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Sustained Release of a Biodegradable Alginate Coating Covering Chemically Unbound Gentamicin on Human Bone Allograft; And a Method for Colorimetric Drug Release Measurement

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Abstract

Antibiotic coating of bone substitutes offers a solution in the prevention and treatment of surgical infections. The primary aims of this study were to use bone allografts for antibiotic delivery, investigate the short-term release profile of gentamicin, and develop a biocompatible coating for long-term sustained release. Spongious human bone allografts were used as the antibiotic vehicle. For the short-term drug release coating, the grafts were incubated in antibiotic solution followed by a freeze-drying step. For long-term release, this protocol was modified by adding a water insoluble alginate film layer. In addition, a novel gentamicin determination method was developed by reacting the drug selectively with ninhydrin to form a chromophore suitable for quantitative spectrophotometric measurements. The short-term release from bone allografts lasted up to 48 hours; the total amount of the released antibiotic was 914±142 µg. With the application of the alginate coating, a 50-day sustained release profile was observed (149±4 µg release, approximately 3µg/day). The determination protocol was fast, accurate, and cost-effective for quantitative measurement of gentamicin. We demonstrated a promising method for increasing the local gentamicin concentration with the application of a widely used bone substitute. By increasing the local drug concentration to a sufficient level, this technique may allow the prevention and treatment of infectious complications in poorly perfused tissues, such as bone replacement sites. Furthermore, local sustained release may extend intravenous administration without prolonged and potentially toxic systemic effects.

Keywords: Allograft; Implant; Sustained release; Alginate; Gentamicin

Introduction

Bone substitutes are widely investigated biomaterials due to their high demand from clinicians performing reconstructive surgery in orthopedics, traumatology and dentistry. Fully rebuilding bone is the main goal of bone replacement therapies. The ideal bone substitute is osteoconductive, osteoinductive and osteogenic, and the search for the ideal material is ongoing [1,2]. Because biocompatibility and cost-effectiveness are also highly important, human allograft is frequently used, even though its osteoinductive potential remains in doubt [3,4]. The addition of biologically active molecules like growth factors and proteins (e.g. albumin) to bone substitutes can enhance regenerative potential and thereby decrease healing time [5,6]. Moreover, antibiotics can be loaded onto biomaterials in order to prevent infectious complications associated with bone surgery [7]. The local use of antibiotics could prevent infections by increasing the local drug concentration, but intravenous administration is still the most frequently applied treatment strategy [8,9]. In our previous work, amoxicillin, ciprofloxacin and vancomycin were tested *in-vitro* as possible drugs for local antibiotic delivery. Our results showed that the sustained release of these drugs provides efficient local concentration with a lower dose than is required intravenously. Therefore, this is a promising method for preventing and treating bacterial infections in poorly perfused tissues, such as bone replacement sites [10].

Gentamicin is another well-known antibacterial drug, applied locally as the biologically active ingredient of gels; beads and bone cement [11-15]. Both *in-vitro* and *in-vivo* studies have been conducted, using gentamicin alone or in combination with other substances, like ciprofloxacin [16-18].

Loading and impregnation are the available methods used to associate the antibiotics with the selected material [18]. Loading is the most frequently applied technique for local sustained release application, however, up to now; there has been no literature data available on sustained release coatings [19]. Sodium-alginate (Na-Alg) is a biodegradable, biocompatible polymer already widely used in beads and for encapsulation [20-23]. This polymer can be converted to water insoluble calcium-alginate (Ca-Alg), which is an important feature for sustained release. Together, these properties suggest that Na-Alg is an ideal coating material for sustained release.

In the present work, we designed a gentamicin delivering system providing efficient local drug concentration in a sustained release fashion from a human bone allograft vehicle with a Na-Alg coating. In addition, we developed a cost-effective, sensitive and

accurate colorimetric determination method based on the amino group present in the gentamicin molecule [24,25].This may prove to be an inexpensive alternative for quantitative gentamicin detection compared to the existing analytical methods, like HPLC, ELISA or fluorometry [26].

Materials and Methods

Materials

The chemicals were purchased from Sigma, with the exception of gentamicin sulphate, which was purchased from Molekula.

The bone blocks were provided by the West-Hungarian Regional Tissue Bank, the blocks originated from the femoral head of human cadavers. Freeze-dried femoral head blocks were cut into 50±5 mg cube-shaped pieces under sterile conditions.

