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# Bacterial safety study of the production process of hemoglobin-based oxygen carriers

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## Abstract

Hemoglobin microparticles (HbMP) produced with a three-step procedure including co-precipitation of hemoglobin with manganese carbonate, protein crosslinking and dissolution of the carbonate template were shown to be suitable for application as artificial oxygen carriers. First preclinical safety investigations delivered promising results. Bacterial safety plays a decisive role during the production of the HbMP. Therefore, bioburden and endotoxin content of the starting materials (especially hemoglobin) and the final particle suspension are intensively tested. However, some bacteria may not have been detected by the standard tests due to low concentration. The aim of this study was to investigate how these bacteria would behave in the fabrication process. Biocidal effects are known for glutaraldehyde and for

ethylenediaminetetraacetic acid, chemicals that are used in the fabrication process of HbMP. It could be shown that both chemicals prevent bacterial growth at the concentrations used during the HbMP fabrication. In addition, the particle production was carried out with hemoglobin solutions spiked with *Escherichia coli* or *Staphylococcus epidermidis*. No living bacteria could be detected in the final particle suspensions. Therefore, we conclude that the HbMP fabrication procedure is safe in respect of a bacterial contamination.

## Keywords

hemoglobin; microparticles; bacterial safety; EDTA; glutaraldehyde

## Introduction

Artificial oxygen carriers used as a red blood cell (RBC) substitute have attracted particular attention in the last years. Many of the different approaches are using hemoglobin as a starting material to produce hemoglobin-based oxygen carriers (HBOC). Thus, human, animal, especially bovine or recombinant hemoglobin is used, which can then be chemically modified, crosslinked, polymerized or encapsulated by various methods [1–3]. We produce biopolymer microparticles as HBOC with the simple CCD technique while utilizing hemoglobin. The three main steps of this procedure are **c**o-precipitation, **c**rosslinking and **d**issolution (CCD). Depending on the biopolymer used, there are also various other possible applications for these microparticles. For example, enzyme particles have been produced that can be used as microreactors or biosensors [4]. This method can also represent a promising approach to the production of drug carriers by precipitation of the favorable

biopolymers and corresponding surface modifications [5, 6]. Thus, it was possible to immobilize vitamin B2, riboflavin, in these particles together with human serum albumin (HSA). This resulted in a drug delivery system with good hemocompatibility and release of riboflavin over a prolonged period [7]. In addition, HSA microparticles could be loaded with doxorubicin, a cytostatic drug used in chemotherapy for cancer treatment. These particles showed higher efficacy in inhibiting metabolic activity in cell culture than free doxorubicin alone [8]. For the use as an artificial oxygen carrier hemoglobin is isolated from bovine blood. Compared to human hemoglobin, it is available in large quantities and free of human pathogens. A comprehensive concept for the biosafety of the pharmaceutical starting material bovine hemoglobin has been developed considering the recommendations of the local authorities (Paul-Ehrlich-Institut, Langen, Germany). Among other things, the focus is on the origin and traceability of the bovine blood back to the individual animal. It is derived in Tyrol, Austria, an area that is free of bovine spongiform encephalopathy (BSE) according to the World Organization for Animal Health (OIE) [9]. In addition, the animals are declared fit for human consumption after a post-mortem inspection. In the geographical area where the blood is collected, many critical viral pathogens do not occur [10]. Nevertheless, the blood is tested for viral contamination. In terms of bacterial safety, the blood and the hemoglobin obtained from it are tested for bacterial load. In addition, the hemoglobin is tested for endotoxins before use as starting material. Only if all specifications are met, the hemoglobin is used for the fabrication of hemoglobin microparticles (HbMP) by means of the CCD-Technique [11, 12]. During the first step, the co-precipitation, two salt solutions and the hemoglobin are mixed. A salt template is created in which hemoglobin is trapped. In the next main step, crosslinking, the individual hemoglobin molecules are polymerized in the salt template. The third step is

dissolution. Dissolving of the salt template by adding EDTA results in sub-micrometer hemoglobin particles. The resulting particles have an advantageous oxygen affinity and show a narrow size distribution around 700 nm. In first pre-clinical investigations it was shown that HbMP meet the requirements as a novel artificial oxygen carrier for application as a blood substitute and are considered non-mutagenic by different *in vitro* and *in vivo* studies [13].

Although all substances used to produce HbMP are of pharmaceutical grade or approved drugs (human serum albumin - HSA) except for hemoglobin, sterility of the final product is not guaranteed. Terminal sterilization of the hemoglobin as well as the particle suspension with standard methods of heat inactivation, UV-C irradiation or gamma irradiation all led to a denaturation of the hemoglobin or to an enormous formation of methemoglobin due to oxidation of the iron in the heme group. Methemoglobin is not able to release oxygen [14, 15]. It is therefore no longer suitable for the use in the production of HbMP applied as an artificial oxygen carrier. Since the aforementioned methods cannot be used, the hemoglobin solution is sterile filtered after production and tested for endotoxin content and bioburden. This way, bacterial contamination is relatively unlikely. Nevertheless, it is conceivable that a minimal amount of bacteria will not be detected by the tests. In this case, it would be advantageous if any additional depletion could be achieved by steps in the production process. In comparison with the manufacturing of human blood products, it should be noted that the starting materials can be tested or are declared by the manufacturer to be sterile or endotoxin-free. The subsequent manufacturing process for human blood products is so safe that random testing of the products is sufficient. Adopting this principle each batch will be tested, but not each single product.

Endotoxin or the lipopolysaccharide (LPS) of the outer membrane of gram-negative bacteria is another important point in the safety concept. Endotoxins could potentially be introduced into the production process by the starting substances. Also, the depletion of any bacteria potentially present in the process could also cause the LPS to be released. One *Escherichia coli* cell has approximately 10 - 50 fg LPS [16, 17]. One endotoxin unit (EU) corresponds to 100 pg *E.coli* LPS or a bacterial count in the range of $10^4$/mL [18, 19]. According to the US and European Pharmacopoeia, the endotoxin limit for intravenous administration of a drug is 5 EU/kg. Taking a model human body of 70 kg, this leads to a dose of 350 EU per administration. If one wants to administer a quantity of 250 mL of HbMP, the suspension must not contain more than 1.4 EU/mL, for an administration of 500 mL, correspondingly 0.7 EU/mL. Our limit for endotoxin load is 0.5 EU/mL and is tested before release of the product.

