and skin regeneration, Molecules, 22 (2017) 1259.
24. L.M. Caballero Aguilar, S.M. Silva, S.E. Moulton, Growth factor delivery: Defining the next generation platforms for tissue engineering, J. Control. Release, 306 (2019) 40-58.
25. M. Abdelhakim, X. Lin, R. Ogawa, The Japanese experience with basic fibroblast growth factor in cutaneous wound management and scar prevention: A systematic review of clinical and biological aspects, Dermatol. Ther. (Heidelb), 10 (2020) 569-587.
26. C.M. Echave, L.S. Burgo, J.L. Pedraz, G. Orive, Gelatin as biomaterial for tissue engineering, Curr. Pharm. Des., 23 (2017) 3567-3584.
27. T. Buie, J. McCune, E. Cosgriff-Hernandez, Gelatin matrices for growth factor sequestration, Trends Biotechnol., 38 (2020) 546-557.
28. L. Lukin, I. Erezuma, L. Maeso, J. Zarate, M.F. Desimone, T.H. Al-Tel, A. Dolatshahi-Pirouz, G. Orive, Progress in gelatin as biomaterial for tissue engineering, Pharmaceutics, 14 (2022) 1177-1196.
29. R. Hama, A. Ulziibayar, J.W. Reinhardt, T. Watanabe, J. Kelly, T. Shinoka, Recent developments in biopolymer-based hydrogels for tissue engineering applications, Biomolecules, 13 (2023) 280.
30. F. Milano, F. Masi, M. Madaghiele, A. Sannino, L. Salvatore, N. Gallo, Current trends in gelatin-based drug delivery systems, Pharmaceutics, 15 (2023) 1499.
31. G. Sezonov, D. Joseleau-Petit, A.D.R. Escherichia coli physiology in Luria-Bertani broth, J. Bacteriol., 189 (2007) 8746-8749.
32. F. Moghtader, S. Egri, E. Piskin, Phages in modified alginate beads, Artif. Cells Nanomed. Biotechnol., 45 (2017) 357-363.
33. F. Moghtader, Nanobio-Approaches for the Detection of Bacteria by SERS, PhD Dissertation (no:547231-Supervised: Prof. Erhan Biskin), Hacettepe Univ., Nanotechnology and Nanomedicine Programme, 2019, Ankara, Turkey.
34. E. Goldman, L.H. Green, Practical handbook of microbiology, CRC Press, Boca Raton, Fl, 2015.
35. M.R.J. Clokie, A.M. Kropinski, Bacteriophages: Methods and protocols, Volume 1: Isolation, characterization, and interactions, Clifton, NJ, 2009.
36. N. Bonilla, M.I. Rojas, G.N.F. Cruz, S.-H. Hung, F. Rohwer, J.J. Barr, Phage on tapa quick and efficient protocol for the preparation of bacteriophage laboratory stocks, PeerJ, 4 (2016) 2261.
37. F. Moghtader, G. Congur, A. Erdem, E. Piskin, Impedimetric detection of pathogenic bacteria with bacteriophages using gold nanorod deposited graphite electrodes, RSC Adv., 6 (2016) 97832-91839.
38. F. Moghtader, A. Tomak, H. Zareie, E. Piskin, Bacterial detection using bacteriophages and gold nanorods by following time-dependent changes in Raman spectral signals, Artif. Cells Nanomed. Biotechnol., 46 (2018) 122-130.
39. A. Mazzocco, T.E. Waddell, E. Lingohr, R.P. Johnson, Enumeration of bacteriophages by the direct plating plaque assay, Methods Mol. Biol., 501 (2009) 77-80.
40. M. Panec, D.S. Katz, Plaque assay protocols, Microbe Library, 2013. Available from: http://www.microlibrary.org/ component/ resources/laboratory-test/3073-plaque-assay-protocols.
41. K.L. Hockett, D.A. Baltrus, Use of the soft-agar overlay technique to screen for bacterially produced inhibitory compounds, J. Visualized Exp., 119 (2017) e55064.
42. M. Ozeki, Y. Tabata, In vivo degradability of hydrogels prepared from different gelatins by various crosslinking methods, J. Biomater. Sci. Polym. Ed., 16 (2005) 549-561.
43. K. Hayashi, Y. Tabata, Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions, Acta Biomater., 7 (2011) 2797-2803.
44. S. Tajima, Y. Tabata, Preparation of cell aggregates incorporating gelatin hydrogel microspheres containing bone morphogenic protein-2 with different degradabilities, J. Biomater. Sci. Polym. Ed., 29 (2018) 775-792.
45. Y. Tabata, A. Nagano, Y. Ikada, Biodegradation of hydrogel carrier incorporating fibroblast growth factor, Tissue Eng., 5 (1999) 127-138.
46. R. Mcgrath, Protein measurement by ninhydrin determination of amino acids released by alkaline hydrolysis, Anal. Biochem., 49 (1972) 95-102
47. A.J. Kuijpers, P.B. van Wachem, M.J. van Luyn, J.A. Plantinga, G.H.M. Engbers, J. Krijgsveld, S.A.J. Zaat, J. Dankert, J. Feijen, In vivo compatibility and degradation of cross-linked gelatin gels incorporated in knitted Dacron, J. Biomed. Mater. Res., 51 (2000) 136-145.
48. C. Tondera, S. Hauser, A. Krüger-Genge, F. Jung, A.T. Neffe, A. Lendlein, R. Klopfleisch, J. Steinbach, C. Neuber, J. Pietzsch, Gelatin-based hydrogel degradation and tissue interaction in vivo: Insights from multimodal preclinical imaging in immunocompetent nude mice, Theranostics, 6 (2016) 2114-2128.
49. T. Sutjaritvorakul, F. Wiwatwongwana, P. Imsuwan, A.J.S. Whalley, S. Chutipaijit, Enzymatic degradation of modified gelatin and carboxymethyl cellulose scaffolds, Mater. Today Proc., 5 (2018) 14813-14817.
50. P.G. Leiman, S. Kanamaru, V.V. Mesyanzhinov, F. Arisaka, M.G. Rossmann, Structure and morphogenesis of bacteriophage T4, Cell. Mol. Life Sci., 60 (2003) 2356-2370.
51. L.W. Black, V.B. Rao, Structure, assembly, and DNA packaging of the bacteriophage T4 head, Adv. Virus Res., 82 (2012) 119-153.

52. A. Fokine, Z. Zhang, S. Kanamaru, V.D. Bowman, A.A. Aksyuk, F. Arisaka, V.B. Rao, M.G. Rossmann, The molecular architecture of the bacteriophage T4 neck, J. Mol. Biol., 425 (2013) 1731-1744.
53. M.L. Yap, M.G. Rossman, Structure and function of bacteriophage T4, Future Microbiol., 9 (2014) 1319-1327.
54. J.D. Childs, H.C. Birnboim, Polyacrylamide gel electrophoresis of intact bacteriophage T4D particles, J. Virol., 16 (1975) 652-661.
55. B. Michen, T. Graule, Isoelectric points of viruses, J. Appl. Microbiol., 109 (2010) 388-397.