MIC measurement

Minimal inhibitory concentration (MIC) was determined on Escherichia coli (ATCC 11303) cells according to Andrews [27]. Briefly, serial dilution of antibiotic from 0 to 25 µg/ml was prepared in molten (50 °C) agar, which were mixed and poured onto Petri dishes. E. coli bacteria from overnight culture were inoculated to the plates so that approximately 104 cfu/spot was applied. Plates were incubated at 37°C for 18 hours. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth of organism.

Antibiotic coating

For the short-term antibiotic release, gentamicin (10 mg/ml) was used in aqueous solution. The bone graft was placed in 1 ml of antibiotic solution and was incubated at 25 °C for 24 hours. Subsequently, the soaked graft, submerged in the antibiotic solution, was frozen at -80 °C followed by lyophilization for 24 hours using a Labconco Freezone 2.5 freeze-drying machine in order to maximize drug content. The alginate coating for long-term release was prepared in two steps. First, the bone grafts were coated with gentamicin as described above; then the alginate film coating was created over the antibiotic layer as follows: Na-Alg solution (1 ml, 4 %) was applied on the surface of the antibiotic coated freeze-dried bone. The graft was dried afterwards at 37 °C for 4 hours on teflon plates. The film coating process was repeated with the dried coated grafts turned upside down, thus a double layer Na-Alg film was formed. Na-Alg was then converted into water insoluble Ca-Alg using CaCl2.To do so, the Na-Alg coated bone grafts were placed in 10 % CaCl2 solution for 60 seconds. In the final step, the grafts were washed with distilled water and dried at 37 °C.

Cromophore introduction

Gentamicin does not have characteristic absorbance in either the ultraviolet(UV) or the visual (Vis) wavelength spectrum, so a Vis absorbing chromophore group specific and selective for gentamicin was introduced in order to measure the antibiotic concentration using a spectrophotometer. It was also important to choose a reagent that does not interfere with the materials present in the test preparation, including the human bone allograft, Na^+ and Ca^{2+} ions and the alginate. For these reasons we used ninhydrin reagent (2,2-Dihydroxyindane-1,3-dione), which is a sensitive reagent for the colorimetric determination of amino groups (Figure 1).

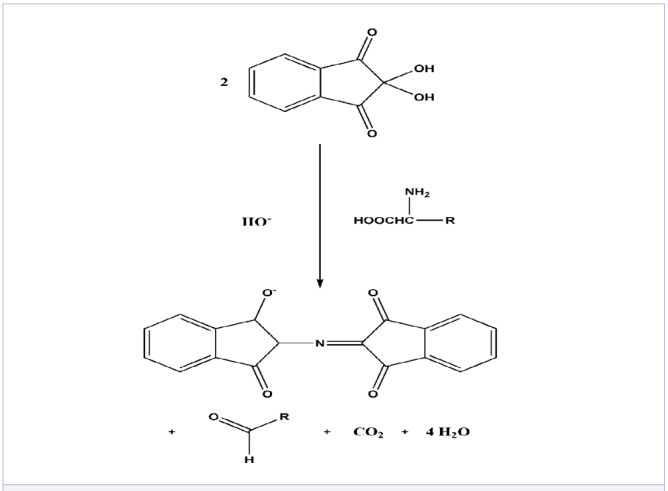

Figure 1: The ninhydrin reaction
The presence of primer amino groups is essential for the ninhydrin reaction.

This highly sensitive reagent is generally used to detect the amine content of fingerprints [28]. Since the ninhydrin reagent is so sensitive, special care was taken not to add any contaminating material containing additional reacting amino groups.

Optimal setup

The following possible effects were studied to optimize the conditions of the reaction:

- Solvent type (amount of EtOH, water and ninhydrin)
- Reaction parameters (time, temperature)
- Gentamicin and ninhydrin concentration
- Linear absorbance-concentration interval
- Interfering materials (Na-alginate, Ca^{2+}, Na-citrate)

During the optimization, the antibiotic concentrations were determined according to Lambert-Beer's law; briefly, optical density was plotted against concentration, and the antibiotic concentration was assessed from the corresponding absorbance in the resulting linear function. Absorbances were plotted in the 1 – 1000 µg/ml concentration range.