The production process includes several washing steps that could contribute to a depletion of the potential bacterial load. Glutaraldehyde (GA) is used for the inter- and, to a certain extent, intramolecular cross-linking of the hemoglobin molecules in a concentration of 0.02 % [20, 21] . It is known to have an anti-bacterial effect and it is used as a disinfectant or for cold sterilization of medical instruments in hospitals in higher concentrations [22–24]. It is also widely used in biochemical applications and as a fixative for electron microscopy [25, 26].

EDTA is used in the HbMP fabrication process to dissolve the manganese carbonate template to produce the pure protein particles. EDTA is widely utilized in medicinal and biological applications. Because of its chelating properties, it is used to anti-coagulate blood samples [27]. It has also long been used to permeabilize the cell wall of gram-negative cells [28, 29]. A certain inhibitory effect of EDTA on the growth of *Staphylococcus epidermidis* could also be shown [30].

Therefore, the aim of this work was to investigate whether the steps of crosslinking with GA or dissolution with EDTA in addition to the washing steps in the particle production process can contribute to a reduction of a potential contamination with gram-positive and gram-negative bacteria. *Staphylococcus epidermidis* and *Escherichia coli* were selected as model organisms for this purpose. Both bacteria have been intensively studied. *E. coli* is mainly found in the intestines of humans and animals, is gram- negative and has an approximate length of 2 µm with a diameter of 1 µm and a cylindrical shape [31]. *S. epidermidis* lives on human skin but is also frequently responsible for infections of immune compromised patients in the hospital [32, 33]. This gram-positive bacterium has a spherical shape and a diameter of 0.5 – 1.5 µm [34]. In addition, both bacteria play a role in adverse transfusion reactions [35]. Due to storage conditions at room temperature, mainly platelet concentrates are affected [36, 37]. Here, contamination with *S. epidermidis* occurs in particular due to colonization of the skin and inadequate disinfection of the puncture site during blood donation [38, 39]. As a result, bacterial contamination that is not detected by testing can occur during the preparation of blood products as well as during the production of HbMP.

To investigate the possible inhibitory effects of the chemicals used in the CCD process we assessed the growth of bacteria with addition of GA and EDTA to the growth medium. In addition, HbMP fabricated with bacteria spiked hemoglobin were produced and the bacterial load was examined at every step of the particle production process.

# Results and Discussion

Various tests were carried out to find out whether any bacterial contamination that may be present is removed during the HbMP production process. For this purpose, the influence of the chemicals glutaraldehyde and EDTA on gram-negative and gram-positive bacteria was investigated in preliminary experiments. Glutaraldehyde is used in the HbMP manufacturing process to cross-link proteins. EDTA is used to dissolve the carbonate template.

In order to check whether glutaraldehyde and EDTA have an influence on the bacterial safety of HbMP, growth tests were first carried out with gram-positive and gram-negative bacterial cultures in the presence of these substances. The model organism *Escherichia coli* was chosen as a representative for gram-negative bacteria, and *Staphylococcus epidermidis* served as an example of a gram-positive bacterium.

Additionally, HbMP were prepared with spiked hemoglobin solution as well as under standard process conditions as a control.

## HbMP – Size, Zeta-Potential, Morphology

In addition to particle preparation with spiked hemoglobin, particles were also prepared using the standard protocol. The CCD method produces nearly uniform, peanut-shaped particles. The size distribution determined by DLS was 759 nm ± 25 nm. CLSM images confirm this size range (Figure 1 C). SEM images of particles produced with the CCD method after precipitation as well as after crosslinking, dissolution and final washing steps are shown in Figure 1 A and B.

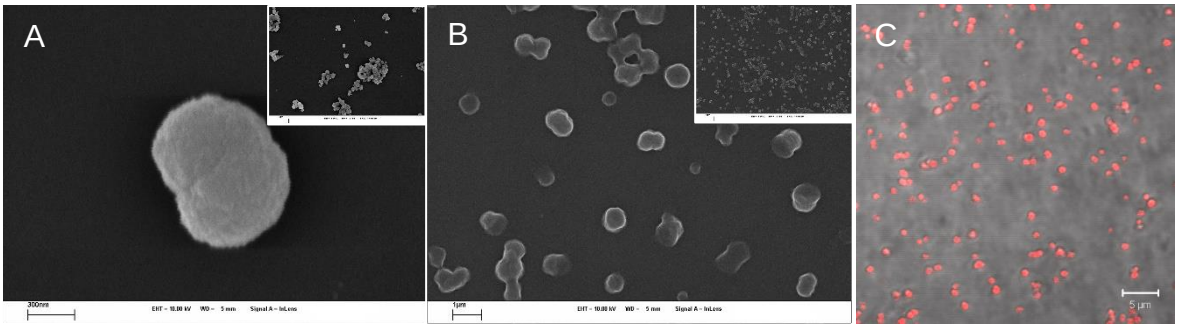


**Figure 1.** Microscopic images of HbMP. A – SEM image of particles after precipitation with carbonate template. B – SEM image of final HbMP after precipitation, crosslinking, dissolution and washing. C - CLSM image with autofluorescent HbMP. Scale bars: (A) 300 nm, (B) 1 µm, (C) 5 µm; insets with overview of the particle samples: 2 µm (A), 3 µm (B).

The zeta potential of HbMP in PBS pH 7.4 was -8.51 ± 0.9 mV. Zeta potential in PBS pH 7.0 of *E. coli* is -16 mV, that of *S. epidermidis* -8 mV [40]. Thus, both the HbMP and the bacteria show a negative zeta potential and a strong aggregation due to different charges seems unlikely. The bacteria have a larger diameter than the approximately 750 nm of the particles. In the CCD process, there are some centrifugation steps in the preparation of the HbMP. Based on the sizes, it can be assumed that the bacteria are thereby in the fraction of the sediment after centrifugation where the particles are also found.

## Influence of glutaraldehyde on bacterial growth

*E. coli* cells cultivated with 0.02 % Glutaraldehyde at 37 °C showed a significantly reduced growth compared to the control in normal growth medium (Figure 2 A). However, the growth of bacteria was stronger than that in the negative control with peracetic acid. This means that multiplication of bacteria still occurred to some degree.

Glutaraldehyde inhibits viable functions so that the bacteria are subsequently unable to proliferate, but it also fixes the cell wall components. Thus, the cells are not lysed. The presence of bacteria was detected in the experiments by determining the optical density. The fixed but dead cells thus explain the higher signal compared to the negative control. The cultivation of *S. epidermidis* with glutaraldehyde at 37 °C delivered similar results (Figure 2 C). Glutaraldehyde inhibits the proliferation of bacteria significantly under the given conditions.

During cultivation of *E. coli* with the addition of glutaraldehyde at room temperature, there were also significant differences in growth rates compared to the control (Figure 2 B). After a small increase in optical density after the measurement point at 30 min, there was no further growth of the cells. In contrast, the cells in the control in normal growth medium continued to grow strongly over the course of the experiment. For better comparability, the optical density values here were normalized to the respective initial value. A similar, albeit not as pronounced, picture emerged when *S. epidermidis* was cultivated at room temperature (Figure 2 D). Here, too, the optical density increased after the measuring point at 30 min, almost to the range of the control. However, it subsequently remained at this level while the control cells continued to grow. The smaller difference between control and cells cultured with glutaraldehyde compared to the *E. coli* growth curves could also be due to the longer generation time of *S. epidermidis*.

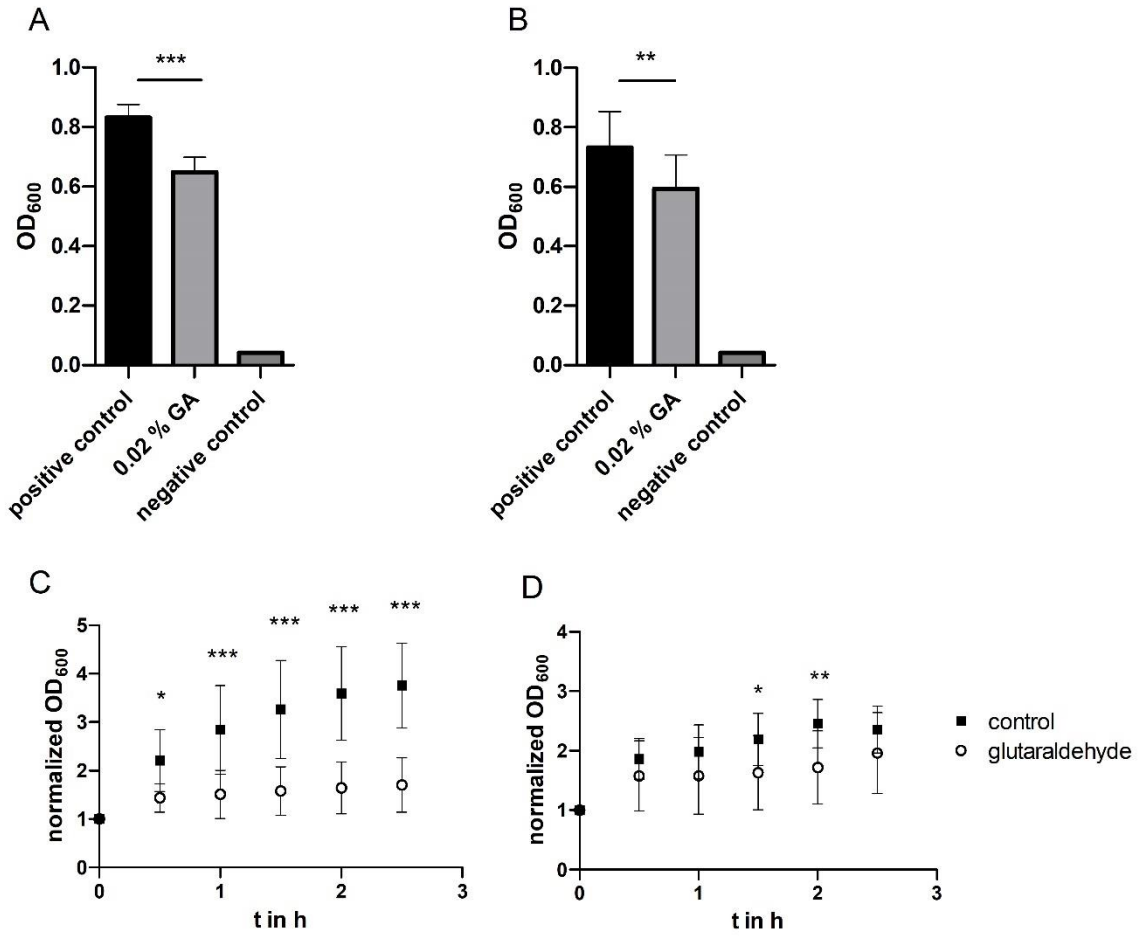


**Figure 2.** Effect of glutaraldehyde on bacterial growth of *E. coli* and *S. epidermidis*. *E. coli* (A) and *S. epidermidis* (B) cells were incubated for 20 h at 37 °C in Mueller-Hinton II-Bouillon (positive control); Mueller-Hinton II-Bouillon + 0.02 % GA or Mueller-Hinton II-Bouillon + 0.4 % peracetic acid (negative control). Error bars are SD (n = 12 biological replicates). Statistical analysis was performed by a one-way ANOVA with Bonferroni's multiple comparison test, SD for negative controls < 0.0015.

*E. coli* (C) and *S. epidermidis* (D) cells were pre-incubated for 20 h at 37 °C in Mueller-Hinton II-Bouillon. Afterwards the growth medium was removed and replaced by fresh Mueller-Hinton II-Bouillon ("control") or Mueller-Hinton II-Bouillon + 0.02 % GA ("glutaraldehyde"). Cells were grown for 2.5 h at room temperature. Error bars are SD (n = 12 biological replicates). Statistical analysis was performed by a two-way ANOVA

with Bonferroni's multiple comparison test; * corresponds to $p < 0.05$; ** corresponds to $p < 0.01$; *** corresponds to $p < 0.001$.