Release measurement

After developing the most cost- and time-efficient analytical protocol, gentamicin release was measured from the samples without the alginate coating. Six samples were put in a 24-well plate and 2 ml water was added to measure the elution at RT. Gentamicin concentration was assessed after 1, 24 and 48

hours. For the long-term release of the alginate coated samples, 11 pieces of prepared bone allografts were used, and samples were taken after 1, 5, 15, 25, 35, 45, and 50 days. The absorbances were assessed in all cases with a Biotek PowerWave XS spectrophotometer. To confirm the accuracy of the measurement, the remaining coating was removed, and unreleased gentamicin was measured after transforming the Ca-Alg coating back to Na-Alg. This was performed using previously described procedures with EDTA or Na-citrate [29,30]. Both methods were tested, but EDTA showed interference with the ninhydrin reagent, therefore, Na-citrate (100 mM) was chosen for this procedure. In order to evaluate possible noise from macroscopic particles, SPE filtering (Supelco, Hybrid SPE), centrifugal membrane filtering (Amicon, Ultra, 3K) and sedimentation of the supernatant (15000 rcf) were also performed and showed no significant change in the purity.

Statistical analysis

The statistical analysis was performed with t-test, simple analysis of variance (ANOVA) and two-way ANOVA with Bonferroni multiple comparison tests using the GraphPad Prism 5.0 statistical software. Probability values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant. All of the values are reported as the mean ± SEM.

Results

Optimal parameters

To choose the appropriate wavelength for the ninhydrin reagent, the absorbance spectrum of the original material and the product were assessed (Figure 2A). The optimized reagent contained 100 µl gentamicin, 60 µl 2% ninhydrin solution and 60 µl water. The finalized reaction parameters were 100 °C (boiling water) for 15 minutes. By comparison of pre- and post reaction absorbances at 560 nm wavelength, linear plots and sufficient accuracy were found in the 10 - 100 µg/ml range (Figure 2B).

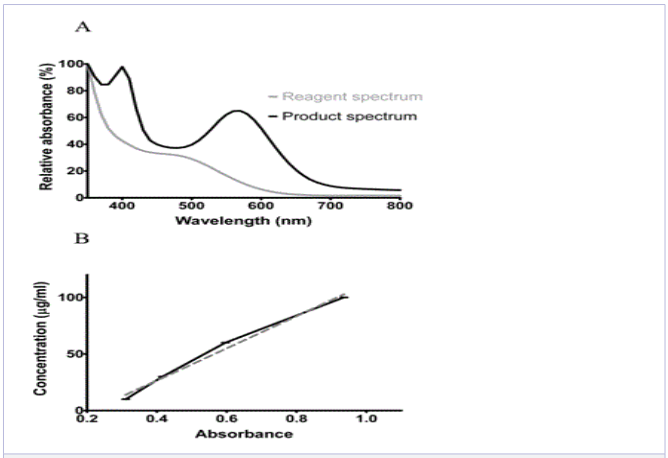

Figure 2: Characterization of the spectrophotometric measurement
Panel A shows the difference between the absorbance spectrum of the original ninhydrin reagent and the product. The appearance of the peak at 560 nm is characteristic for the product. Panel B shows the linear interval of the gentamicin concentration – absorbance diagram, between 10 and 100 µg/ml.

Interfering materials

During the optimization procedure Ca^{2+} and Na-citrate were tested to determine whether they change the accuracy of the measurement. We found that if the solution contains 10 w/w% of Ca^{2+}, it significantly modifies the accuracy of the detection (Figure 3A). In the experiments, however, the Ca^{2+} concentration remained under 1 w/w%, which would have negligible effect on the detection. Na-citrate in the concentration of 10 mM was not found to have any significant effect on the reaction, while 25 mM significantly increased the absorbance (Figure 3B).

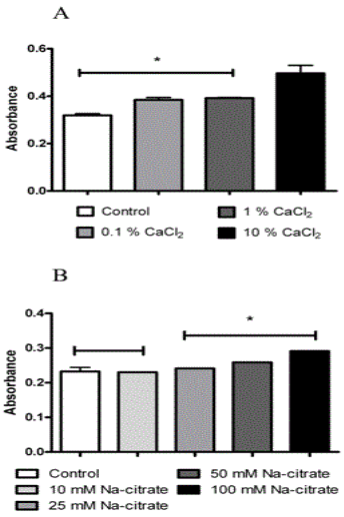

Figure 3: The effects of interfering materials
Panel A shows the effect of the increasing Ca^{2+} concentration on the ninhydrin reaction. There is no significant difference when the Ca^{2+} content is between 0 to 1%. Significant interference can be seen at 10 % Ca^{2+} content. Panel B shows the effect of Na-citrate on the gentamicin measurement. Determination is unaffected in the 0 to 10 mM interval, but from 25 mM, the interference becomes significant.