Glutaraldehyde in higher concentrations is widely used as a biocide. It is mainly applied to disinfect surfaces or medical instruments [41–43]. Glutaraldehyde is applied in the manufacturing process of HbMP to crosslink hemoglobin molecules. This crosslinking is also the main reason for the biocidal effect. Glutaraldehyde reacts strongly with proteins and can inhibit DNA synthesis in bacteria, similar effects are also seen on RNA and protein syntheses [44, 45]. In addition, glutaraldehyde acts particularly on the outer layers of *E. coli* and crosslinks lipoproteins and proteins there as well. This fixation of the bacteria prevents the bacterial cells from multiplying. Permeabilization of the cell wall and leakage of intracellular material does thereby not take place [46]. Similar effects have also been shown for *S. epidermidis*. Glutaraldehyde can also kill the bacteria here but does not permeabilize the cell wall [47, 48]. In the manufacturing process of HbMP the GA concentration of 0.02% was used. A study of stability testing was carried out to confirm that this GA concentration was sufficient for the production of HbMP. Three batches of HbMP were aliquoted and stored at 2 – 8 °C. Every month an aliquot per batch was analyzed for the amount of released hemoglobin. As shown in Figure 3, the concentration of free hemoglobin remained almost constant over the measurement period in the range of 1 to 1.5 mg/mL and thus in a similar range as the amount of free hemoglobin allowed for erythrocyte concentrates during their storage period [49, 50]. No additional release of hemoglobin was observed so the particles prepared with 0.02% GA are stable for at least six months. A higher concentration of GA was not used in the particle fabrication process since they caused higher phagocytosis rate of HbMP and led to an increased amount of methemoglobin [51].

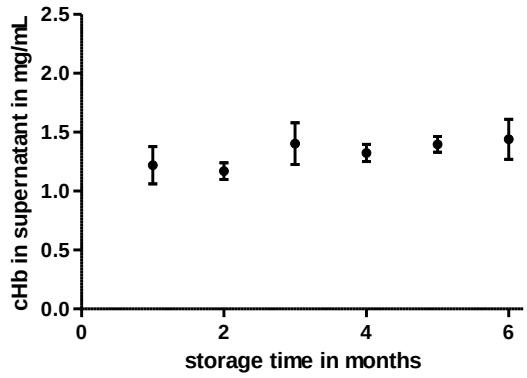


**Figure 3.** Concentration of free hemoglobin in the supernatant of HbMP suspensions. Measuring points are means with SD from three batches of HbMP.

## Effect of EDTA on bacterial growth

*E. coli* (Figure 4 A) and *S. epidermidis* (Figure 4 C) were cultivated in growth medium containing 0.2 M EDTA at 37 °C. In contrast to the experiments with an addition of glutaraldehyde, here both bacterial strains showed no growth compared to controls in medium without additives. The growth, as determined by the optical density of the sample, was equivalent to that of the negative control in peracetic acid. The addition of EDTA to the growth medium leads to complete inhibition of bacterial proliferation.

There was also no growth during incubation of *E. coli* with EDTA addition at room temperature (Figure 4 B). The optical density did not increase at any time point compared to the initial value. In contrast, the control in normal medium grew strongly. Again, optical density values were normalized to the respective initial value for better comparability. The cells of the control while cultivating *S. epidermidis* showed similar growth behavior as in the experiment with glutaraldehyde addition (Figure 4 D). The cells with EDTA addition showed some increase in optical density at the measurement point after 30 min. However, this drops back to the initial value in the further course of

the experiment. Thus, there is no growth of either bacterial strain in this experimental arrangement.

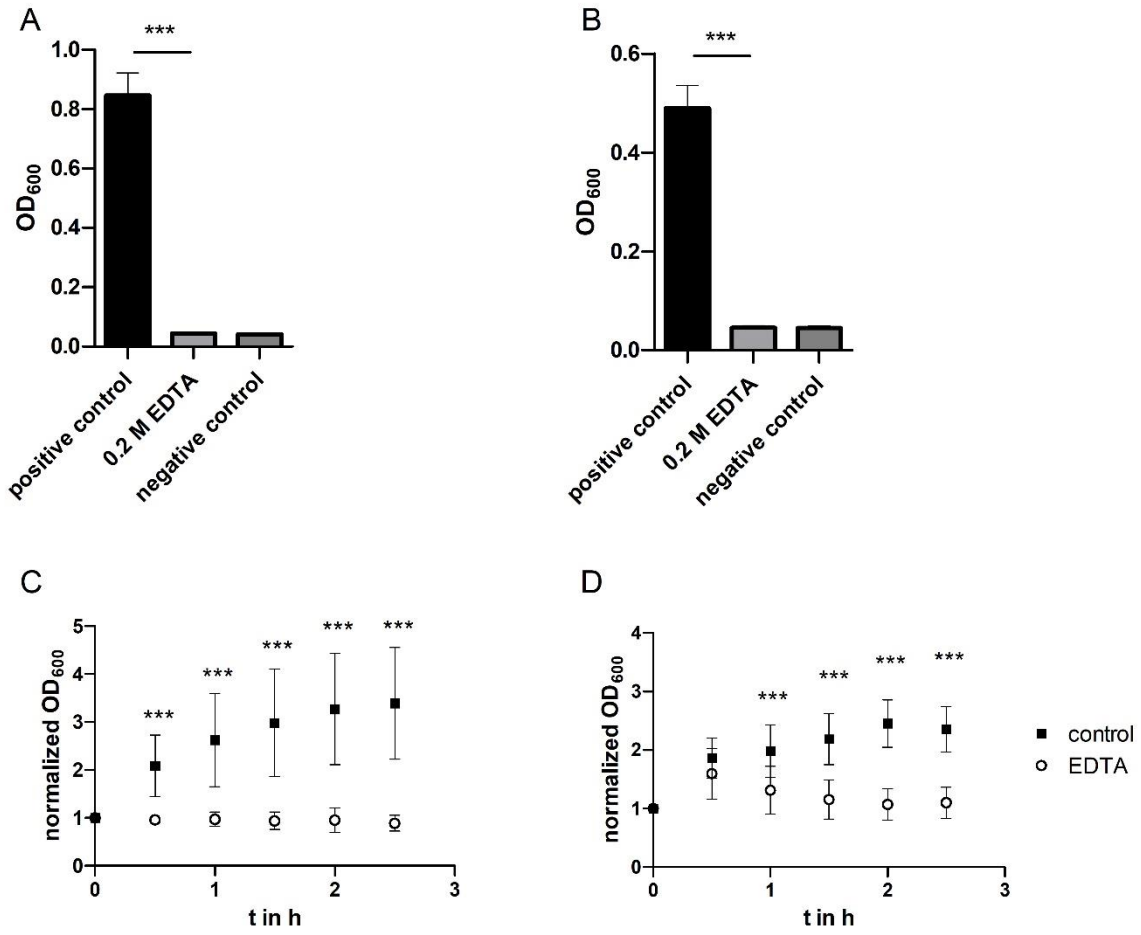


**Figure 4.** Effect of EDTA on bacterial growth of *E. coli* and *S. epidermidis*. *E. coli* (A) and *S. epidermidis* (B) cells were incubated for 20 h at 37 °C in Mueller-Hinton II-Bouillon (positive control); Mueller-Hinton II-Bouillon + 0.2 M EDTA or Mueller-Hinton II-Bouillon + 0.4 % peracetic acid (negative control). Error bars are SD (n = 12 biological replicates). Statistical analysis was performed by a one-way ANOVA with Bonferroni's multiple comparison test. SD for negative controls and 0.2 M EDTA < 0.005.

*E. coli* (C) and *S. epidermidis* (D) cells were pre-incubated for 20 h at 37 °C in Mueller-Hinton II-Bouillon. Afterwards the growth medium was removed and replaced by fresh

Mueller-Hinton II-Bouillon (“control”) or Mueller-Hinton II-Bouillon + 0.2 M EDTA (“EDTA”). Cells were grown for 2.5 h at room temperature. Error bars are SD (n = 12 biological replicates). Statistical analysis was performed by a two-way ANOVA Bonferroni’s multiple comparison test; *** corresponds to $p < 0.001$.

EDTA as a chelating agent is known to bind divalent cations. Especially the chelation of $Mg^{2+}$ results in a destabilization of the negative charges of the outer membrane of gram-negative bacteria [45]. The cells thus become permeable. This effect can be exploited to make the cells more receptive to antibiotics, biocides or other substances [28, 52]. Depending on the concentration of EDTA and the bacterial strain, there is a release of membrane components, proteins and finally lysis of the cell [53–55]. The treatment of gram-negative cells with EDTA can lead to a release of up to 50% of the LPS from the cell wall of the bacterium [56, 57].These effects have been demonstrated for gram-negative bacteria and especially *E. coli* [29, 58]. However, EDTA can also lead to inhibition of growth and cell lysis in gram-positive bacteria [59, 60].

In the study presented here, EDTA is used to dissolve the carbonate template during particle preparation at a concentration of 0.2 M. The results of our experiments (Figure 4) are in agreement with the above-mentioned literature.

## Combination of GA and EDTA according to the particle preparation routine

In addition to the experiments described above, in which the effect of glutaraldehyde and EDTA was separately examined, both substances were combined with one another in further experiments. It should be investigated whether the effect of the two substances can cancel or strengthen each other. The concentrations of GA and EDTA

corresponded to those that are used in the fabrication process. The results are shown in Figure 5.

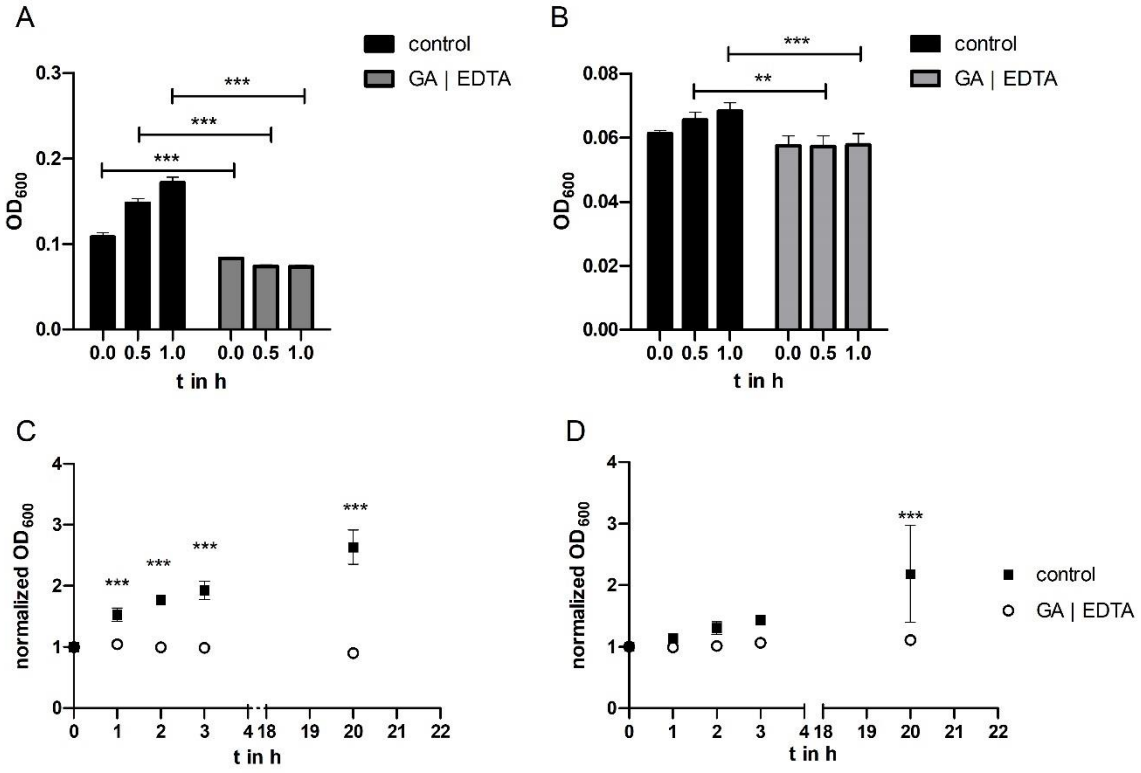


**Figure 5.** Effect of a combination of glutaraldehyde and EDTA on *E. coli* and *S. epidermidis*. *E. coli* (A) and *S. epidermidis* (B) cells were grown in Mueller-Hinton II-Bouillon ("control") and Mueller-Hinton II-Bouillon supplemented with 0.02 % glutaraldehyde and 0.2 M EDTA (GA | EDTA) for one hour at room temperature. SD for GA | EDTA in (A) is 0.005. C and D: Cells grown as described in (A) and (B) were subsequently incubated at 37 °C for 20 h. Error bars are SD (n = 4 biological replicates) Statistical analysis was performed by a two-way ANOVA with Bonferroni's multiple comparison test; ** corresponds to $p < 0.01$; *** corresponds to $p < 0.001$.

When *E. coli* were cultured with the addition of EDTA and GA at room temperature for one hour, there was no increase in optical density and thus no growth of bacteria (4A).

In contrast, the control cells grew normally. In the corresponding growth experiments with *S. epidermidis*, there was also no increase in optical density after addition of EDTA and GA (4C). The cells of the control did not grow as much here, but the differences in growth were significant during the experiment compared to the cells cultured with additives.