In our setup, 100 mM Na-citrate solution was used to remove the coating, but this was diluted 10 times for the actual measurement. Consequently the interference of Na-citrate in the applied concentration can also be disregarded. In addition, the bone itself might interfere with the measurement because of the amino groups in the bone collagen. However, since collagen is water insoluble, a simple centrifugation step is enough to prevent its interference in the present setup.

Short-term release

The results from the short-term release profile showed that nearly all the gentamicin was dissolved in 48 hours in the absence of alginate coating. The total amount of the dissolved gentamicin was914±142 µg per bone graft (Figure 4).

Long-term release

The release profiles from alginate-coated specimen have showed a much slower rate of release. The total amount of gentamicin used for the process was 1156±254 µg.Only 149±4 µg (33.65 %) of the starting gentamicin was released during the 50-day sustained release period, 372±73 µg gentamicin was under

the coating (Figure 5). This means that 521±76 µg was the total gentamicin amount under the Ca-Alg coating (Figure 6).

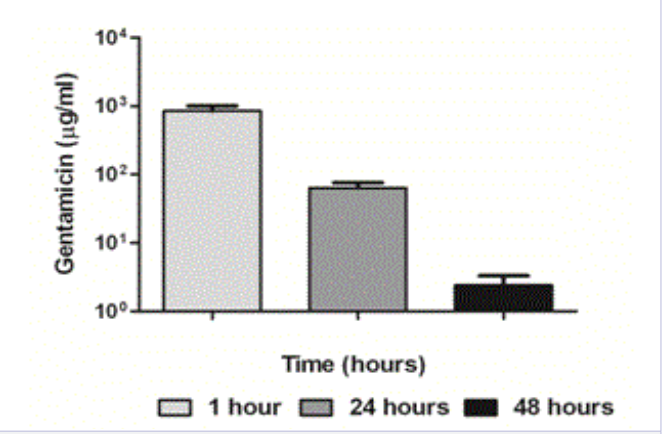

Figure 4: Short-term antibiotic release
The release profile of simple freeze-dried antibiotic coatings. The total amount of gentamicin is released within 48 hours; 6 samples were used.

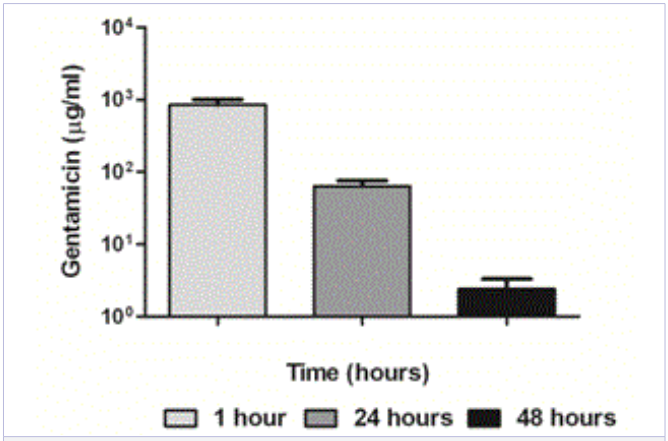

Figure 5: Long-term antibiotic release
The release profile of the gentamicin alginate coating. The Ca-Alg film layer releases 149±4 µg gentamicin during the 50-day interval, 11 samples were used.

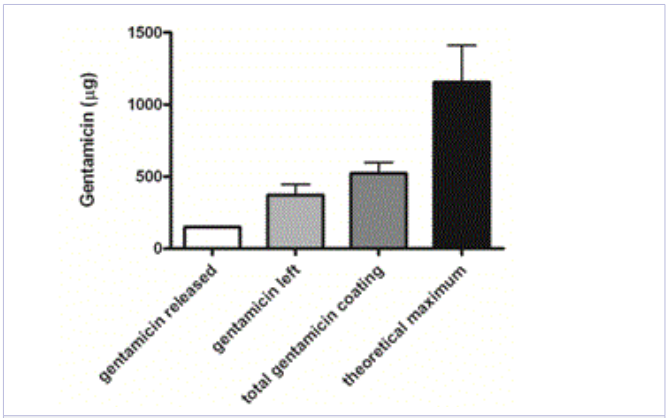

Figure 6: Summary of the sustained release coating
After the 50-day period, only 33.65 % of the starting gentamicin was released, 372±73 µg gentamicin remained under the coating. 521±76 µg was the total amount of gentamicin in the antibiotic coating, 1156±254 µg was the total amount used for the process, and 11 samples were used

MIC results

Minimal inhibitory concentration (MIC) was determined on Escherichia coli (ATCC 11303) cells with the result of 3 µg/ml for the gentamicin that was used in the experiments.