There was also no detectable growth of *E. coli* after the addition of EDTA and GA and cultivation at 37 °C (4B). The optical density remained almost constant during the experiment compared to the initial value. The control cells grew strongly, so there were significant differences in growth at each measurement time point in this experiment. When culturing *S. epidermidis* after addition of EDTA and GA, no growth was seen either. However, again the control did not grow as much. Nevertheless, at the end of the experiment, the difference in growth compared to the control was significant.

These experiments also confirm the growth-inhibiting effect of EDTA and GA, also in combination, in the concentrations used in the routine HbMP manufacturing process.

## Particle preparation with the addition of bacteria

The experiments described above have shown the inhibitory effect of glutaraldehyde and EDTA on the growth of *E. coli* and *S. epidermidis* when the substances are added to the growth medium. Regarding the production of HbMP, however, it is of particular interest whether bacterial contamination can also be removed during the production process. To investigate the growth of both bacteria during the HbMP production process, the initial hemoglobin solution was spiked with *E. coli* and *S. epidermidis*, respectively, and particle production was performed. Samples were taken after each production step and checked to see if viable bacteria were still present. The samples were centrifuged and the amount of bacteria in the total sample suspension (before

centrifugation) as well as in the supernatant (after centrifugation) was examined (Figure 6).

After precipitation, most of the original bacterial count of both bacteria was still present in the suspension. In the experiment with *E. coli*, a small proportion was detectable in the supernatant of the sample (upper row A). This means that a large part of the bacteria was in the sediment, i.e. in the particle fraction. After the first washing step in the process, the detectable bacterial count was further reduced to about one quarter of the initial value. In this series of experiments, the proteins in the particles were not cross-linked, but the carbonate templates were immediately dissolved with EDTA resulting in no particle formation (see Figure 7 - sample A). After resuspension of the particles in EDTA, only a few of the originally used bacteria were still present. The results were similar for particle preparation with hemoglobin spiked with *S. epidermidis* (lower row A). However, after the final dissolution step with EDTA, the sample still contained about a quarter of the initial number of viable bacteria. In another series of experiments, the particles were cross-linked with glutaraldehyde before dissolution with EDTA. This corresponded to the actual HbMP production process (Figure 7 - Sample B). Here it could be seen that after cross-linking with glutaraldehyde, no viable bacteria of either strain were detectable (Figure 6 B).

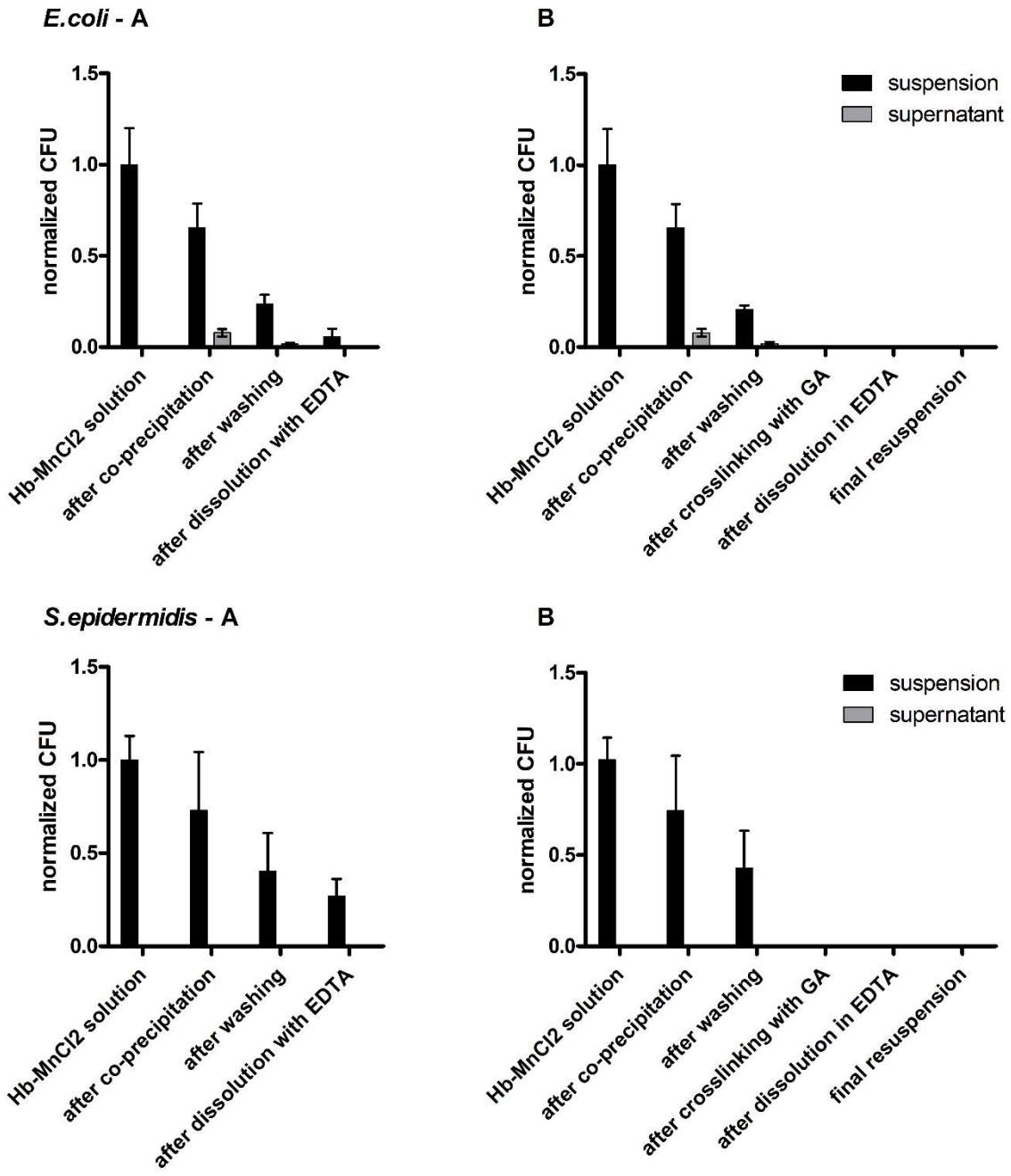


**Figure 6.** Particle preparation with *E. coli* and *S. epidermidis*. The Hb-$MnCl_2$ solution was spiked with *E. coli* (upper row) or *S. epidermidis* (lower row) and co-precipitated with $Na_2CO_3$. The resulting particles were washed with NaCl and either the salt template was directly dissolved (Sample A) or the particles were crosslinked with glutaraldehyde and then dissolved with EDTA (Sample B). At each given production

step, the number of colony-forming units was determined. Error bars are SD, n=8 except for “Hb-$MnCl_2$ solution” (n = 4).

In these experiments, the above observations could be confirmed. The concentrations of GA and EDTA used in the CCD process are sufficient to crosslink the hemoglobin molecules and dissolve the carbonate template. Furthermore, both chemicals resulted in no detectable viable bacteria at the end of the particle production process. In the above experiment, the EDTA concentration optimized for the CCD process alone was not sufficient to remove all bacteria from the solution without crosslinking the particles with GA. EDTA forms chelate complexes with metal ions, which are thereby incorporated into a ring structure. The formed complexes with manganese ions are stronger than the complexes formed with magnesium [61]. For this experiment, it should be noted that the EDTA here complexes both the manganese from the manganese carbonate template and magnesium ions from the cell wall of the bacteria. The former leads to dissolution of the carbonate template of the particles, while the latter leads to prevention of bacterial proliferation. It is possible that the stronger binding of EDTA to manganese results in insufficient EDTA for all the magnesium from the cell walls of the bacteria. However, if the actual production process of the HbMP was performed, i.e. including the cross-linking with glutaraldehyde, no more bacteria were detectable after the cross-linking step.

Thus, the use of GA and EDTA together with washing effects may be part of a comprehensive biological safety concept for the production of HbMP for the potential use as an artificial oxygen carrier and blood substitute.

# Conclusions

In conclusion, we could show that the HbMP can be produced safely with respect to bacterial contamination. Biopolymer particles can be produced with the simple CCD technique and promise a wide range of biomedical applications, depending on the biopolymer used. Especially the application as artificial oxygen carriers came into focus. Initial preclinical studies yielded promising results. In these particles, the HbMP, hemoglobin is used for production and EDTA and glutaraldehyde are applied in the standard production process. The effect of these chemicals in the concentration range used, together with washing effects during production, ensure that any previously undetected bacterial contamination is removed. After production and a final determination of the bioburden and endotoxin content, (and of course if all other quality control parameters are met) the produced batch of HbMP can be released for further use. These findings are an important part of our extensive safety concept.

# Experimental

## Materials

Ringer´s acetate solution was purchased from Serumwerk Bernburg AG, Bernburg, Germany; Ampuwa (aqua ad iniectabilia) from Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany and sodium chloride (NaCl) from B.Braun Melsungen AG, Melsungen, Germany. Human Albumin 200 g/L Baxalta (HSA) was obtained from Takeda Pharma Vertrieb GmbH & Co. KG, Berlin, Germany. Ethylenediaminetetraacetic acid (EDTA), Phosphate-buffered saline (PBS) and glycine were obtained from AppliChem GmbH, Darmstadt, Germany. Manganese dichloride

($MnCl_2$), sodium carbonate ($Na_2CO_3$), glutaraldehyde (GA), sodium borohydride ($NaBH_4$), sodium hydroxide (NaOH) and Mueller-Hinton II-Bouillon were purchased from Merck KGaA, Darmstadt, Germany.

*Staphylococcus epidermidis* (own cultivation) and *Escherichia coli* (ATCC 25922) were kindly provided by the Bereswill Lab, Institute of Microbiology, Infectious Diseases and Immunology, Charité – Universitätsmedizin Berlin, Berlin, Germany. Columbia agar 5 % sheep blood was purchased from Thermo Fisher Diagnostics GmbH Microbiology, Wesel, Germany.

Hemoglobin (Hb) was prepared by hypotonic hemolysis with slight adjustments as described earlier [62, 63]. Briefly, fresh bovine whole blood (anticoagulated with 2 g/L EDTA, provided by Biophyll GmbH, Dietersburg, Germany) was centrifuged for 20 min at 2500 *g* at 2 – 8 °C. The resulting packed red blood cells (RBC) were washed at least three times with sterile 0.9 % NaCl and lysed over night with four to five volumes of a low osmotic sodium chloride solution (100 mOsmol/kg). The lysate was centrifuged at 5500 *g* and 2 – 8 °C for 4 h and the supernatant was processed by means of tangential flow filtration (TFF). A KrosFlo KR2 system with a 500 kDa mPES hollow fiber module (Repligen Europe B.V., Breda, Netherlands) was used similar to the process described earlier [64]. Processed Hb was stored at -80 °C until use.

## Preparation and characterization of HbMP

Hemoglobin Microparticles (HbMP) were fabricated by the co-precipitation crosslinking dissolution (CCD) technique [65, 51]. Shortly, 0.25 M $Na_2CO_3$ and 0.25 M $MnCl_2$ including 10 mg/mL Hb and 1 mg/mL HSA were mixed rapidly at room temperature (co-precipitation). After the co-precipitation, 2.5 mg/mL HSA were added and after 5 min the particles were separated by centrifugation and washed three times with 0.9 %

NaCl. The particles were resuspended in a 0.02 % GA solution and incubated for 1 h at room temperature on a shaker (crosslinking). After another centrifugation, the excess GA was quenched with 0.1 M glycine. 0.2 M EDTA solution pH 7.4 was added to dissolve the $MnCO_3$ template (Dissolution) and the resulting protein particles were treated with $NaBH_4$ in 0.1 M NaOH. Lastly, the particles were washed three times with 0.9 % NaCl and resuspended in Ringer´s acetate until further use.

## Characterization of HbMP

For the scanning electron microscopy (SEM) analysis, one drop of the sample was applied to a glass slide, dried overnight, and sputtered with gold. A Gemini Leo 1550 (Carl Zeiss AG, Oberkochen, Germany) instrument was utilized for the measurements at an operation voltage of 10 kV.

The particle size was measured by dynamic light scattering (DLS) applying a Zeta Sizer nano ZS instrument (Malvern Panalytical Ltd., Malvern, U.K.). Additionally, CLSM (Confocal laser scanning microscopy) images were taken with a confocal microscope LSM 510 Meta (Carl Zeiss AG, Oberkochen, Germany) and the size was measured from the images. The microscope was used with a 100 x oil-immersion objective (numerical aperture 1.3) while utilizing excitation wavelength of 488 and a 505 nm long pass emission filter.

Zeta potential of HbMP in 0.9 % NaCl (pH 7.4, conductivity 17.2 ± 0.9 mS/cm) was measured using the Zeta Sizer nano ZS.