Discussion

Cromophore evaluation

In the present work, we developed are liable colorimetric method to measure gentamicin concentration with visible light spectroscopy. The two characteristic functional groups present in gentamicin are the aminoglycoside group and the amino group. These groups can be converted to chromophores absorbing visible light between 400 and 800 nm to make the gentamicin molecule detectable by a spectrophotometric analysis. After preliminary studies, the amino group was chosen because neither the short-term release, nor the sustained release mixture contained water-soluble amino groups. Using the ninhydrin reagent as a chromophore, the functional groups in gentamicin were converted to products absorbing visible light with a specific absorbance spectrum.

Release profiles

This method allows us to quantify elution of gentamicin from coated bone allografts. Two types of gentamicin applications were tested: The first method was successful in creating a short-term release coating by freeze-drying gentamicin on the allograft surface. In the second method, we developed a sustained-release coating by turning Na-Alg on bone allografts into a water insoluble calcium-alginate coating. The colorimetric determination was complicated as the number of possibly interfering materials increased in the experiment. Therefore, only specific and sensitive reagents were used, and all of them were tested for interference.

Functional group evaluation

It is important to note that the aminoglycoside group is another specific functional group present in the molecule of gentamicin. This part of the molecule is more specific when compared to the amino group, but according to a previous study, the reaction is not sensitive enough, meaning that the detectable limit is below 200 µg/ml [25]. Therefore, the ninhydrin reagent could be safely chosen for our measurement, which was optimized based on Frutos's data [24]. With ninhydrin, the linear absorbance interval fell in the 10 - 100 µg/ml range, so any sample containing over 100 µg/ml gentamicin had to be diluted.

Reagent decomposition

One disadvantage of this method is the rapid decomposition of the ninhydrin reagent. In order to test this phenomenon, 200 µl fresh 2% ninhydrin reagent was added into a 96-well plate, and the decomposition was detected by measuring the absorbance every 10 minutes at 500 nm wavelength. Significant decomposition was detected after 12 hours, with only 71.07 % of the starting material remaining at room temperature at atmospheric air with light exclusion. This indicates that freshly prepared reagent stored at 4°C under inert

gas with light exclusion should be used for optimal results.

Theoretic therapeutical application

Taking into consideration that the minimal inhibitory concentration of gentamicin against P. aeruginosa is 2 μg/ml and 0.5 μg/ml for MRSA, we calculated that approximately3 μg gentamicin should be released daily from a 50 mg allograft over the 50-day interval [31,32]. The systemic effects of this amount should be negligible, since a higher dosage of intramuscular injections (1.5 mg/kg, twice a day for 3 weeks) were tested and showed no negative side effects in a rat model [33]. The main example for the human therapeutical use of local antibiotics is PMMA beads, which is considered to be a valuable product and compared to PMMA is was also discovered that the antibiotic uptake of human bone exceeds the vancomycin uptake of PMMA 10 times [34,35]. According to this, sustained release is a safe method that provides an efficient local antibiotic concentration.

Limitations of the study

The limitations of this current study come from its *in-vitro* nature, since it does not take into account possible enzymatic reactions or other in-vivo processes affecting the degradation process of the sodium-alginate coating. Thus there is still the need of finding a model, which would bring the in-vivo release closer, however our group has only validated the ninhydrin method to our simplified model and we assume that the rate of the release will certainly increase among more realistic circumstances. Introducing the gentamicin-coated bone allograft in cell cultures and in-vivo models would be the next step to strongly support the present findings.

Conclusion

We have successfully prepared an antibiotic bone coating, which may prevent bacterial infections after bone replacement therapies. In addition, we have developed both a short-term and long-term antibiotic release formulation. The short-term formulation maybe effective in preventing primary infections, while the long-term antibiotic release may be an effective treatment in revision bone surgery. Intravenous application may at least partially be replaced by this coating, which has the benefit of reducing treatment time and sparing potentially toxic prolonged systemic administration. We also developed a cheap, fast, selective and accurate method to cost-effectively determine gentamicin concentration *in-vitro.*

Acknowledgement

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