For the determination of the concentration of free hemoglobin in the HbMP suspension aliquots of three batches of HbMP, produced with 0.02% GA were stored at 2 – 8 °C for up to six months. Every month an aliquot was taken and centrifuged at 20 000 *g* for 30 min (Hettich Mikro 22R, Hettich GmbH & Co. KG, Tuttlingen, Germany). Released

hemoglobin in the supernatant was measured with standard alkaline haematin detergent (AHD) [66].

## Preparation of HbMP Spiked with Bacteria

HbMP spiked with bacteria were produced by adding *E. coli* or *S. epidermidis*, respectively to the hemoglobin solution (see Figure 7). The bacteria were spread on agar plates three days prior to particle preparation and incubated at 37 °C. Then, about one third of a bacterial colony was transferred into Ampuwa, the optical density at 600 nm ($OD_{600}$) was measured (Spectra Classic, Tecan Group Ltd., Maennedorf, Switzerland) and adjusted to correspond to 1.5 * $10^8$ CFU/mL (CFU – colony-forming unit). The bacterial suspension was diluted to 2.5 * $10^5$ CFU/mL and mixed with the hemoglobin solution. The concentration of bacteria in the hemoglobin solution was determined for every particle batch and used as an initial value to evaluate bioburden at different steps in the particle preparation process.

## Determination of Bacterial Growth

### Influence of GA and EDTA

The bacteria plus EDTA or GA were filled in the wells of a microtiter plate. The samples contained bacteria at a concentration of approximately 7.5 * $10^5$ CFU/mL, 0.2 M EDTA or 0.02 % GA. The positive control consisted of bacteria and growth medium (Mueller-Hinton II-Bouillon), the negative control of bacteria and 0.4 % peracetic acid and the sterile control of medium only. The bacteria were pre-incubated for 20 h at 37 °C in Mueller-Hinton II-Bouillon. Afterwards the growth medium was removed and replaced by fresh Mueller-Hinton II-Bouillon (control) or Mueller-Hinton II-Bouillon + 0.02 % GA or 0.2 M EDTA. Cells were grown for 2.5 h at room temperature. At the start and at

different time points, the optical density at 600 nm was determined to assess bacterial growth. In a further experiment, a mixture of EDTA and GA was examined simultaneously.

**Determination of Bioburden during Preparation of HbMP**

To assess the amount of viable bacteria samples were taken from the initial Hb-$MnCl_2$ solution and after co-precipitation, washing, crosslinking, dissolution and from the final particle suspension (Figure 7 - Sample B). Two samples for determining the bioburden were analyzed. One from the particle suspension and one from the supernatant after centrifuging (3000 x *g*, 3 min) the suspension.

In addition to this standard protocol, test series were also carried out in which coprecipitated particles were dissolved with EDTA without prior crosslinking with glutaraldehyde. Therefore, the last sample here was taken after resuspension in EDTA solution (Figure 7 - Sample A).

The respective samples were serially diluted (undiluted to 1:1000), 100 µL of each dilution were spread on agar plates and incubated for one day at 37 °C. Possibly growing colonies were counted.

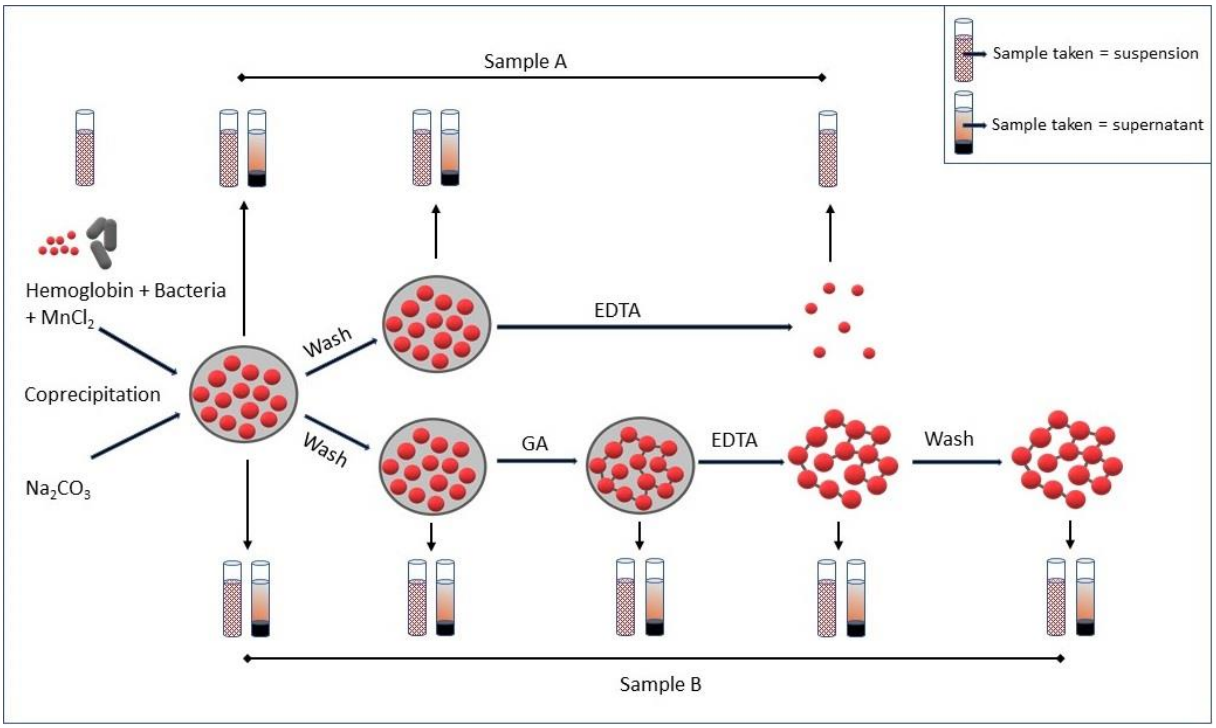


**Figure 7.** Simplified scheme of the experimental approach. The hemoglobin-$MnCl_2$ solution was spiked with *E. coli* or *S. epidermidis*, respectively and coprecipitated with $Na_2CO_3$. The resulting particles were washed with NaCl and either the salt template was directly dissolved with EDTA (Sample A) or the particles were crosslinked with glutaraldehyde (GA) and then dissolved with EDTA (Sample B). At various production steps, the number of colony-forming units (CFU) was determined in the complete sample (suspension) and in the supernatant after centrifugation, respectively.

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